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Spatial Epidemiology of Bovine Tuberculosis in domestic animals and Evaluation of Surveillance

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PhD Thesis

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Space-time epidemiology of bovine Tuberculosis in domestic animals in Spain. Evaluation of the surveillance system.

Tesi doctoral presentada per Ariadna García Sáenz per accedir al grau de Doctora en Veterinària dins del programa de Doctorat en Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la dirección del Dr. Alberto Allepuz Palau i el Dr. Sebastian Napp Avelli.

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ALBERTO ALLEPUZ PALAU, profesor del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona i investigador del Centre de Recerca en Sanitat Animal (CReSA), i SEBASTIAN NAPP AVELLI, investigador del Centre de Recerca en Sanitat Animal (CReSA).

Certifica:

Que la tesi doctoral titulada "Space-time epidemiology of bovine Tuberculosis in domestic animals in Spain. Evaluation of the surveillance system" presentada per Ariadna García Sáenz per l'obtenció del grau de Doctora en Veterinària, s'ha realitzat sota la seva direcció a la Universitat Autònoma de Barcelona i al CReSA.

I per tal que consti als efectes oportuns, signem el present certificat a Bellaterra, a 22 de setembre de 2015.

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Summary

The present PhD Thesis aimed to provide information about the epidemiology of bovine tuberculosis in cattle and relevant data for the implementation of a risk based surveillance system. The studies included in this PhD Thesis are summarized below:

1. In the first study the space-time variation of the risk of bovine tuberculosis (bTB) in cattle between 2006 and 2011 was analyzed. The results indicated that at country level, there were no significant temporal changes between years, but, at county level bTB evolution was more heterogeneous. In some counties, between some years, the prevalence and the incidence of the disease was higher as compared to the global rate in the rest of the counties of Spain.

   The analysis of potential risk factors indicated that both, a large number of movements from counties with high incidence (>1%), and presence of bullfighting cattle herds increased the bTB risk. Red deer abundance, number of goats and number of mixed cattle-goat farms were not significantly associated with the prevalence/incidence of bTB.

2. In the second study we describe a risk-based approach for bTB surveillance that is under development in New Zealand. Given that the presence of bTB in a herd is driven by a number of factors including previous infection history, the amount of testing carried out on individual herds, geographic location or herd movement behavior, the objective was to use routinely recorded data to derive a 'risk score' for each of these factors and then to combine them to return a composite bTB risk score for each herd. By this way, herds could be ranked and

this would enable to focus surveillance in those herds with the highest risk score, providing effective surveillance coverage at a reasonable overall cost.

3. In the third study the individual sensitivity of bovine tuberculosis surveillance in Catalonian slaughterhouses of cattle was assessed. The probability of detection of a bTB-infected cattle by the slaughterhouses in Catalonia was estimated as the product of three consecutive probabilities: P1) the probability that a bTB-infected animal arrived at the slaughterhouse presenting Macroscopically Detectable Lesions (MDL); P2) the probability that MDL were detected by the routine meat inspection procedure, and P3) the probability that the veterinary officer suspected of bTB and sent the sample for laboratory confirmation.

   The first probability was obtained from data collected through the bTB eradication program carried out in Catalonia between 2005 and 2008, while the last two were obtained through the expert opinion of the veterinary officers working at the slaughterhouses who fulfilled a questionnaire administered during 2014.

   The mean individual bTB surveillance sensitivity of the different cattle slaughterhouses in Catalonia obtained in this study was 31.4% (CI 95%: 28.6-36.2), and there were differences among them. This low sensitivity was mainly related with the low probability that a bTB-infected animal presented MDL (44.8%). The sensitivity of the slaughterhouse detection was significantly associated with some variables included in the questionnaire such as attendance to training courses, number of meat technicians or speed of the slaughter chain. These variables were responsible for the variability of the sensitivity observed among Catalonian slaughterhouses. Technical and policy

efforts should be focused on the improvement of these factors in order to maximize the slaughterhouse sensitivity for bTB detection.

4. In the fourth study the within-herd transmission dynamics of bovine tuberculosis in Spanish herds was evaluated. A stochastic compartmental SEI (Susceptible, Exposed (latent), and Infectious) model was developed to mimic Bovine tuberculosis (bTB) within-herd transmission dynamics.

   This model was used to infer several parameters related to bTB spread within Spanish cattle farms, in particular the cattle-to-cattle transmission rate (β) and the rate at which infected cattle become infectious (α). Also, given the controversy over the sensitivities of the single intradermal tuberculin test (SIT) application in field conditions, the probability of detection of both infected and infectious cattle (φ and ρ, respectively), were also evaluated.

   Data for parameter inference was obtained from farms where there were epidemiological evidences of bTB introduction into the herd through the purchase of infected animals, which allowed us to have data on: a) the date of introduction of infection into the herd, b) initial number of infected animals introduced and c) final number of infected animals (when infection of the herd is detected).

   A Markov Chain Monte Carlo-Approximate Bayesian Computation (MCMC-ABC) method was used to generate posterior distributions of bTB transmission parameters and sensitivities of SIT test in Spanish cattle farms.

   The mean within herd transmission rate (β) estimated in 33 Spanish herds varied between 0.0001 and 0.0002 per day, and the mean rate at which infected cattle become infectious (α) varied between 0.011 and 0.0001.

The results from the studies I, II and III included in the present PhD Thesis have been published or submitted for publication in international scientific peer-reviewed journals:

Study I:

Garcia-Saenz A, Saez M, Napp S, Casal J, Saez J.L, Acevedo P, Guta S, Allepuz A. *Spatio-temporal variability of bovine tuberculosis eradication in Spain (2006-2011)*. Spatial and Spatio-Temporal Epidemiology (2014) 10:1-10.

Study II:

Garcia-Saenz, Bosson M, Dawson K, Stevenson MA. *Finding needles in a haystack: bTB surveillance in New Zealand.* Under review in Preventive Veterinary Medicine.

Study III:

Garcia-Saenz A, Napp S, Lopez S, Allepuz A. *Estimation of the individual slaughterhouse surveillance sensitivity for bovine tuberculosis in Catalonia (North-Eastern Spain).* Preventive Veterinary Medicine (2015) 121:332–337.

List of abbreviations

ABC: Approximate Bayesian Computation
AC: Autonomous Community
bTB: Bovine Tuberculosis
CMI: cell-mediated immune
CR: credible intervals
DIC: Deviance Information Criterion
DR: direct repeat
EFSA: European Food Safety Authority
ELISA: enzyme-linked immunosorbent assay
GLMM: generalized linear mixed model
HPD: Highest Posterior Density
IDT: intradermal tuberculin
IFN-γ: gamma-interferon assay
LIC: Livestock Improvement Corporation
MAGRAMA: the Spanish Ministry of Agriculture, Food and Environment
MCMC Markov Chain Monte Carlo
MDL: Macroscopically Detectable Lesions
MIRU: mycobacterial interspersed repetitive unit
MTC: Mycobacterium tuberculosis-complex
NAIT: National Animal Identification and Tracing System
OIE: World Organization for Animal Health
OTF officially tuberculosis free
PCR: Polymerase chain reaction
PPD: purified protein derivative
RFLP: restriction fragment length polymorphism
RRI: relative risks of the incidence
RRP: The relative risks of the prevalence
SD: standard deviations
Se: sensitivity
SICCT: single-intradermal comparative cervical tuberculin
SIT: single-intradermal tuberculin
SMC: Sequential Monte Carlo
Sp: specificity
SPDE: stochastic partial differential equation
UTM: Universal Transverse Mercator
VNTR: variable number tandem repeat

Chapter 1: Introduction

Section 1. Background of bovine tuberculosis

I. Etiology

Bovine Tuberculosis (bTB) is defined as a chronic infectious disease of cattle (including all *Bos* species, and *Bubalus bubalus*) and bison (*Bison bison*) caused by any of the disease-causing mycobacterial species within the *Mycobacterium tuberculosis*-complex (MTC) (Anonymous, 2013a). *Mycobacterium bovis* is the most frequent MTC isolated in cattle followed by *Mycobacterium caprae* (Aranaz et al., 2006; Rodriguez et al., 2009; Rodriguez-Campos et al., 2011; Muller et al., 2013). Both are Gram positive, acid-fast bacterium of the family Mycobacteriaceae (OIE., 2009) with a thick and lipid-rich cell-wall which protects DNA from the attack of lytic enzymes after the autolysis and necrosis of the host cell, and are characterized by a slow growth in culture.

Other livestock species, besides cattle, can also be affected by bTB (Pesciaroli et al., 2014). Both companion and wild animals are also susceptible (Aranaz et al., 2004), and it may cause zoonotic disease in humans (Müller et al., 2013). The disease is endemic in many European countries and constitutes a significant economic burden to the agricultural industries (Schiller et al., 2011).

II. Pathology: clinical signs and lesions

Bovine TB is a chronic disease, and clinical signs are generally not present, and if present, they may take months or even years to develop depending on the functional impairment of the affected organ, due to the evolution of the granulomatous lesions. In countries under an eradication campaign for many years, clinical symptoms are even less common, as the affected animals are usually detected (and eliminated) at the very

early stages of infection, and therefore, lesions are rarely seen at slaughterhouse inspection (Cassidy et al., 2006; OIE, 2009).

On the other hand, in endemic countries with no control programs, animals may show hacking cough and tracheal oppression (due to the enlargement of the regional lymph nodes), pneumonia, weakness, loss of appetite and weight, fluctuating fever or diarrhoea (OIE, 2009).

Regarding the lesion distribution, the route of transmission will determine the location and the degree of extension (Domingo et al., 2014). After the entry of the mycobacteria in the animal, the inflammatory signal pathways will be activated, leading to the phagocytosis of the bacteria by macrophages (Domingo et al., 2014), which are the primary host cell for intracellular growth (Pollock et al., 2006). It is assumed that this initial infection will lead to a "primary complex" which is defined as the combination of lesions in the initial focus together with the lesion in the regional lymph node (Neill et al., 2001; Domingo et al., 2014).

The inhalation of infected droplets is described as the most common route of infection in cattle (Neill et al., 2001) causing initial granulomatous lesions in the nasopharynx and lower respiratory tract, including the lungs, and associated lymph nodes (Neill et al., 1991; Pollock et al., 2006; Liebana et al., 2007; Domingo et al., 2014). If the initial immune response is ineffective, a post-primary stage will take place leading to a chronic infection and after that, mycobacteria might spread via pre-existing anatomical channels to different organs where multiple small granulomas may be found in numerous organs as the liver, spleen and surfaces of body leading to a generalised tuberculosis (Neill et al., 1994; Domigo et al., 2014).

If animals get infected through the ingestion of contaminated materials, the lesions will occur most commonly in the mesentheric lymph nodes but few lesions in the intestinal wall may also appear (Menzies and Neill et al., 2000). On the other hand, oropharyngeal mucosa and retropharyngeal lymph nodes can be considered as a common pathway for both respiratory and digestive infections (Domingo et al., 2014), although studies have shown the relevance of the palatine tonsils as an infection pathway as well (Palmer et al., 1999; Neill et al., 2001).

III. Diagnosis

The following section gives an overview of the current ante- and post-mortem diagnostic techniques.

a. Ante-mortem diagnostic tools

Given that *M. bovis* is an intracellular pathogen of macrophages and other monocytic cell types (De la Rua et al., 2006), cell-mediated immune (CMI) response is the main target for the ante-mortem diagnosis of the disease (Pollock et al., 2005).

The intradermal tuberculin (IDT) test has been used for routine field detection of infected animals since nearly a century ago, and it is the official test in most countries (Monaghan et al., 1994). In Spain, the official ante-mortem diagnostic techniques regulated by RD 2611/1996 and RD1047/2003, and approved by the EU regulation (De la Rua-Domenech et al., 2006; OIE, 2009), are the single-intradermal tuberculin (SIT) and the single-intradermal comparative cervical tuberculin (SICCT).

Both are delayed hypersensitivity tests, which can be applied in animals from 6 weeks of age and involve measuring skin thickness before and after the intradermal injection of a purified protein derivative (PPD) of *M. Bovis*. In the SIT, the PPD of *M. Bovis* is

inoculated, and if the animal has been previously in contact with *M. bovis*, its immune system will generate an inflammatory reaction on the site of injection, which will be evident 48-72 hours after the inoculation of bovine PPD (Pollock et al., 2005). Evidence of pain, heat or oedema at the site of inoculation may also result in a positive interpretation of the test.

In the SICCT both bovine and avian PPD (prepared with *Mycobaterium avium* strains), are injected simultaneously in two different locations, and the inflammatory reactions to both PPDs are then measured and compared. A larger reaction to bovine PPD than to avian PPD would be indicative of *M. Bovis* infection, while a larger reaction to avian PPD than to bovine PPD would be indicative of exposure to other mycobacteria related genera.

Diagnostic blood tests are now available and can be used as complementary techniques to the IDT test (Pollock et al., 2005). The more extended is the gamma-interferon assay (IFN-γ), described by Wood et al. (1990) and introduced in the EU legislation (Council Directive 64/432/EEC) as an ancillary test to enhance the sensitivity of the bTB diagnostic. One advantage of the IFN-γ, is that it reduces the handling of animals (animals must be restrained only one time as compared with twice, with the IDT test), and that is particularly relevant in the case of bullfighting cattle (Rodríguez-Prieto et al., 2012; Aranaz et al., 2006)

This test uses an enzyme-linked immunosorbent assay (ELISA) as the detection method for the gamma-interferon released by the lymphocytes stimulated with bovine PPD. Moreover, this technique allows the comparison of the amount of interferon released through the stimulation with avian PPD, which enables the differentiation of infections

caused by other mycobacteria such as *Mycobacterium avium subspecies paratuberculosis*. The IFN-γ assay can be applied in animals from 6 months of age.

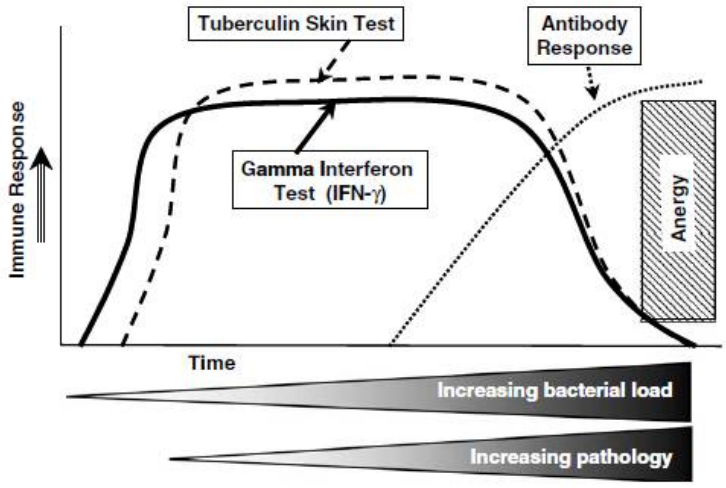
Compared with the tuberculin skin test, the IFN-γ is considered to have a higher sensitivity, allowing the detection of animals in the very early stages of infection (Wood and Rothel, 1994; Aranaz et al., 2006; Gormley et al., 2006) but also a lower specificity (Cagiola et al., 2004; Vordermeier et al., 2004). Alvarez et al. (2011), estimated through a Bayesian approach, the sensitivities (Se) and specificities (Sp) of SIT and IFN-γ with data from the eradication campaign in Spain. The median estimates of Se were 66-69% for SIT (lower compared with those reported in the review of De la Rua et al. (2006), with a median result of SIT Se of 83.9%) and 83.5-90% for IFN-γ, while median values of Sp were over >99% for SIT and 85.7-90.3% for IFN-γ.

In **Figure 1** the immune responses of infected cattle and their relation with the diagnostic tests are summarized. In the first stages of infection the immune response will be predominantly cell mediated (Schiller et al., 2010), but as the disease progresses it will shift towards an antibody-based response. Tuberculin skin test and IFN-γ responses are correlated with the immune response but do not necessarily correlate with lesion severity whereas antibody responses generally are correlated with lesion severity (Waters et al., 2014).

In advanced and generalized infections, infected cattle might enter into an "anergic" stage, where there is no detectable cell-mediated immune response (De La Rua-Domenech, 2006) and then, antibody-based bTB assays (mainly ELISA-type) might be helpful to detect infected animals (Neill et al., 2001; Pollock and Neill., 2002).

In the last decades, the use of more specific "sero-dominant" *M. bovis* antigens, like MPB70 and MPB83, has allowed to increase the test specificity of antibody-based bTB assays, but their sensitivity is still suboptimal (De la Rua-Domenech., 2006).

Figure 1. Response of bovine immune system to various ante-mortem diagnostic tests for bTB (De la Rua-Domenech et al., 2006).



b. Post-mortem diagnosis

Post-mortem inspection at the slaughterhouse, is based on Regulation (EC) No 854/2004, and relies on observation, palpation and incision (EFSA, 2013a). If bTB compatible lesions are detected during the inspection, samples will be collected and sent to the laboratory for a histopathological exam and Ziehl Neelsen staining and finally, culture isolation for confirmation (Courcoul et al., 2014).

The isolation of mycobacteria on selective culture media is considered as the gold standard diagnostic technique by the World Organization for Animal Health (OIE), and has to be used to confirm the infection (OIE, 2009). However, even in samples with visible bTB lesions, bacteriology has a low sensitivity and can take several months to yield a result Courcoul et al. (2014). This has negative consequences as suspected herds have to maintain movement restrictions for several months until their bTB status

is clarified. An alternative to obtain a faster confirmation might be the use of molecular methods such as the Polymerase chain reaction (PCR). Courcoul et al. (2014), estimated the sensitivity and the specificity of the bacterial culture and PCR using field samples from cattle with compatible bTB lesions. By using latent class analysis they showed that the sensitivity of PCR was higher than that of bacteriology (on average 87.7% [82.5–92.3%] versus 78.1% [72.9–82.8%]) while the specificity of both tests was very good (on average 97.0% for PCR [94.3–99.0%] and 99.1% for bacteriology [97.1–100.0%]). Therefore, PCR might have the potential to replace bacteriology to confirm the infection.

On the other hand, techniques based on bacterial DNA sequencing and exponential amplification of genetic targets via PCR has become very useful for epidemiological and phylogenetic studies. In the last years they have been used for epidemiologic investigations (like determining potential links between outbreaks and their likely source of infection), or to examine the evolution of *M. bovis* infection in space and time (Milian-Suazo et al., 2008; De la Cruz et al., 2014).

One of the most widely used molecular techniques for the identification of *M. tuberculosis* complex isolates, is the spoligotyping. This technique detects the presence or absence of spacers in the direct repeat (DR) locus of the *M. bovis* genome, a characteristic that is used to determine genetic similarity among strains and clustering in different groups called spoligotypes (Aranaz et al., 1998; Javed et al., 2007; Milian-Suazo et al., 2008; Rodriguez-Campos et al., 2011).

Additional techniques to study genetic differentiation of isolates and their phylogenetic relationships are the mycobacterial interspersed repetitive-unit-variable number tandem repeat (MIRU-VNTR), restriction fragment length polymorphism

(RFLP), and high throughput whole genome sequencing of mycobacterial DNA (Waters et al., 2014; Hauer et al., 2015).

IV. Public health and economic impact of bovine tuberculosis

Zoonotic TB is mainly caused by *Mycobacterium bovis* and *Mycobacterium caprae* (Cosivi et al., 1998; Rodriguez et al., 2009). The disease is transmitted to humans by the inhalation of aerosols or by the ingestion of unpasteurized milk (Rodriguez et al., 2009). People who are working in close contact with animals, such as farmers, or veterinarians are more likely to become infected due to the inhalation of infected droplets released by animals. Also meat inspectors and slaughterhouse personnel can be infected by contact with mucous membranes and broken skin (De la Rua-Domenech et al., 2006).

Zoonotic tuberculosis still represents an important public health problem in developing countries (O'Reilly and Daborn., 1995) however, with the introduction of milk pasteurization and control programs in bovines, the prevalence has dramatically reduced and nowadays, around 1-3% of the clinical cases of human tuberculosis is considered to have a zoonotic origin (EFSA, 2013a).

Evidence of human-to-human bTB transmission is limited and anecdotal. For instance, in Paris, a patient from a hospital with pulmonary tuberculosis, due to a multidrug-resistant strain of *M. bovis,* led to active disease in five patients (Cosivi et al., 1998). Also humans infected by *M. bovis* may act as a source of infection for cattle (Prodinger et al., 2002; Krajewska et al., 2012).

Even though the major economic impact of bTB relies in the livestock sector, due to cost of surveillance, movement restrictions and slaughter of large numbers of cattle (Riviere et al., 2014), these control programs are justifiable also in terms of food safety and public health. Therefore, bTB eradication is considered an important objective to be achieved in the EU (Reviriego-Gordejo and Vermeersch., 2006; Schiller et al., 2011).

Section 2. Epidemiology of bovine tuberculosis

I. Geographical distribution

Control programs, mainly based on the slaughter of animals positive to the tuberculin skin test, have substantially reduced or nearly eradicated the disease from farm animals in many industrialized countries (Reviriego-Gordejo and Vermeersch., 2006). However, bTB is still widespread in Africa, Central and South America, parts of Asia and some Middle East countries (Figure 2).

In Europe, countries are classified as officially tuberculosis free (OTF) or not officially tuberculosis free (non-OTF) (Commission Decision 2003/467/EC). The OTF status is achieved after reporting less than 0.1% infected herds during 6 consecutive years (Council Directive 64/432/EC). Despite the intensive eradication efforts applied over the years, bTB continues to be present in countries such as the United Kingdom, Ireland, Spain, Greece, Portugal or Italy. Besides, in countries such as France, that was declared OTF in 2001, the incidence has recently increased in some areas and that may result in the loss of the OTF-free status (Dommergues et al., 2011; Bekara et al., 2014). Other examples of OTF countries with recent outbreaks include Germany, with 46 outbreaks in cattle notified in 2013, mostly in the region of Bavaria and Lower Saxony

(Moser et al., 2014) or in Austria, where several red deer infected with *M. caprae* were found in regions along the border with Germany and Switzerland (Schiller et al., 2011)

OTF and non-OTF countries, as reported by the European Food Safety Authority (EFSA) in their last update (2013), are represented in figure 3. Non-OTF countries include Ireland, Spain, Greece, Bulgaria, Croatia, Cyprus, Hungary, Lithuania, Malta, and Romania. Portugal, Italy and the United Kingdom are non-OTF "regionalized" countries, in which only some areas are OTF (Anonymous, 2012a).

Figure 2. Bovine Tuberculosis world distribution map according to reports submitted to the World Organisation of Animal Health (OIE) in the first semester of 2015. Available at:

http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasedistributionmap

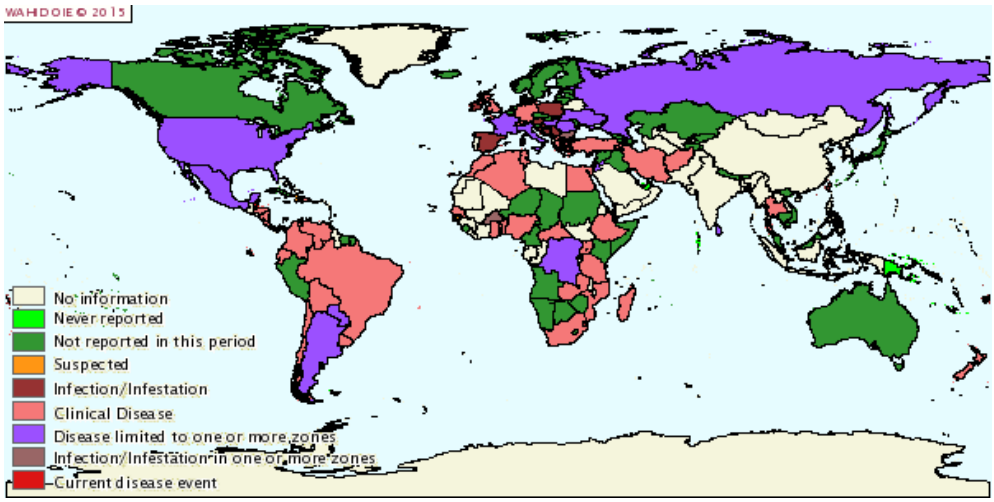
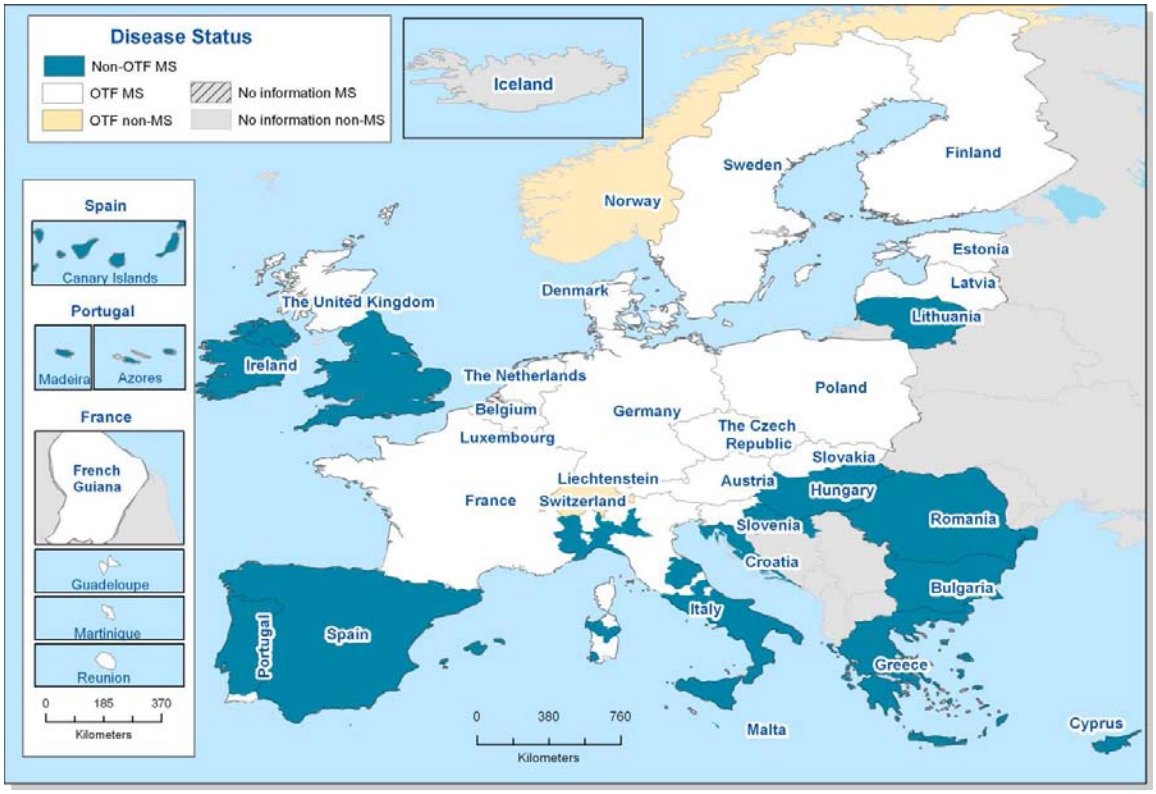


Figure 3. Status of countries regarding bovine tuberculosis, 2013 in Europe. Available at: http://www.efsa.europa.eu/en/efsajournal/pub/3991.htm



II. Disease transmission

a. Cattle to cattle transmission

The pattern of lesions observed in slaughtered animals represents a good evidence of the transmission route of *M. bovis* to cattle (Philips et al., 2003). Therefore, the high frequency of lesions found in the nasopharynx, lower respiratory tract and associated lymph nodes in cattle (Goodchild and Clifton-Hadley, 2001; Phillips et al., 2003; Courtenay et al., 2006; Johnson et al., 2007; Domingo et al., 2014), is indicative that the respiratory route (via the inhalation of infected aerosol droplets) can be considered as the primary mechanism of infection, (Menzies and Neill, 2000; Courtenay et al., 2006). Moreover, experimental studies have shown that low numbers of bacilli are needed to experimentally infect animals via the respiratory tract,

as compared to the large doses needed to infect animals via the digestive route, which appears to be a less effective method of disease transmission (Phillips 2003; Palmer et al., 2002). In addition, it has been suggested that some intestinal lesions in cattle may be the result of swallowing their own *M. bovis*-contaminated sputum (Menzies et al., 2000).

Indirect horizontal transmission through ingestion or by inhalation of infected aerosols present in the environment relies on the survival of the mycobacteria as it must remain viable in order to be infectious. The survival of the mycobcateria in the environment is dependent mainly on the temperature, moisture and exposure to ultraviolet light. Low temperature, high humidity and protection from sunlight will provide an ideal environment for persistence of the mycobacteria (Phillips et al., 2003). Infection through the ingestion of infected milk from tuberculous udders together with vertical transmission, as a result from tuberculous endometritis, can be described also as a way of direct transmission in calves (Neill et al., 1994; Goodchild and Clifton-Hadley, 2001). However, those transmission routes are very uncommon in developed countries.

b. Transmission from other domestic and wild species

Even though most of the domesticated species seem to act as spill-over hosts (i.e. the animals get infected but are not able to transmit the disease), in some particular epidemiological scenarios, domestic animals may play a role in the inter-species transmission of the disease (Pesciaroli et al., 2014). For instance, in Spain, an outbreak of *M. caprae* in a dairy cattle herd was detected, and the epidemiological investigations revealed a neighbouring goat herd as the most likely source of infection (Napp et al., 2012). In fact, goats are not tested within the Spanish national eradication

program; unless goats are cohabiting with bTB infected cattle, or when there is an epidemiological link with cattle outbreak. However, in some regions of Spain such as Murcia or Catalonia, compulsory and voluntary control programs have been established, respectively, in goat herds, applying similar ante-mortem diagnostic assays that are used for cattle.

On the other hand, although bTB is not diagnosed in sheep very often, a study carried out in Galicia (North-western Spain) by Muñoz-Mendoza et al. (2015), revealed the infection of 23 flocks in which sheep cohabited with bTB infected cattle and/or goats. Therefore, the epidemiological role of sheep in the epidemiology of bTB might need to be reconsidered as a potential source of bTB.

Domestic and wild pigs are also susceptible to get the infection (O'Reilly and Daborn., 1995; Parra et al., 2003). In the case of the Iberian pigs, tuberculous lesions have been described in pigs sharing spaces with other domesticated species or wildlife (Parra et al., 2003) in areas of high risk of bTB in Spain (Allepuz et al., 2011). Moreover, Di Marco et al. (2012) found tuberculous lesions in the black pig in Sicily, compatibles with a reservoir host rather than a spill over host. In addition, in Spain and Portugal it has been reported the circulation of the same *M. bovis* spoligotypes in domestic pig, wild boar and cattle populations (Parra et al., 2003).

In many countries, the presence of wildlife reservoirs endemically infected poses a challenge to bTB eradication schemes. Examples of such reservoirs include the European badger (*Meles meles*) in Great Britain and Ireland (Delahay et al., 2001) or the brushtail possum (*Trichosurus vulpecula*) in New Zealand (Porphyre et al., 2007). In Spain, the Eurasian wild boar (*Sus scrofa*), the red deer (*Cervus elaphus*) and the fallow

deer (*Dama dama*) have been identified as bTB maintenance hosts (Parra et al., 2005; Naranjo et al., 2008; Gortazar et al., 2011). In the case of the wild boar, it has been described as a reservoir of bTB in Mediterranean ecosystems and in central and southern Spain it has been implicated in the maintenance of the disease (Naranjo et al., 2008).

c. States of infection

When an animal is infected, it will go through different stages of infection as the disease progresses through time. The first state of infection is defined as the latency or exposed (E) period, which according to Barlow et al. (1997) could last from 87 to 226 days, and could be divided into two sub-stages: the unresponsive or occult period, and the responsive or reactive period. During the unresponsive period (U), the infected animal has not yet mounted a cell-mediated response, and therefore will not react to the intra- dermal tuberculin test or the gamma-interferon assay and therefore, in this state, the animal presents a null responsiveness to the CMI response diagnostic tests (IDTB and/or IFN-γ). Once the animal has mounted the cell-mediated immune response, it will start the reactive period (R), in which the animal will be detectable by the ante-mortem diagnostic tests, but no shedding of mycobacteria will take place and therefore, the animal will not be infectious for other animals.

The times of transition between those states has been recently reviewed by Alvarez et al. (2014). Reported values for the "U" state shows an interval from 30 to 54 days and for the "R" state a range from 14 to 22 months, although estimates of 34 months have also been reported. The duration of these states will vary depending on several factors that may affect the immunological response of the animal, such as the physiological

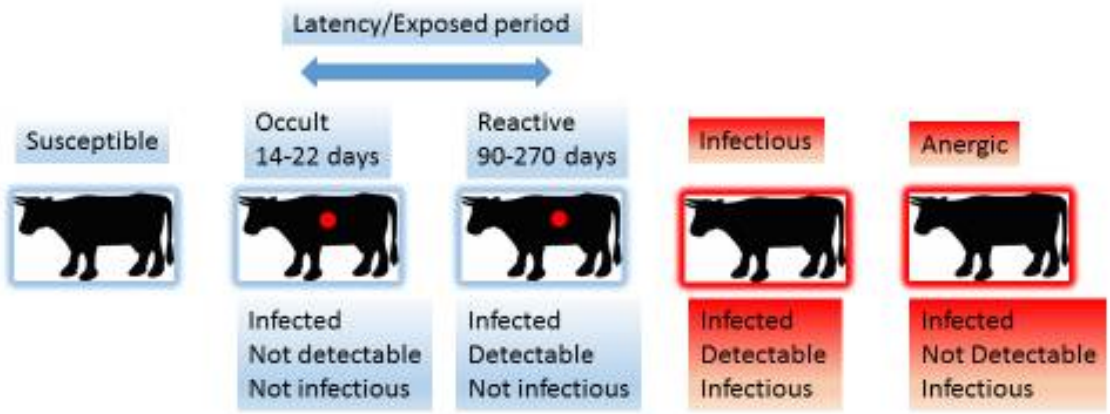
stages of the animal (nutrition, pregnancy, etc.) or other infections that might compromise the immune system (Barlow et al., 1997).

The infectious state (I) will start when the animal, besides reacting to the intra-dermal tuberculin test or the gamma-interferon assay, is capable of infecting other animals. The animal will remain in this stage until is detected and removed from the herd. However, if the animal is not detected and remains infected for a long time, it may enter in a state of depressed cell-mediated immune response that is called 'anergy'. In this state the animal will continue spreading the disease to other animals, but will not react to the intra-dermal tuberculin test or the gamma-interferon assay. In contrast, it may develop a higher antibody response (De la Rua-Domenech et al., 2006).

d. Within-herd transmission dynamics

When bTB enters the herd, the transmission of infection between animals is generally considered to be a relatively slow process (Menzies et al., 2000). For instance, Alvarez et al. (2012) reviewed the transmission rates reported in the literature form the las decade. For instance, the reported values of β, which is the average number of individuals that are newly infected from an infectious individual per unit of time, ranged between 0.02 and 5.2 (from 9 studies). Therefore, dynamics of bTB transmission might depend on different factors such as management systems, animal density or herd size (Barlow et al., 1997; Perez et al., 2002; Alvarez et al., 2012; Conlan et al., 2012).

Figure 4. Transition stages of bTB infection and the infectious status and responsiveness to in vivo diagnostic tests, as the disease progresses through time (Adapted from Barlow et al., 1997).



Section 3. Eradication of bovine Tuberculosis

I. bTB Surveillance components

The surveillance of bTB is challenging, due to its underlying complex epidemiology, which involves multiple hosts in domestic and wild populations. Therefore, adaptations to EU legislation were required to deal with the heterogeneity of epidemiological situations, which explain the various surveillance systems implemented (Riviere et al., 2014).

Test and slaughter policy and/or abattoir surveillance are the main tools for the control and eradication of bTB. Other strategies like whole herd depopulation is not so frequent for economic reasons and due to animal welfare concerns (Schiller et al., 2011).

On the other hand, due to the increasing of live animal trade, both at national and international level, pre-movement testing is becoming increasingly important as a

central tool for eradication and for protection against reintroduction of bTB (Schiller et al., 2011). Moreover, as proposed by international organizations (Anonymous, 2008), trade agreements increasingly utilize concepts like regionalisation, zoning, and compartmentalisation as principles of disease control.

Riviere et al. (2014) reviewed the different surveillance components implemented within the different EU Member States (MS), through an online survey (Table 1) with a total of 16, from the 26 participating countries. All the non-OTF MS and regionalized participating countries, except Macedonia (non-MS) Malta and Ireland, indicated that they applied all 3 recommended surveillance components by the EU which are: routine screening test in herds, pre-movement tests (to ensure safe trading between farms) and post-mortem examinations at the slaughterhouse.

Table 1. Surveillance components of bTB in cattle in EU Member States, Switzerland, Norway and Macedonia during 2013, by official country status (adapted from Riviere et al., 2014).

		OTF countries	*Regionalized countries*	*Non-OTF countries*
1 Surveillance component	Screening tests	Poland		
	Slaughterhouse	Germany, Latvia, Luxembourg, The Netherlands, Norway, Sweden		
2 Surveillance components	Screening tests, Pre-movement tests			Macedonia
	Screening tests, Slaughterhouse	Slovakia, Slovenia		Ireland, Malta
	Pre-movement tests, Slaughterhouse	Austria, Belgium, Finland, Switzerland		
3 Surveillance components	Screening tests, Pre-movement tests, Slaughterhouse	Czech Republic, Estonia, France	Italy, Portugal, United Kingdom	Croatia, Cyprus, Romania, Spain

II. Eradication program in Spain

In Spain, the first official bTB testing was performed in 1950 in a dairy herd in the north of Spain, but it was not until 1993 when most dairy and beef herds were included in the national control program. During the application of this program, mainly based on IDT testing and culling of reactor animals, the cattle herd prevalence decreased from 5.9% in 1993 to 1.72% by the end of 2014 (Anonymous, 2014).

The Spanish national bTB eradication program is compulsory for all bovines as defined in the Directive 64/432/EEC, incorporated into the domestic legal system by RD 2611/1996 and RD 1716/2000, which establish the national programs for ruminant diseases eradication, and the health standards for the intra-community exchange of animals.

In accordance with the requirements laid down in the Community legislation, Directives 97/12/EC and 98/46/EC, and national legislation (RD 51/2004), herds are qualified according to their disease status in one of the following categories:

T3 = officially bTB free herds (at least two consecutive negative tests).

T2- = Last test was negative, but the previous one was positive.

T2+ = herds with one or more positive cattle in the last test.

T1 = herds with unknown status.

Between 60 and 90 days after the detection of bTB positive animals in a herd, a second test must be carried out. If all animals tested are negative, the herd achieves the T2- status, and the officially free status (i.e., T3) will be reached if all animals are also

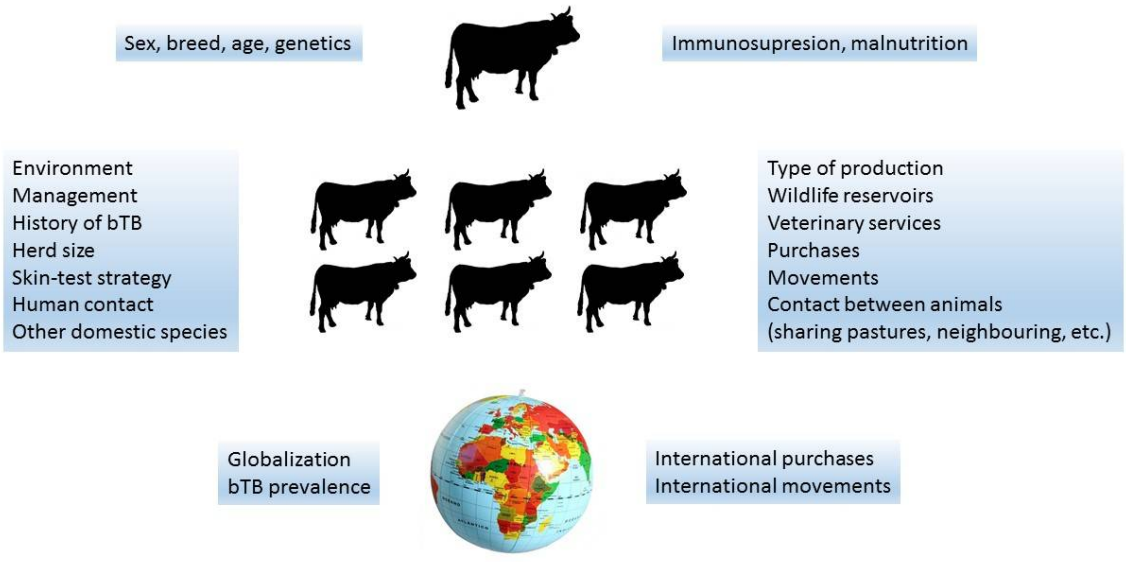
negative in the next bTB test.

Once the herd reached the T3 status, depending on the prevalence of the belonging Autonomous Community (AC), it will be tested with a required frequency. For instance, if the AC prevalence is <1% the herd will be tested at least once a year by IDT. If the AC prevalence is >1%, the herd will be tested two times per year.

III. Factors that difficult the bTB eradication

Due to the political-economic situation and other factors like farming practices, pasturing systems and contacts between animals, the difficulties to eradicate the disease differ substantially in developed and developing countries. For instance, Humblet et al. (2009) reviewed the most important worldwide risk factors of bTB infection. In Figure 5, adapted from Humblet et al. (2009), are summarized the main bTB risk factors classified into three levels: animal, herd and region or country:

Figure 5. bTB risk factors divided first at animal level, secondly at herd level and lastly at global level.



In countries where bTB eradication programs are established, different factors such as the cattle movement behaviour (i.e. trading, sharing pastures, etc.), contact between infected neighbouring farms, bTB historic cases (i.e. residual infection) in previous years, interactions with potential wildlife reservoirs or other domestic species, environmental persistence of the mycobacteria due to favourable climatic conditions, limited sensitivity of the skin test or herd size might hinder the eradication of the disease, (Humblet et al., 2009; Skuce et al. 2012).

In Spain, different studies have also been performed in order to assess factors that compromise the progress of the eradication of the disease. For instance, Guta et al., (2014a) analysed the most likely cause of 687 bTB herd breakdowns, concluding that residual infection followed by interaction with wildlife reservoirs were the most frequent causes of bTB breakdowns. The high percentage of residual infection might be the result of a combination of different risk factors such as lack of sensitivity of the ante-mortem diagnostic tests, difficulties in the testing of all the animals due to the fact that animals are reared in large pasture areas in extensive systems or lack of good veterinary practice. Other studies performed in different regions of Spain have identified other risk factors for bTB like the management in large pasture areas, the presence of bTB infected neighbours (Guta et al., 2014b), the herd size and animal density (Alvarez et al., 2012), high bTB prevalence in wild boar and red deer and type of production (dairy, beef and bullfighting) (Rodriguez-Prieto et al., 2012).

Chapter 2: Objectives

The general aim of this PhD Thesis was to assess the evolution of the bTB eradication campaign in Spain and to identify factors that might hamper the eradication of the disease. This PhD aimed also to provide relevant data for the implementation of a risk based surveillance system in order to optimize resources. In order to accomplish this general aim several specific objectives were developed through the PhD:

1) To develop a model useful to analyze the space-time variation in the risk of bTB in Spain and to identify factors related with this pattern.
2) To evaluate a risk-based approach of bTB surveillance using routinely recorded data of the eradication campaign.
3) To estimate the slaughterhouse surveillance sensitivity for bTB.
4) To estimate parameters related with bTB transmission within a herd.

Chapter 3: STUDY I

Spatio-temporal variability of bovine tuberculosis eradication in Spain (2006–2011)

Introduction

Bovine tuberculosis (bTB) is a chronic infectious disease of cattle (including all *Bos* species, and *Bubalus bubalus*) and bison (*Bison bison*) caused by any of the disease-causing mycobacterial species within the *Mycobacterium tuberculosis*-complex (Anonymous, 2013a). Cattle are mainly affected by *M. bovis* and *M. caprae* which can also affect other domestic and wild animals as well as humans (Aranaz et al., 2004; De la Rua-Domenech et al., 2006). Due to its zoonotic nature and the high economic impact in livestock production, eradication of bTB in the EU is the final target (Reviriego and Vermeersch, 2006) through the development of bTB eradication programs.

In Spain, the first official bTB testing was performed in 1950 in a dairy herd in the north of Spain, but it was not until 1993 when most dairy and beef herds were included in the national control program. During the application of this program, mainly based on intradermal tuberculin testing (IDT) and culling of reactor animals, the cattle herd prevalence has decreased from 5.9% in 1993 to 1.3% by the end of 2011 (Anonymous, 2012b). Despite that important progress, during the last 12 years, the herd prevalence in Spain has only declined from 2.5% in 2000 to 1.3% in 2011, and the herd incidence has been fluctuating between 0.8 and 1.0%.

Spatio-temporal disease mapping models are a useful tool to describe the pattern of diseases and to identify regions with unusual levels of disease, time trends or both (Meliker and Sloan, 2011; Schrödle and Held, 2011). Moreover, space-time models can contribute to the assessment of the stability of the risk of infection, which cannot be evaluated just by spatial models (Abellan et al., 2008). When analyzing the observed counts of disease within different areas for a sequence of time periods, four groups of components can be considered (Lawson et al., 2003): i) the spatial structured component: due to area-specific risk factors such as contact with infected wildlife, ii) the unstructured spatial component: due to characteristics of the farms within the counties (e.g. herd size or livestock rearing practices), iii) the temporal component: due to time risk factors such as national changes in the national eradication program, and finally iv) the space-time component: due to time-area specific risk factors, such as changes between years in the counties due to new personnel, diagnostic procedures or movements of animals. These different levels of variability can be accounted for by the use of multilevel (i.e., hierarchical) models (Beale et al., 2008). Moreover, the decomposition of the risk variability by using different (and appropriate) random effects allows the formulation of hypotheses about the role of factors potentially related to the risk of infection at different levels.

In a previous analysis of the variability of the geographical risk of bTB infection across Spain, it was evidenced that counties located in the central and south of Spain had a risk more than three times higher than the rest of the country (Allepuz et al., 2011). However, in that study the temporal evolution of the disease across areas was not assessed. Furthermore, specific explanatory variables were not included in the model so their relationship with the risk of bTB could not be quantified.

The objective of the present work was to analyze the space-time variation in the risk of bTB in Spanish cattle between 2006 and 2011, and to identify factors related with the variability of the risk of infection.

Materials and Methods

1. Data management

For the period 2006–2011, annual data at county level, of the total number of herds stratified by type (dairy, beef or fighting bulls), disease status (i.e., herds that became positive/new positive), number of cattle-goat mixed herds, number of goat farms and animal movements, were provided by the Spanish Ministry of Agriculture, Food and Environment (MAGRAMA).

The abundance of red deer (*Cervus elaphus*) in Spain was obtained from Acevedo et al. (2010) at Universal Transverse Mercator (UTM) 10x10 km^2 grid cells. This variable was aggregated at county level by calculating the average abundance of the different cells that intersected with the county. This data aggregation was carried out through Quantum GIS (Quantum GIS Development Team, 2012) by joining the attributes based on their spatial location.

For each year, three variables related to animal movement were created by using R version 2.15.1, (R Development Core Team, 2008) and the 'statnet' package (Handcock et al., 2003): i) in-degree, i.e., the number of contacts that a given county received from other counties ii) the number of movements to a given county (including intra-county movements), and iii) the number of animals moved to a given county (including animals from intra-county herds).

In addition, for each of these movement-related variables, a differentiation was made between high risk movements (e.g., in-degree from counties with an incidence >1%),

and low risk movements (e.g., in-degree from counties with an incidence ≤1%), resulting in a total of six explanatory variables.

Even though all information was provided on an annual basis, all explanatory variables were included into the model as fixed effects, which imply that the value of each of these variables remained the same during the years.

Moreover, in order to avoid problems derived from non-linear relationships with the dependent variable and the possible concurvity between explanatory variables and random factors (Saez M.; Personal communication), all explanatory variables were categorized before including them in the model. For this purpose, a reclassification into four categories following their quartile distribution was carried out. In the cases of red deer abundance and number of mixed cattle-goat farms, as their first quartile was zero, they were also divided into four categories, but the first one included all the zeroes, and the remaining values were divided into terciles. In addition, as half of the values of bullfighting cattle were zeroes, this variable was included as dichotomous (absence and presence). In table 1 are showed the distribution of the explanatory variables after the categorization.

Table 1. Distribution of the covariates included in the model. Dairy, beef, and goat herds together with number of movements were categorized in quartiles. Red deer abundance and mixed cattle-goat farms were categorized in terciles including all zeros in the first category and bullfighting herds were divided in presence or absence.

Distribution of explanatory variables at county level				
Explanatory variables	**Category 1**	**Category 2**	**Category 3**	**Category 4**
Dairy herds	0.0-1.0	1.0-5.8	5.8-31.4	31.4-1698.0
Beef herds	0.0-8.1	8.1-44.2	44.2-249.2	249.2-2601.2
Goat herds	0.0-596.5	0.0-1527.0	1527.0-4432.5	4432.5-224597.0
High risk movements	0.0-17.6	17.6-63.0	63.0-169.3	169.3-2624.6
Low risk movements	0.0-24.6	24.6-95.6	95.6-264.6	264.6-2624.6
Red deer abundance	0.0-0.0	0.0-5.5	5.5-8.2	8.2-14.1
Mixed cattle-goat herds	0.0-0.0	0.0-3.0	3.0-21.5	21.5-814.0
	Category 1	**Category 2**		
Bull herds	0.0-0.0	2.2-55.5		

Figures 1 and 2 show the spatial distribution of the categorized explanatory variables included in the model.

Figure 1. Geographical distribution of the explanatory variables in Spain during 2006. Categories of variables included in the model divided in quartiles: Beef herds, Dairy herds, Bull herds, Goat herds, Number of low and high risk movements. Category 1=first quartile, category 2=second quartile, category 3=third quartile and category 4=fourth quartile. Bull herds are divided in Category 1=presence and 2=absence. Canary Islands are represented in the box underneath the map.

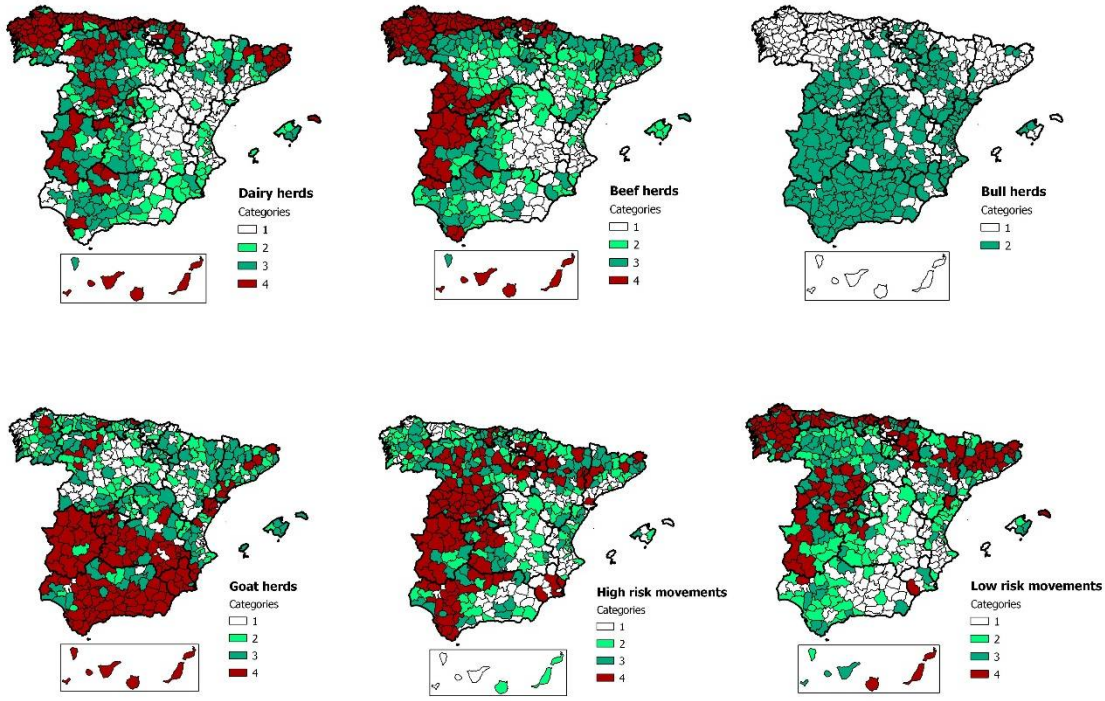
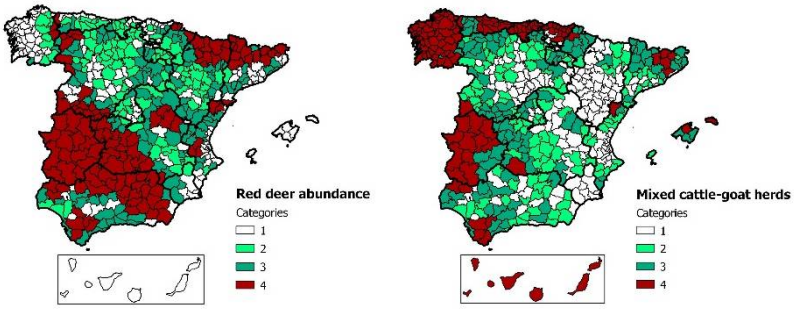


Figure 2. Geographical distribution of the explanatory variables in Spain during 2006. Categories of variables included in the model divided in terciles: Red deer abundance and mixed cattle-goat farms. Category 1=0 values, category 2=first tercile, category 3=second tercile and category 4= third tercile.

Canary Islands are represented in the box underneath the map.



2. Model specification:

We assumed that the total and the new bTB positive herds at county level followed a Poisson distribution centered on λ_{ij} and γ_{ij}, respectively, being *i* the county (i.e., *i*=1 to 483) and *j* the year (i.e., *j*=2006 to 2011):

$$\lambda_{ij} = P_{ij} \, x \, H_{ij}$$

$$\gamma_{ij} = I_{ij} \, x \, H_{ij}$$

Where, P_{ij} and I_{ij} are the prevalence and incidence respectively, and H_{ij} the total number of herds, in each county and year.

Due to the hierarchical structure of the data (i.e., space, time and space-time), a generalized linear mixed model (GLMM) approach was implemented (Zuur et al., 2009).

Within the Poisson regression and GLMM context, $\hat{P}_{ij}$ and $\hat{I}_{ij}$ were parameterized as a function of random and fixed effects:

$$\hat{P}_{ij} \text{ or } \hat{I}_{ij} = \exp(\beta_0 + S_i + eta_i + g_j + psi_{i,j} + Z\beta)$$

Where, β_0 represents the intercept, S_i was the structured spatial random effect for the spatial dependence between counties, which was defined by a stochastic partial differential equation (SPDE) (Lindgren et al., 2011), and eta_i was the unstructured spatial component. The temporal component g_j and the space-time interaction $psi_{i,j}$ random effects were defined by an autoregressive model of order 1. Finally, $Z\beta$ represented the matrix of explanatory variables.

The SPDE was calculated from a matrix of Euclidean distances between centroids of each county using Delaunay triangulation (Simpson et al., 2011; Cameletti et al., 2012). The relative risks of the prevalence (RRP) and incidence (RRI) across the country were calculated dividing the estimated prevalence ($\hat{P}_{ij}$) or incidence ($\hat{I}_{ij}$) in a given county and year, by the national prevalence or global incidence in that year, respectively.

As a first step bTB prevalence and incidence were modelled just in terms of random effects. Explanatory variables were added to this model one at a time by the following procedure: in a first step, the model with random effects and just one fixed effect at a time were evaluated. Then the resulting model with the lowest Deviance Information Criterion (DIC) value was selected. Secondly, all possible combinations of the first selected model, plus a new variable were analyzed, and the model with the lowest DIC value was selected. This process was repeated by adding new explanatory variables until the final model (i.e., with the lowest DIC value) was achieved (Spiegelhalter et al., 2003; Held et al., 2010).

To assess the statistical significance of the variables in the model, 95% credible intervals (CR) were obtained from the exponential of the posterior probability distribution. We considered that a relation was significant if the probability of association was over 95%: i.e., if the 95% CR was greater or lower than 1. If greater, the variable increases the risk of bTB infection, and if lower it decreases the risk.

All statistical analyses were performed using R-INLA package (Schrödle and Held, 2011) to avoid the algorithm problems of convergence and mixing of the Markov Chain Monte Carlo (MCMC) based sampling methods (Bisanzio et al., 2011).

In this context we used the default specification of R-INLA for the distribution of the hyper-parameters (i.e., the prior distribution of the random effects precision) that is a logGamma (1, 0.0005) (Blangiardo et al., 2013).

Within R-INLA, the marginal variance for the random effects (mean and standard deviation) can be obtained by applying the "marginals.hyperpar" function implemented in the program. However, in the case of the spatial structured random effect, it is only the variance that is obtained, and therefore they are not comparable. In order to make them comparable we empirically estimated the marginal variance of the spatially structured component following the procedure described in Blangiardo et al. (2013).

Results

1. Descriptive results

The number of positive and new positive herds by year and type of herd are presented in Tables 2 and 3. Moreover, maps of the geographical distribution of prevalence and incidence during the studied period are shown in Figures 3 and 4.

Table 2. Bovine tuberculosis (bTB) positive herds (Pos), number of herds (Herds) and percentage of bTB positive herds (%), between 2006 and 2011.

Year	Positive herds									Total		
	Beef			Dairy			Bullfighting					
	Pos	Herds	%	Pos	Herds	%	Pos	Herds	%	Pos	Herds	%
2006	2108	105,164	2.0	203	30,568	0.7	98	1184	8.3	2411	136,916	1.8
2007	1809	99,236	1.8	199	29,012	0.7	120	1815	6.6	2128	130,063	1.6
2008	1703	97,067	1.8	173	26,454	0.7	108	1220	8.9	1984	124,741	1.6
2009	1683	92,684	1.8	159	25,767	0.6	128	1211	10.6	1971	119,662	1.6
2010	1504	90,174	1.7	123	25,012	0.5	128	1209	10.6	1759	116,395	1.5
2011	1304	86,083	1.5	97	24,219	0.4	84	1153	7.3	1488	111,455	1.3

Table 3. Bovine tuberculosis (bTB) new positive herds (Pos), number of herds (Herds) and percentage of bTB new positive herds (%), between 2006 and 2011.

Year	New positive herds									Total		
	Beef			Dairy			Bullfighting					
	Pos	Herds	%	Pos	Herds	%	Pos	Herds	%	Pos	Herds	%
2006	987	105,164	0.9	136	30,568	0.4	43	1184	3.6	1166	136,916	0.9
2007	1126	99,236	1.1	160	29,012	0.6	91	1815	5.0	1377	130,063	1.1
2008	950	97,067	1.0	122	26,454	0.5	63	1220	5.2	1135	124,741	0.9
2009	1039	92,684	1.1	119	25,767	0.5	73	1211	6.0	1231	119,662	1.0
2010	837	90,174	0.9	96	25,012	0.4	57	1209	4.7	990	116,395	0.9
2011	822	86,083	1.0	74	24,219	0.3	42	1153	3.6	938	111,455	0.8

For all the years of study, dairy cattle had the lowest percentage of positive and new positive herds, while fighting bulls had the highest proportion. Approximately 50% of the positive herds each year were new positives.

Figure 3. Geographical distribution of bTB prevalence in Spain at county level between 2006 and 2011. Counties in grey colour were not tested on that year.

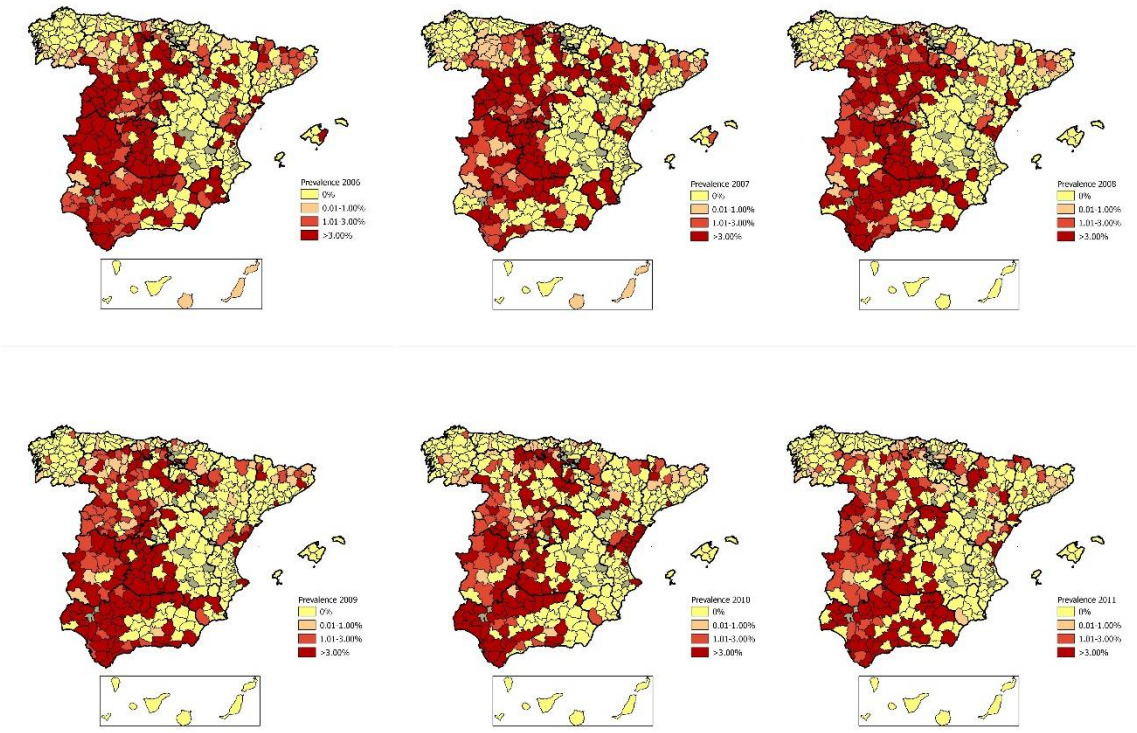
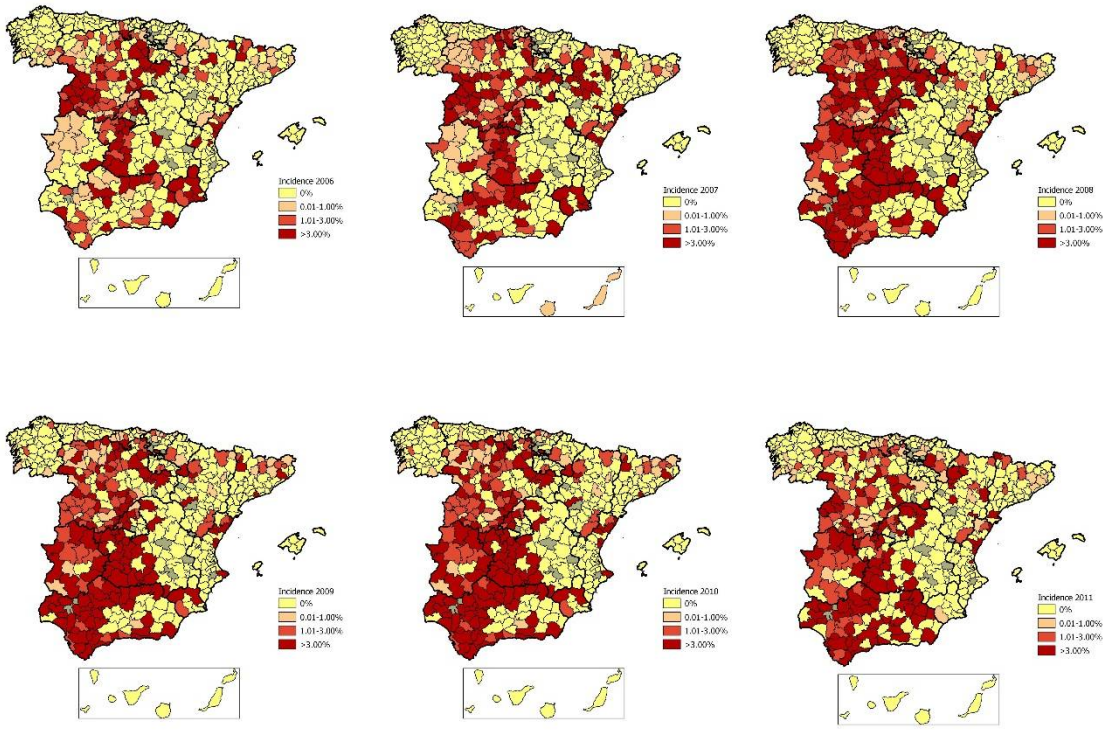


Figure 4. Geographical distribution of bTB incidence in Spain at county level between 2006 and 2011. Counties in grey colour were not tested on that year.



2. Space-time model results

The 95% CR for the temporal random effect included the number 1 in all the years, indicating that in Spain as a whole, there were no significant changes between consecutive years in the risk of becoming infected or newly infected. Even though at country level there were no significant temporal changes, at county level it was more heterogeneous. For some years, some counties presented a significant increase (or decrease), in the risk of being infected or newly infected, compared to the temporal changes of all the counties in Spain (Figures 5 and 6, respectively).

Figure 5. Geographical distributions in Spain, at county level, of the space-time interaction of bTB prevalence (2006-2011). Space-time interactions (Int) of bTB prevalence (P) between the years of study, as compared with the year before: Int P07-06 to Int P11-10. Counties in blue colours represent a significant decrease of the prevalence (interaction coefficient <1) and counties in red colours represent a significant increase of the prevalence (interaction coefficient >1). Counties in white colour did not show a significant space-time interaction. Canary Islands are represented in the box underneath the map.

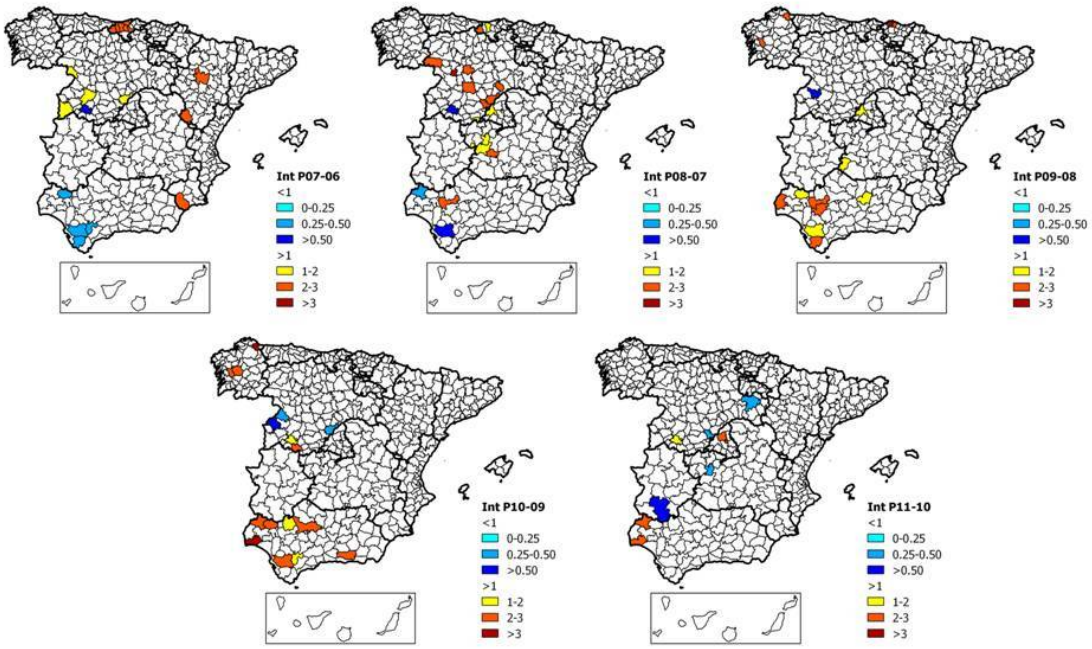
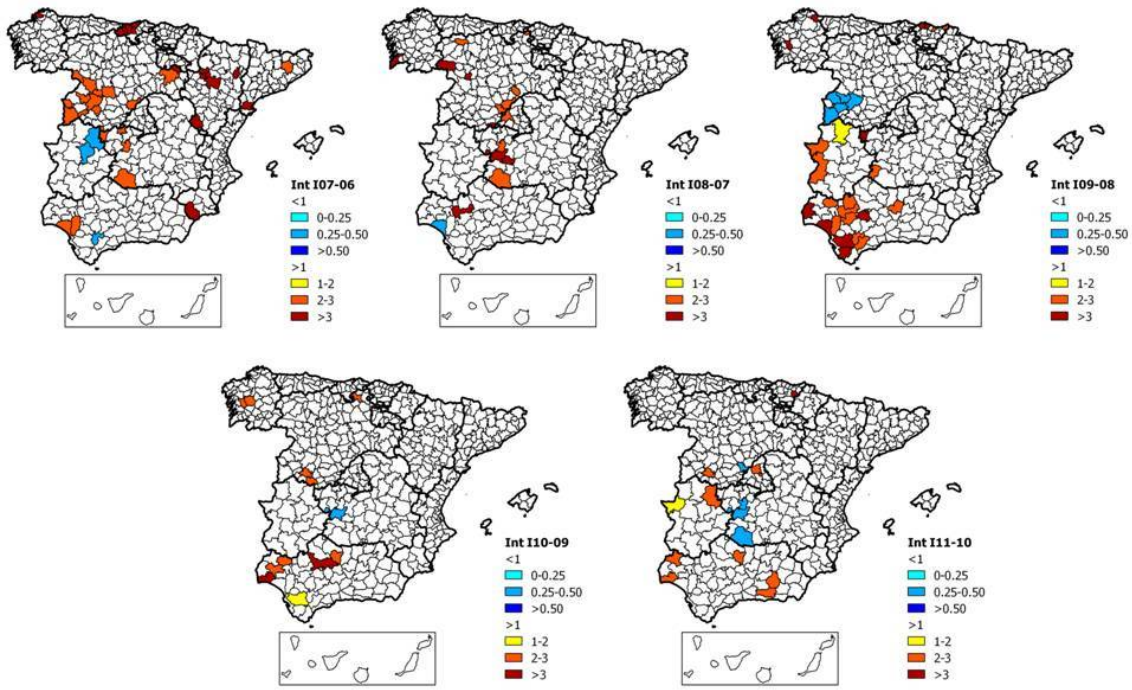


Figure 6. Geographical distributions in Spain, at county level, of the space-time interaction of bTB incidence (2006-2011) in Spain. Space-time interactions (Int) of bTB incidence (I) between the years of study, as compared with the year before: Int I07-06 to Int I11-10. Counties in blue colours represent a significant decrease of the incidence (interaction coefficient <1) and counties in red colours represent a significant increase of the incidence (interaction coefficient >1). Counties in white colour did not show a significant space-time interaction. Canary Islands are represented in the box underneath the map.



The final models for prevalence and incidence included 2 and 3 variables respectively (Table 4). The number of movements included on each contact from counties of high risk (incidence >1%) and presence of bullfighting cattle herds had a positive relation with both the high risk of positives and the high risk of new positives. Specifically, we found that herds located in counties with the highest number of risk movements

(categories 3 and 4) had a high risk, between 1.5 and 1.7 times higher, compared to those counties with the lowest number of risk movements (category 1) for prevalence and incidence, respectively. Moreover, counties with presence of bullfighting herds had a prevalence and incidence between 1.6 and 1.3 higher respectively as compared with counties with no bullfighting herds. On the other hand, the abundance of red deer improved the model for new infections by means of a decrease of the DIC, explaining more proportion of the risk heterogeneity; even though the relationship was not statistically significant.

The relative risks of the prevalence (RRP) and incidence (RRI) in Spain, throughout the years of study, are represented in Figures 7 and 8. The results of the model showed that the RRP and RRI were higher in counties located in central and southern Spain, throughout the whole period of study.

Table 4. Fixed and random effects included in the final model, statistical coefficients and their standard deviations (SD) and 95% credible intervals. Coefficients are relative to the lowest values (categories 1). Significant categories are marked with an (*). S and eta: structured and unstructured spatial random effects respectively; g: temporal random effect; psi: space-time random effect.

Model	Variable	Category	Coefficient	SD	Credible interval 2.5%	Credible interval 97.5%
Space-time prevalence	High risk movements	2	1.07	1.16	0.79	1.46
		3*	1.71	1.17	1.25	2.33
		4*	1.66	1.18	1.19	2.33
	Bullfighting	2*	1.56	1.14	1.19	2.05
	S		1.16	0.36		
	eta		0.63	0.05		
	g		0.04	0.01		
	psi		0.51	0.02		
Space-time incidence	High risk movements	2	1.03	1.15	0.78	1.38
		3*	1.67	1.15	1.25	2.23
		4*	1.58	1.17	1.16	2.16
	Bullfighting	2*	1.33	1.13	1.05	1.70
	Red deer abundance	2	0.92	1.14	0.71	1.20
		3	1.11	1.15	0.84	1.46
		4	0.95	1.18	0.69	1.32
	S		1.08	0.34		
	eta		0.45	0.08		
	g		0.01	0.01		
	psi		0.72	0.03		

Figure 7. Geographical distributions in Spain, at county level, of the relative risks of bTB prevalence (2006-2011) in Spain. Relative Risks (RR) of bTB prevalence (P) between 2006 and 2011 (P06 to P11). Canary Islands are represented in the box underneath the map.

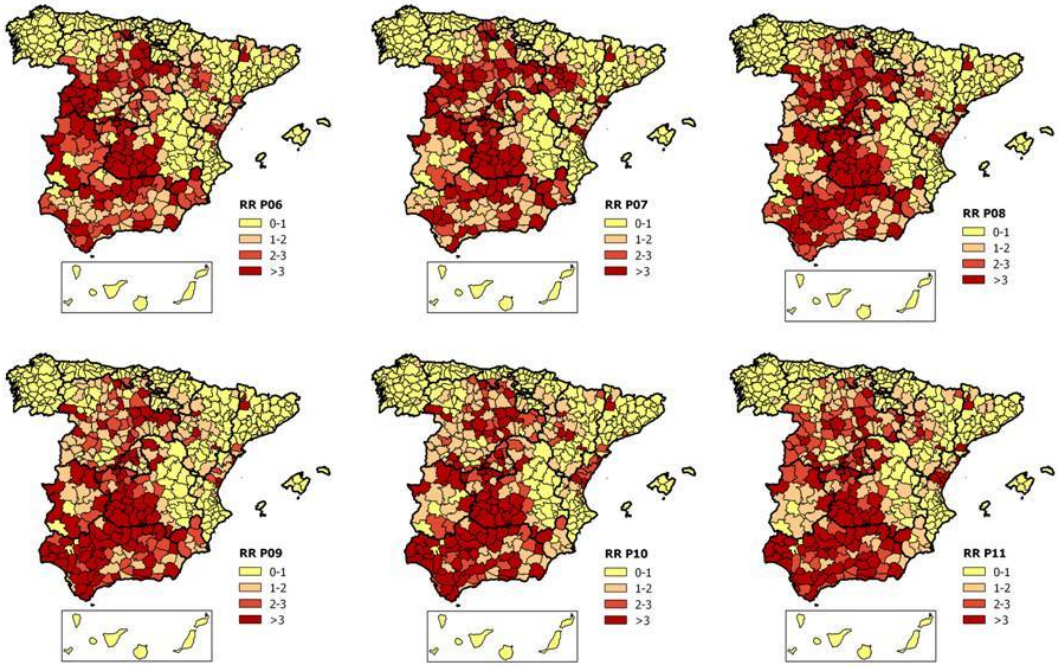
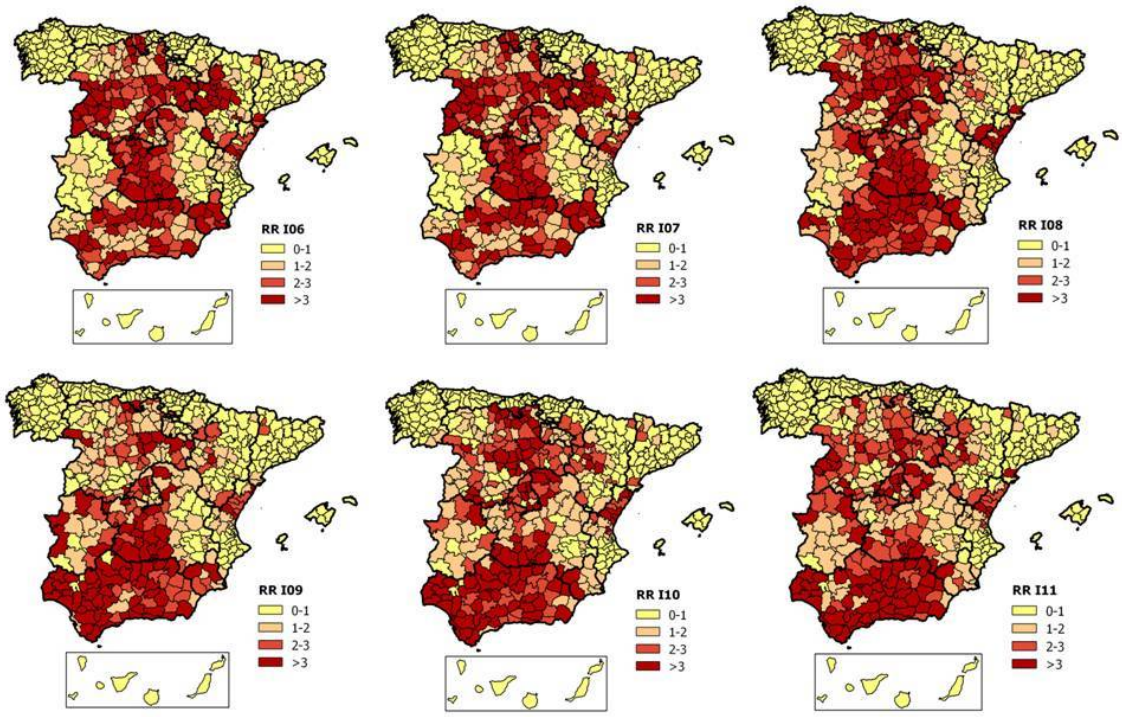


Figure 8. Geographical distributions in Spain, at county level, of the relative risks of bTB incidence (2006-2011) in Spain. Relative Risks (RR) of bTB incidence (I) between 2006 and 2011 (I06 to I11). Canary Islands are represented in the box underneath the map.



Discussion

Our results showed that nationally there were no statistically significant changes in bTB prevalence or incidence between any of the years of study. However, the result of the temporal random effect in our models has to be interpreted based on its specification (i.e., autoregressive of order 1). Therefore, the random effect indicates the increase or decrease in the probability of disease compared with the previous year, and not the temporal trend for the whole period of study. The temporal trend in herd prevalence has been reported to decrease significantly, from 1.8% in 2006 to 1.3% in 2011 (Anonymous, 2011a). Therefore, both results are not necessarily contradictory, as there may be no significant improvements in the bTB eradication campaign between consecutive years, but a significant temporal change between 2006 and 2011. At county level, the evolution has been much more heterogeneous, as some counties presented significant space-time interactions. This result indicates an increase or a decrease in the risk in that area in that specific year that might be related with the presence of short-latency underlying factors (i.e. not occurring in a regular manner over time) (Abellan et al., 2008). It is difficult to have reliable information about which short-latency uncontrolled factors could be responsible for those significant space-time interactions, but we speculate that factors such as changes in the personnel in charge of the implementation of the diagnostic tests, intensification of controls, or the application of immediate depopulation of infected herds, may be related to them.

Our results indicate that movements of animals from counties with high incidence (>1%) were positively associated with increased prevalence/incidence. This is in agreement with studies conducted in the United Kingdom, where movements of animals were identified as a significant risk factor for bTB (Gilbert et al., 2005; Gopal et

al., 2006; Green et al., 2008; Bessell et al., 2012a). Data on movements used in our analyses included different types of movements: to shared grasslands, transhumance, to livestock markets and to other farms. According to the Spanish eradication programme, and with the exception of some movements which are considered of low risk (e.g. to slaughterhouses), animals are subjected to pre or post-movement controls (Anonymous, 2012a). However, because of the limited sensitivity (52-100%) of the IDT test (De la Rua-Domenech et al., 2006), or because the animals may be within a pre-allergic, or anergic state (Álvarez et al., 2012), controls may fail resulting in the spread the disease to other farms.

The results indicated that the risk of a herd being infected or newly infected was higher in counties which had bullfighting herds. The type of production, in particular fighting bulls, has been described as a potential risk factor for bTB infection in Spain by other authors (Anonymous, 2011a; Allepuz et al., 2011; Boadella et al., 2011; Rodríguez-Prieto et al., 2012). Fighting bulls are difficult to handle because of their vigor and the fact that they are managed in extensive areas with difficult access, which implies that some of the animals of the herd may not be tested (Aranaz et al., 2006; Rodríguez-Prieto, 2012). Moreover, according to the Spanish Royal Decree (RD 1939/2004) bullfighting herds were introduced gradually into the eradication program context since 2004. These reasons may explain why the presence of bullfighting herds increases the risk of a herd becoming infected or newly infected. In fact, to tackle this problem, a new legislation (RD 186/2011), which reinforces the controls in bullfighting herds, has been introduced in 2011.

Our results showed a positive association, although not significant, between the red deer abundance and the risk of new infections. The possible role of wild animals,

mainly wild boar (*Sus scrofa*) and red deer, as reservoirs of bTB in Spain, has been suggested in different studies (Aranaz et al., 2004; Vicente et al., 2006; Naranjo et al., 2008; Gortázar et al., 2011; Rodríguez-Prieto, 2012). In central and southern Spain, high prevalences are reported in these species, particularly in areas of high density (Acevedo et al., 2007, 2008; Gortázar et al., 2008; Boadella et al., 2011; Castillo et al., 2011; García-Bocanegra et al., 2012). Molecular typing has shown that different wildlife species such as red deer, fallow deer (*Dama dama*) and wild boar are infected with the same *M. bovis* spoligotypes as cattle, and that they may be maintained in the same area over time, even in the absence of contact with domestic ruminants (Aranaz et al., 1996; Gortázar et al., 2005; Parra et al., 2005; Hermoso de Mendoza et al., 2006; Naranjo et al., 2008; Romero et al., 2008; Rodríguez et al., 2009). As only data for red deer abundance was available for the whole Spain, this was the species whose role was assessed. However, as wild boar and red deer have similar ecological requirements (Acevedo et al., 2011), their distributions are highly correlated, and therefore red deer abundance may be considered as a proxy measure of wild ungulates abundance.

Even though there is no official data on the prevalence of caprine tuberculosis, the disease is considered to be endemic in Spain (Liébana et al., 1998), and therefore the presence of goats has also been suggested as a potential risk for bTB (Álvarez et al., 2008; Rodríguez-Prieto et al., 2012). In a recent study, the fact that goat herds infected with tuberculosis may pose a threat to neighboring bovine herds was found (Napp et al., 2013). Nevertheless, our results did not show any significant association between number of goat herds or mixed cattle-goat farms and the risk of a herd being infected or newly infected.

In the interpretation of the variables included in the space-time model, it is important to keep in mind the spatial scale at which the analyses were done, as the relationship between the variables may change with the selection of different areal units (Meliker and Sloan, 2011). While models which use aggregated data are useful to identify broad-scale spatio-temporal trends, they hide farm level heterogeneity, a phenomenon known as the ecological fallacy. Therefore, relations found at the aggregated level do not have to be the same at the individual level, and therefore, caution has to be taken when drawing conclusions from disease data summarized at the area level and should be corroborated by analysis at local scale.

The prevalence and the incidence of disease were the parameters used to evaluate the risk of bTB infection instead of the standardized mortality/morbidity ratio (SMR). SMR describes the odds of being in the disease group rather than the background group (Lawson et al., 2003) by calculating the ratio between the observed number of cases and the number that would have been expected in a standard population. It is very useful for disease mapping of aggregated (e.g. county) data (Pfeiffer, 2008), and it was previously used to describe the risk of bTB in Spain (Allepuz et al., 2011). However, as we developed a space-time model, aimed at comparing the risk of bTB from one year to the previous year, a ratio between prevalences or incidences seemed easier to interpret than a ratio between SMRs.

Regarding, the application of space-time models, there are a variety of formulations that have been proposed for the spatiotemporal analysis of the pattern of the risk of a disease, and it is not totally clear which one would be the most useful (Lawson et al., 2003). Different approaches, such as the use of mixed models (Held et al., 2005), different specifications for the random temporal and space-time temporal components

(Bernardinelli et al., 1995; Knorr-Held, 2000; Abellan et al., 2008; Martínez-Beneito et al., 2008) and the use of non-separable space-time interactions (Knorr-Held, 2000), have been proposed. In our model, the correlated and uncorrelated spatial components were defined as constant in time, and there were separate temporal and space-time interaction terms. Within this specification, an autoregressive prior distribution allowing a non-parametric temporal and space-time trend was used. We did not formally compare the performance of different space-time models specifications, as it was beyond the scope of this study. The model specification that we used has been reported to give a parsimonious representation of the space-time behavior in risk (Knorr-Held, 2000; Lawson et al., 2003), and therefore we believe it gives an accurate account of the variability of bTB across Spain during the study period. Space-time models have been usually solved by the use of Markov chain Monte Carlo (MCMC) algorithms. However, they are computationally expensive and may induce large errors in parameter estimates (Schrödle and Held, 2011). An alternative method to improve the approximation to the marginal posterior density for the hyperparameters, is the integrated nested Laplace approximations (INLA), recently proposed by Rue et al. (2009). The major advantage of this method is that it is computationally much faster than MCMC, returning precise parameter estimates (Blangiardo et al., 2013). The application of space-time models using INLA is becoming more common and, among others, has been used to assess the space-time evolution of bovine viral diarrhea eradication in Switzerland (Schrödle and Held, 2011), to predict the areas with the highest potential for West Nile Virus introduction and amplification in Italy (Bisanzio et al., 2011), or to estimate air quality also in Italy (Cameletti et al., 2013).

Chapter 4: STUDY II

Finding needles in a haystack: bTB surveillance in New Zealand

Introduction

In New Zealand the control of bovine tuberculosis (bTB) has concentrated on elimination of the major wildlife reservoir, the brushtail possum (*Trichosurus vulpecula*), and regular tuberculin skin testing of susceptible stock with subsequent slaughter of individuals that return a positive test. Pre-movement testing rules are applied in areas with a high-risk of wildlife disease. Additional control measures include inspection of carcasses processed through abattoirs and application of quarantine-like 'movement control' period for bTB-positive herds. To date, this strategy has been effective with a steady reduction in the period prevalence of bTB-positive herds since 1995. In 2013 the national herd-level period prevalence of bTB in New Zealand was 0.21% (Anonymous, 2014b).

The final stages of any disease eradication program present a number of challenges for animal health authorities. A common scenario is that a reduction in disease prevalence is associated with a corresponding reduction in the level of funding allocated for control efforts. A second common scenario is that as the prevalence of disease decreases herd managers begin to question the need for intensive testing procedures, particularly if their herds have been shown to be continuously free of infection. To mitigate the negative effects of these two 'threats' to a successful eradication program, it makes sense that in the latter phase of such programs, surveillance strategies are revised so that they specifically target those herds with the highest risk of infection.

In this paper we describe a risk-based approach for bTB surveillance in New Zealand. We acknowledge that the presence of bTB and the ability to detect bTB in a herd is driven by a number of factors including previous infection history, the amount of testing carried out, geographic location (as a reflection of wildlife risk) and herd movement behavior (herds receiving large numbers of stock from other herds are hypothesized to be at higher risk of bTB compared with those that receive no incoming movement events). This being the case, our objective is to use routinely recorded data to derive a herd-specific 'risk score' for each of these factors and then to combine them to return a composite bTB risk score for each herd which would be used to assign a test policy believed to be appropriate for that level of risk.

Materials and methods

The eligible population for this pilot study was New Zealand dairy herds that supplied milk for human consumption in January 2010. The study population comprised those members of the eligible population that used the herd testing facilities of the national dairy herd improvement authority, Livestock Improvement Corporation (LIC) whose details could be matched with bTB testing information held by TBfree New Zealand.

Details related to the following areas were retrieved for each herd from the TBfree and LIC databases: (1) previous infection history, (2) the results of herd-level bTB test activities carried out since 2001, (3) geographic location and (4) herd movement behaviour, as described below.

Previous infection history

Analyses of dates of detection and dates of herd clearance held by TBfree New Zealand, has shown that those herds with a history of bTB are at greater risk of

experiencing subsequent bTB breakdowns (Dawson et al., 2014). On the basis of these findings, it was reasoned that a history of previous herd breakdown from 2001 (set as a 0 or 1 flag for each herd) was an indicator of future infection risk.

Herd-level bTB testing

A scenario tree approach (Martin et al., 2007) was used to quantify the probability of a herd being free of bTB based on the amount and frequency of herd-level testing.

The estimated probability of a herd being bTB free was calculated using a two-step process. Firstly, the herd-level sensitivity of detection in year y , SeH_y was calculated as a function of an animal-level design prevalence P_A , a weighted average (by herd size) estimate of the sensitivity of detection for abattoir and herd testing Se , the number of animals tested in year y, n_y, and herd size in year y, N_y. The estimated number of bTB-positive animals in a herd in year y , dy was given by:

Equation 1: $dy = N_y \times P_A$

So

Equation 2: $SeH = 1-[1-Se \times (n_y \div N_y)]^{dy}$

The second step was to calculate the prior probability of freedom for a herd in year y , $Prior\ (PFree)_y$, which was a function of the probability of being bTB free in year $(y-1)$, $PFree_{y-1}$ and a user-defined estimate of the risk of bTB introduction into a given herd type in year y , $HerdRisk_{[herd\ type,y]}$. Estimates of the risk of bTB introduction were calculated using details of the number of herds of a given type at risk in a given year (as the denominator) and the number of herds detected as bTB positive during the same time frame (as the numerator).

Equation 3: $Prior(PFree)_y = 1- [(1-PFree_{y-1}) + HerdRisk_{[herd\ type,\ y]} – (1-PFree_{y-1}) x He$

With *SeH* and $Prior(PFree)_y$ known the probability of a herd being free of bTB in year *y* , $PFree_y$ was equal to:

Equation 4: $PFree_y = [(Prior(PFree)]_y ÷ [1-SeH x (1 – [Prior (PFree)]_y]$

For each herd, the prior probability of being bTB free in the first year of testing was 0.5. Our justification for using 0.5 was to acknowledge the fact that when TBFree New Zealand staff test a herd for the very first time they are doing so without any pre-conceived ideas about the presence or absence of bTB. This allows the accumulated test data to be the main driver of the posterior probability of freedom rather than the prior that has been set.

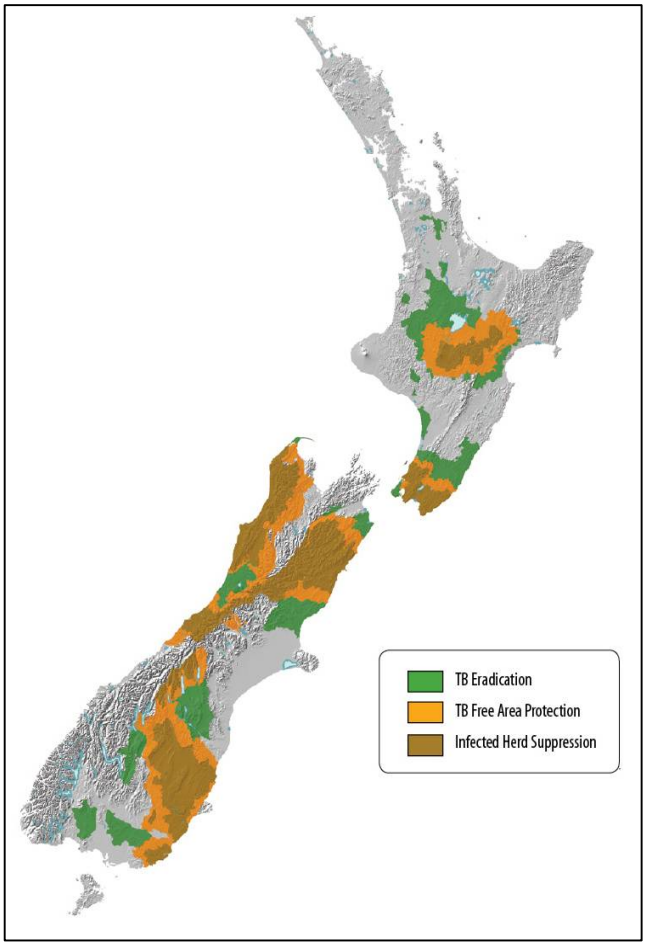
Details of herd bTB testing carried out over a number of years by TBfree New Zealand, accounting for the risk of re-introduction of infection were used to estimate the probability of each of the study herds being bTB free in 2013.

Geographic location

For the purpose of bTB control TBfree New Zealand divides the country into Vector Risk Areas. In 2010 there were multiple Vector Risk Areas throughout New Zealand separated by tracts of land where prolonged testing failed to identify established infection in wildlife populations, called Vector Free Areas. The prevalence of bTB is known to vary within Vector Risk Areas and, as a result, surveillance for bTB within a Vector Risk Areas is allowed to vary (Figure 1). Movement Control Areas are the highest risk category; where testing is carried out on all animals over 3 months of age every 12 months (annual testing) and prior to off-farm movement events. On the boundaries of Vector Risk Areas testing is carried out every 24 months (biennial

testing). Biennial Test Areas usually extend into neighbouring Vector Free Areas to enhance the ability to detect extension of disease beyond the current Vector Risk Areas boundaries. Herds in Vector Free Areas are tested every 36 months (Triennial Test Areas). Herds in defined dairying areas of the country comprise a special category. Prior to 2009 dairy herds in these areas were tested every 12 months. After 2009 the frequency of testing was reduced to every 24 months.

Figure 1. Territorial divisions for the bTB eradication campaign in New Zealand. In green, the triennial testing area of surveillance. In orange, the biennial special testing area. In brown, the vector risk areas with annual testing and pre-movement controls.



A study quantifying the risk of bTB infection in each of the defined risk areas for the period 2005 to 2010 (Stevenson., 2013) identified a progressive increase in the odds of bTB for herds in Dairy Test Areas, Biennial Test Areas, Annual Test Areas and Movement Control Areas, compared with herds in Triennial Test Areas. Based on these findings, a geographic risk score was assigned: Firstly, 0 for herds in Triennial Test Areas and then, 1, 2, 3, and 4 for herds in Dairy Test, Biennial Test, Annual Test and Movement Control Areas, respectively.

Herd movement behaviour

Although the main activity of Livestock Improvement Corporation (LIC) is the provision of herd recording facilities, an additional service is the MINDA system which allows herd managers to record the dates and details of calving, service, disease, treatment, and dry-off events for individual cows. An important feature of this facility is that when individual cows are transferred (i.e. moved) from one MINDA herd to another, the date and reason for the movement event are recorded in addition to their accumulated set of MINDA event records.

Details of herd-to-herd movement records of dairy cows for the period 1 July 2009 to 30 June 2010 (inclusive) were retrieved from LIC's MINDA system. LIC herd identifiers were then matched with herd details held by TBfree New Zealand. Social network analyses using the igraph package (Csardi and Nepusz, 2006) implemented in R (R Development Core Team, 2012) were carried out at the individual herd level. Here, each herd comprised the vertices (nodes) of a social network of herds and the recorded movement events that occurred between herds formed the edges (ties) between two nodes. Two forms of in-degree were calculated for each herd: unweighted and weighted. Unweighted in-degree enumerated the total number of

individual herds that sent cattle to a given destination herd. Weighted in-degree enumerated the total number of cattle received by a given destination herd.

Putting it all together

The analyses described above resulted in a five-column data frame listing, for each herd: (1) the unique herd identifier, (2) a 0 or 1 flag to indicate the absence or presence of a previous herd bTB breakdown event, (3) a number ranging from 0 to 1 representing the estimated probability of the herd being bTB free in 2013 based on herd-level testing, (4) an integer set to 0 for herds in Triennial Test Areas and 1, 2, 3, and 4 for herds in Dairy Test, Biennial Test, Annual Test and Movement Control Areas, respectively, and (5) a number representing the total number of cattle received by a given herd over the period 1 July 2012 to 30 June 2013 (weighted in-degree).

Our intention was to provide, based on the four risk measures described above, a composite herd-level score that would allow herds to be ranked in terms of their risk of being bTB positive. We stress that this derived measure does not provide an absolute estimate of herd-level bTB risk, simply a number used for the sole purpose of ranking herds.

To develop a composite herd-level risk score each of the component measures were expressed on a 0 to 1 scale. For the five levels of geographic location (0 = Triennial Test, 1 = Dairy Test, 2 = Biennial Test, 3 = Annual Test and 4 = Movement Control Areas) the levels 0.00, 0.25, 0.50, 0.75, and 1.00 were used. The weighted in-degree estimates for each herd were divided by the maximum weighted in-degree estimate for the entire data set.

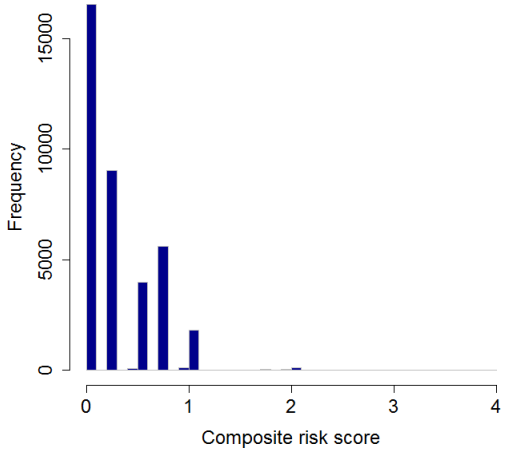
Each of the four risk score measures calculated for each herd were multiplied by equal weights and then summed to provide a composite risk score for each herd.

Results

The study population was comprised of 38,466 dairy herds. Of this group 16,898 were in Triennial Test Areas, 9258 were in Dairy Test Areas, 4214 were in Biennial Test Areas, 5757 in Annual Test Areas and 2099 in Movement Control Areas. It should be noted that the number of herds represented in this study was greater than the number of dairy farms in New Zealand (which was around 11,000 in 2010, Anonymous., 2011b) because of the common practice for up to 5 herd groups to managed as a single unit at a single geographic location.

The median composite risk score was 0.25 out of a maximum possible score of 4.0 (Q1 0.0002; Q3 0.5001). A frequency histogram of composite risk scores is shown in Figure 2.

Figure 2. Frequency histogram showing the distribution of bTB composite risk scores computed for New Zealand dairy herds in 2009-2010.



While the majority of herds had risk scores of less than 0.5 there were smaller numbers with relatively high scores of 1.5 or greater. While most of the 'high' composite risk score herds were located in Movement Control Areas (data not shown) a considerable number were located in other areas of the country. This supports the need for surveillance strategies to be based on objective estimates of risk, as opposed to simpler coarse criteria such as the geographical region in which the herd is located.

Discussion

The aim of this study was to describe a methodology to derive a composite bTB risk score for New Zealand cattle herds using details of previous infection history, individual animal bTB test details, the geographic location in which a herd is located and details of recent herd movement behaviour. While the input data may not provide a perfect indication of bTB risk, a key requirement was to use data routinely recorded for all herds. In doing so we believe this approach reduces the likelihood of serious selection of misclassification bias in risk score estimates both geographically and over time.

Ospri NZ Ltd currently hold historical testing data for all herds and historical information on wildlife risk but had no data on animal movement for all herd types until NAIT was initiated in 2012. However, the MINDA data set on movement between dairy herds was available and this allowed this pilot study to be conducted in this livestock production sector with sufficient input data (collected over a number of years) and allowed meaningful risk score estimates to be developed. A natural extension of this work is to extend eligible population to include beef and deer herds. As of March 2013 the National Animal Identification and Tracing System (NAIT,

Anonymous, 2012c) requires herd managers to declare on- and off-farm movements of livestock across the dairy, beef and deer sectors. A logical approach would be to use NAIT data for all three sectors to inform bTB risk score estimates in the future when this data is sufficiently comprehensive.

The idea of risk-based scoring methodologies is not new in animal health. For instance, during the latter stages of the bTB eradication campaign in Australia, risk management policies were applied mainly to increase the sensitivity of abattoir monitoring, through the National Granuloma Submission Program (Radunz., 2005). In Spain Guta et al. (2014a) adapted a risk-score approach using decision trees, and based on the results of an expert opinion workshop, assigned the most possible sources of infection for incident bTB-infected herds.

Previous studies of bTB carried out in other countries have identified issues that should be taken into account in the final stages of control and eradication programs. Recurrence of infection has been identified as the most important cause of infection of bTB in Spain (Guta et al., 2014a) and Ireland (Wolfe et al., 2010; Gallagher et al., 2013). In New Zealand the relative importance of recurrence as a determinant of incident herds has also been documented (Dawson et al., 2014). A number of explanations for bTB recrudescence have been proposed including the presence of false negatives to skin testing, as a consequence of imperfect test performance, anergy in animals being tested (De la Rua-Domenech et al., 2006) and errors in the application and interpretation of the caudal skin fold test (Humblet et al., 2009).

The effect of herd-to-herd movement of cattle as a risk for bTB in New Zealand dairy herds has been quantified using social network analyses, where Stevenson (2013) identified an association between weighted herd in-degree and bTB infection risk. This

agrees with studies conducted in the United Kingdom and Spain where movements of animals were identified as a significant risk factor for bTB infection (Gilbert et al., 2005; Green et al., 2008; Bessell et al., 2012a; Garcia-Saenz et al., 2014).

The derived risk score measure obtained in the present work does not provide an absolute estimate of herd-level bTB risk, simply a number used for the sole purpose of ranking herds. Acknowledging the idea that risk is not static and can change over time, an extension of this work will be to use expert opinion to assign appropriate weights to each of the four factors contributing to the overall risk estimate and then to assign the intensity of bTB testing to each herd accordingly. Consideration should also be given to how this system might be validated. One approach might be to select two study areas with equivalent bTB risk: routine surveillance would be applied in the first area and risk-based surveillance in the second. Over time incident bTB herds would be recorded (as usual) in addition to the total amount of money spent on surveillance. This would allow the amount of money spent to detect a bTB positive herd to be quantified and compared for the two surveillance approaches. Further work would be to compare the bTB detection cost per herd using the risk score estimates based on expert opinion weights with those computed using equal weights.

An additional improvement on this methodology would be to adopt the concept of a 'risk matrix' as opposed to estimation of a single bTB risk score for each herd. Here, herds with particular combinations of each of the four risk score estimates might be assigned a specific surveillance strategy. Management of wildlife disease identifies contract areas for planning wildlife control and recording results, TBFree New Zealand hopes in the future to utilise this information to better predict the risk of wildlife disease and use this as a more granular indicator of geographical risk.

In conclusion, the methodology proposed in this paper provides a numeric indicator of bTB risk in a given herd at a given time which contributes to the overall herd-level bTB risk.

Chapter 5: STUDY III

Estimation of the individual slaughterhouse surveillance sensitivity for bovine tuberculosis in Catalonia (North-Eastern Spain).

Introduction

Bovine tuberculosis (bTB) is a chronic infectious disease of cattle caused by any of the mycobacterial species within the Mycobacterium tuberculosis-complex (Anonymous, 2013a) and it is one of the biggest challenges facing the cattle farming industry in some Member States of the European Union (EU) (EFSA, 2013a). In the EU, countries are classified as Officially Tuberculosis Free (OTF) if they maintained the herd prevalence below 0.1% for a minimum period of 6 consecutive years, and as non-OTF otherwise (Council Directive 98/46/EC). In non-OTF countries such as Spain, surveillance of bTB is based on a) periodic testing of herds with tuberculin skin test and removal of reactor animals, b) pre-movement testing and c) meat inspection at the slaughterhouses (Council Directive 77/391/EEC).

When bTB eradication is achieved, the transition to a surveillance based only in meat inspection could be considered (EFSA, 2013b). However, OTF countries should continuously demonstrate their status, and therefore the sensitivity of the surveillance system should be high enough to substantiate that the bTB prevalence is below the required level. Besides, bTB infected herds should be detected early enough to prevent further dissemination to other herds. Therefore, the recognition of bTB during meat inspection is still a very important component for the surveillance and control of the disease, in either OTF or non-OTF countries (Domingo et al., 2014).

Some negative experiences have been reported in OTF countries that relied exclusively on meat inspection for bTB surveillance. For example, Fischer et al., (2005) questioned the efficiency of meat inspection to detect infected cattle early enough to limit spread to other herds after they had an epidemic in the Netherlands which affected 10 herds. On the other hand, in regions with low bTB herd prevalence, the achievement of the OTF status might be a likely option in the medium term and within this context, risk-based approaches could be an alternative surveillance strategy to the costly current strategy. However, before any change in the surveillance system may be contemplated, a reliable estimate of the sensitivity of the current surveillance system is needed. In this study, we focused on the slaughterhouse component, and the aim was to estimate the individual slaughterhouse surveillance sensitivity for bTB in Catalonia (North-Eastern Spain), where herd prevalence was 0.16% in 2014 (Anonymous, 2013b).

Materials and Methods

In order to estimate the probability of a bTB-infected animal being detected at the slaughterhouse, we considered three consecutive steps each of them with an associated probability:

P1: probability that a bTB-infected animal arrived at the slaughterhouse presenting bTB Macroscopically Detectable Lesions (MDL).

This probability was estimated using data collected through the bTB eradication program carried out in Catalonia (North-eastern Spain) between 2005 and 2008. An animal was considered as infected by bTB if it resulted positive to either the single intradermal tuberculin skin test (SIT) or the single cervical comparative tuberculin skin test (SICCT), routinely applied by the control program. In order to take into account the existence of false positives (i.e. animals that tested positive to either SIT or SICCT but

were not infected), we took into account the specificities of SIT and SICCT extracted from the metanalysis published by EFSA (2012). Therefore, P1 was calculated following the equation 1:

$P1 = posMDL / ((posSIT - posSIT * (1-Sp_{SIT})) + (posSICCT - posSICCT * (1-Sp_{SICCT})))$ [equation 1]

Where: posMDL represented the number of cattle positive to either SIT or SICCT which presented MDL; posSIT and posSICCT represented the number of cattle test-positive to those tests, and Sp_{SIT} and Sp_{SICCT} represented the specificities of SIT and SICCT, respectively. In order to incorporate the uncertainty associated with the skin test specificities, Pert distributions were assigned to SIT specificity (Sp_{SIT}) and SICCT specificity (Sp_{SICCT}), with the minimum, mode and maximum values extracted from the metanalysis published by EFSA (2012):

$Sp_{SIT} \sim Pert(0.7, 0.9, 1)$

$Sp_{SICCT} \sim Pert(0.9, 1, 1)$

Further steps were:

P2: probability that MDL, from cattle belonging to bTB negative farms, were detected by the routinely meat inspection procedure carried out in the slaughterhouse.

P3: probability that the veterinary officer suspected of bTB and sent the sample to the laboratory for confirmation, or notified directly to the authorities.

P2 and P3 were estimated by means of a questionnaire, administered during the first semester of 2014, to the slaughterhouse veterinary officers. The objective was to obtain these values based on their personal experience and expert opinion. In order to reduce bias, in those slaughterhouses where more than one veterinarian was working,

the interview was performed with the two or three more experienced veterinarians working in the plant. Data was obtained by personal interview, except in some slaughterhouses, where the questionnaire was administered by phone interview due to logistical reasons. The questionnaire (available upon request) was structured in 5 different blocks: i) general data about the slaughterhouse: location, number of veterinarians, meat technicians and number of inspection points; ii) training received to detect MDL; iii) condition of the facilities regarding lighting, space and speed of the slaughter chain; iv) organs and lymph nodes examined by visual inspection, palpation and/or incision and v) sampling protocol in case of identification of MDL.

At the end of the questionnaire, the veterinarians were asked to provide an estimate (in the scale of 0 to 10) for P2 and P3, taking into account all those factors that were mentioned during the interview. Additionally, their opinion regarding which aspects could be improved in order to increase the probability of detecting MDL was recorded.

Moreover, associations between the results from the questionnaires and the estimated probability of detecting MDL during meat inspection (P2) were evaluated using ANOVA test or Wilcoxon rank test, for categorical variables, and linear regression for continuous variables. A p-value of 0.05 was used as a significance level for associations.

According to the National bTB eradication program (Anonymous, 2014a) when MDL are detected and samples are sent to the laboratory for confirmation, a preliminary evaluation is carried out through histopathological examination and Ziehl Neelsen staining. If the preliminary result is positive, a tuberculin skin test is carried out in the herd of origin without waiting for the mycobacterial culture result, which still remains the gold standard method for confirmation of infection (OIE, 2009). Due to the high

sensitivity of histopathology in MDL (Courcoul et al., 2014) we did not included this probability into the model.

For each slaughterhouse, the sensitivity of the surveillance was calculated as the product of P1, P2 and P3. Monte Carlo simulations, with 10,000 iterations, were performed using the mc2d package (Pouillot et al., 2010), implemented in R (R Development Core Team, 2013). Finally, the average sensitivity was calculated taking into account the sensitivities estimated from each slaughterhouse.

Results

1. Descriptive results

A total of 409 cattle were positive to the SIT and 231 to the SICCT. From those 640 positive animals, 282 presented MDL. In table 1 the frequencies of the locations where MDL were detected during the meat inspection procedure, are shown. Approximately, 39% of the animals with MDL (112 out of 282) presented lesions in more than one cavity at the same time and 95% of the lesions where found in the thoracic cavity and in the head. Answers to the questions about training programs, human resources and sampling procedures are shown in Table 2. Remarkably, only 30% of the interviewed veterinarians considered that the current training program was adequate in order to fulfil all the requirements to recognize MDL during the slaughter process. Also, about 70% highlighted that sometimes the number of meat inspectors had not been enough in order to assure a reliable inspection during those periods of the year with high volumes of animals slaughtered. Finally, most of the veterinarians knew the protocol to collect and send the suspected samples to the laboratory and also knew about the existence of the Slaughterhouse Support Network called SESC (Vidal et al., 2015).

Table 1. Number and proportion of Bovine tuberculosis Macroscopically Detectable lesions (MDL) presented in one or more locations during the meat inspection. Data obtained from the eradication campaign (2005-2008) in Catalonia (North-Eastern Spain).

Lesion location		Single location (%)	Multiple locations (%)
Head	Retropharyngeal lymph nodes	61 (35.8)	58 (21)
	Submandibular lymph nodes	1 (0.6)	0 (0)
Toracic cavity	Lungs	19 (11.1)	58 (21)
	Tracheobronchial lymph nodes	21 (12.3)	69 (25)
	Mediastinal lymph nodes	60 (35.2)	79 (29)
Abdominal cavity	Liver	1 (0.6)	0 (0.0)
	Liver lymph nodes	0 (0)	5 (1.8)
	Mesenteric lymph nodes	7 (4.1)	7 (2.5)
N total of lesions		170	276
N total of animals		170	112

Table 2. Answers to the categorical questions from the questionnaires administered to the veterinary officers (*MDL: bovine tuberculosis Macroscopically Detectable Lesions)

Categorical questions			
Training of veterinary officers	**Yes**	**Total answers**	**%**
Specific training to detect MDL* when started working at the slaughterhouse	5	36	13.8
Assistance to continuous training programs to detect MDL	17	36	47.2
Is the training considered to be enough?	11	36	30.5
Human resources			
Is the number of inspectors generally considered to be enough for the detection of MDL?	11	19	57.8
Has the number of inspectors not been enough at some point?	14	19	73.6
Sampling procedures			
Knowledge of the protocol for collecting and sending samples to the laboratory	31	36	86.1
Knowledge of the Catalonian platform of support for slaughterhouses (SESC)	34	36	94.4

On the other hand, the mean scorings for optimal slaughter chain speed and for facilities were 8.4 (sd: 1.4, min: 4, max: 10) and 7.2 (sd: 1.9, min: 2, max: 10), respectively. Around 43% of the improvements suggested by veterinary officers were mainly centred on improving the facilities of the slaughterhouses (i.e. lighting, cleaning and workspace), also to organize more training courses (30%), either for veterinarians or meat inspectors, and to increase the number of technicians (20%). Other improvements (7%) were related with interdepartmental communication and with the logistics to send samples to the laboratory

2. Model results

The estimated probability of an infected animal presenting MDL (P1) was 44.8% (sd: 1.7%, 95%CI: 41.9-48.0). The probability that a MDL was detected by the meat inspection procedure carried out in the slaughterhouse (P2), and that the veterinary officer suspected of bTB and sent the sample to the laboratory for confirmation (P3) were 85% (sd: 8.6, 95% CI: 79.6-85.7) and 91.7% (sd: 7.0, 95% CI: 89.1-97.5), respectively. The score values for P2 and P3 obtained from each of the 36 slaughterhouses are represented in figure 1. The surveillance sensitivity for each slaughterhouse is represented in figure 2. The average sensitivity of the 36 slaughterhouses was 31.4% (CI 95%: 28.6-36.2).

Significant associations between some variables included in the survey and the probability of detecting MDL (P2) were found. For instance, a lower score for P2 was recorded in those slaughterhouses that suggested the organization of more training courses (p=0.01) and to increase the number of technicians in the team (p=0.04). On the other hand, those slaughterhouses with higher P2 values were the ones with the most optimal culling speed (p=0.03, regression coefficient=0.28).

Figure 1. Histograms of the scores obtained from the questionnaires, expressed as per-unit, for the probability detecting an animal with MDL (P2 values), and sending samples for laboratory confirmation or notification (P3 values).

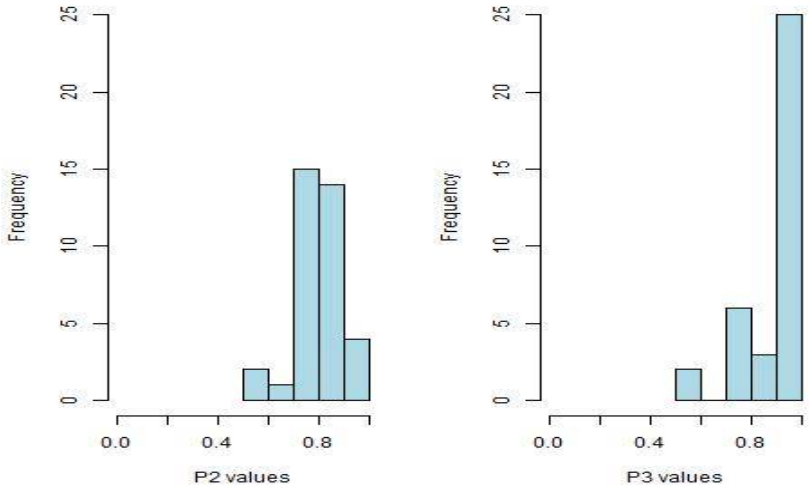
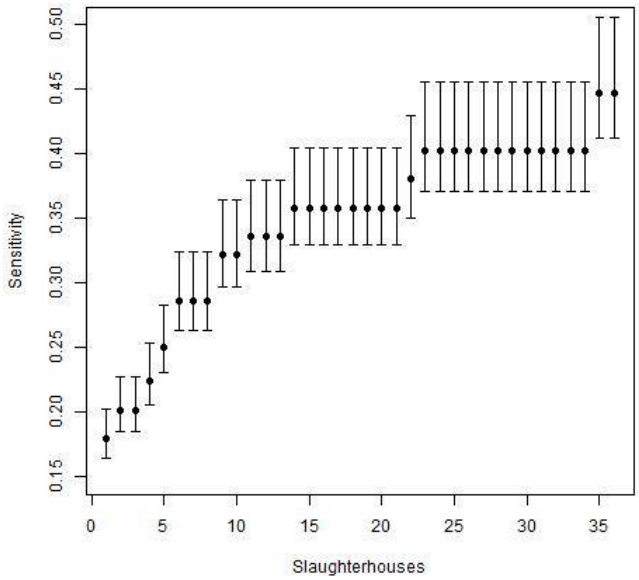


Figure 2. Mean, minimum and maximum values of the surveillance sensitivity of each slaughterhouse.



Discussion

In this study we estimated the probability that an infected animal will present visible lesions at slaughter based on data from skin test positive animals within the eradication campaign. Therefore, this estimate might present some bias as the sensitivity of the skin tests is not 100% and the population of skin test-positive animals might not represent an accurate picture of the bTB-infected animals in the whole population. Different causes of false negative reaction to skin test have been described (De la Rua-Domenech et al., 2006). For instance, animals at a very early stage of infection do not have a full response to the skin test until 3 to 6 weeks post-infection (Morrison et al., 2000); or the state of depressed cell-mediated immune response to tuberculin ('anergy'), which may develop mainly in those animals with an advanced or generalized bTB infection (Pollock and Neill, 2002). However, we did not have data from skin test negative animals and therefore we could not take into account the number of false negative animals for P1 estimation. Nevertheless, and keeping in mind the possible bias of P1, the estimate obtained in this study (around 45%) is in agreement with other studies (Corner et al., 1990; Asseged et al., 2004) and we consider that it might be a good estimate for P1. In order to validate P1 values, other approaches might have been possible. For example, a study could have been designed in order to perform secondary inspections in positive animals to SIT or SICCT to determine failure rates. However, such an approach would have implied a great effort and high expenses and in our opinion, would not have been justified. We believe that meat inspection in test-positive animals is well performed as these animals arrived to

the slaughterhouse with a notification about their bTB status and there was a need to detect CVL in order to confirm the infection.

Probabilities of MDL detection (P2) and sending samples to the laboratory for posterior confirmation or notification (P3) were estimated directly through expert opinion by means of interviewing the veterinary officers of each slaughterhouse and therefore, they are subjected also to bias. In order to try to reduce it, the veterinarians were asked about different factors that might influence P2 and P3 before giving their estimate of the probabilities, so they all took into account the same criteria before giving their estimates. Corner (1994) described some of these factors, like the intensity of inspection, the skills and dedication of the inspector and other variables related with the facilities of the slaughterhouse. On the other hand, P2 could be influenced by the prevalence in the region. In low prevalence regions meat inspectors may not perform the inspection rigorously as they do not expect to find positive animals. In order to reduce this bias, during the questionnaire we tried to make very clear that they had to give the estimate in the scenario of cattle arriving from negative herds. In Catalonia, herd prevalence is very low and we assume that the estimate given by the veterinarians might reflect the real probability of MDL detection by the meat inspection procedure in a low prevalence situation. Nevertheless, it would be desirable to repeat the study in the future in order to update these values.

During the study, it was not possible to survey all the cattle slaughterhouses in Catalonia, as in 4 out of 40 their personnel could not be interviewed due to lack of time to perform the interview. Nevertheless, they were small slaughterhouses (with only 7 cattle slaughtered on average per week) and we assume that the impact on the average sensitivity might not be significant.

Through this study we identified several aspects that could be improved in order to maximize P2 and as a consequence the bTB slaughterhouse surveillance sensitivity. For instance, it would be desirable to put more efforts in the training process of the veterinarians and also to take into account that the lack of human resources and optimal facilities might result in a lack of compliance with the meat inspection procedure, which could result in the under-detection of bTB compatible lesions during the slaughter.

Detailed examination (including palpation and incision) of thoracic and head lymph nodes followed by the lungs, may detect between 70 to 80% of animals with a single tuberculous lesion (primary sites of infection) (Corner, 1994; Asseged et al., 2004). Accordingly, our results showed that MDL presented in head and thoracic locations, resulted around 95% of single site and multisite cases, which highlights the importance of these parts of the carcass in order to improve the detection.

Different studies have described the important role of the slaughterhouse-bTB surveillance in endemic situations. For instance, in England, between 5 and 15% of the new breakdowns detected from 2002 to 2010 were identified by meat inspection (Abernethy et al., 2013). In Germany, several bTB infected herds were identified after the detection of the index case through the slaughterhouse surveillance (Probst et al., 2011) and in Spain, 14% of the 687 new herd breakdowns included in the study of Guta et al., (2014a) were also detected through the meat inspection. In addition to highlight the importance of the slaughterhouse in bTB surveillance, the key point would be to quantify which is the sensitivity of the slaughterhouse surveillance in our area of study (i.e. North-Eastern Spain) in order to assess how confident we could be in a context of risk based surveillance. Estimates of slaughterhouse surveillance sensitivities have

been compiled by the European Food Safety Authority (EFSA, 2012). In this report, results of six different studies were reported and the mean sensitivity obtained was 71% with a wide 95% confidence interval (i.e. from 38 to 92%). In our study the mean sensitivity was lower (i.e. 31.4%) and the confidence interval was narrower (i.e. from 28.6 to 36.2%). The lower sensitivity obtained in our study as compared to the ones included in the meta-analyses could be due to a combination of two factors i) the intensification of the control strategies, like the incorporation of the gamma-interferon in the last years in Catalonia that would enable positive animals to be detected at a more early stage of infection (De la Rua-Domenech et al., 2006) ii) some of the countries included in the EFSA analysis are not under an eradication campaign so the probability of infected animals showing MDL is higher.

Finally, through this study it was evidenced the heterogeneity of the sensitivity of the different slaughterhouses in Catalonia. This result highlights that the assumption of a uniform performance of the sensitivity of all the slaughterhouses is not a realistic assumption and this should be taken into account when performing risk-based models.

In further studies, bTB spread within a farm will be modelled with the final objective to estimate how much time would take an infected herd to be detected by the slaughterhouse under different levels of infection scenarios in order to develop risk-based approaches for the control of bTB in this area.

Chapter 6: STUDY IV

Modelling bovine Tuberculosis within-herd transmission in Spanish herds

Introduction

Bovine Tuberculosis (bTB) is defined as a chronic infectious disease of cattle (including all Bos species, and Bubalus bubalus) and bison (Bison bison) caused by any of the disease-causing mycobacterial species within the Mycobacterium tuberculosis-complex (Anonymous, 2013a). Cattle are mainly affected by Mycobacterium bovis and Mycobacterium caprae which can also affect other domestic and wild animals as well as humans (Aranaz et al., 2004; De la Rua-Domenech et al., 2006). Due to its zoonotic nature and the high economic impact in livestock production, the objective within EU countries is the elimination of bTB (Reviriego and Vermeersch, 2006) through the development of bTB eradication programs.

In Spain, it was not until 1993 when most dairy and beef herds were included within the bTB national control program (64/432/EEC), mainly based in the diagnosis of bTB by routinely screening cattle herds with intradermal tuberculin test (IDT) and more recently, pre-movement control tests for safe trading (Riviere et al., 2014), ending with culling all IDT positive cattle, followed by slaughterhouse post-mortem examination. Due to the application of this program, the cattle herd prevalence decreased from 5.90% in 1993 to 1.80% by the end of 2004 (Anonymous, 2014a). However, in the last 10 years the herd prevalence has only declined from 1.80% in 2004 to 1.72%, in 2014. This stagnation on the prevalence and the number of outbreaks evidences the need to re-evaluate the measures that are currently being applied.

Between 2006 and 2011, it was estimated that approximately 50% of the positive herds were new infected herds (Allepuz et al., 2011; Garcia-Saenz et al., 2014). Detection of infected herds relies on the sensitivity of the tests, which in the case of bTB are mainly based in the detection of the cellular mediated immune (CMI) response (De la Rua et al., 2006). The single intradermal tuberculin (SIT) test is the most widely used as the screening technique following European and Spanish legislation (EU Council Directive 64/432/CEE and RD 2611/1996). However, the imperfect sensitivity of the SIT test could be one of the possible causes of the failure in the eradication (Alvarez et al., 2011; Humblet et al., 2009; Skuce et al. 2012). Besides the sensitivity of the test, detection of infected herds is dependent on the progress of the disease within those herds. The probability of detection of infected animals is affected by the stage of the infection (De la Rua-Domenech et al., 2006), as recently infected animals generally do not react to the intradermal injection of tuberculin, and probability of detection increases with the time since the infection (Vordermeier et al., 2004; De la Rua-Domenech et al., 2006). On the other hand, animals in more advanced stages of the disease might enter into a state of anergy with a depressed cell-mediated immune response, and not detected either (De la Rua-Domenech et al., 2006). Detection of infected herds will also be dependent on the frequency with which herds are tested, and which varies among Autonomous Communities depending on the prevalence in the region.

The other 50% of the herds that were infected between 2006 and 2011 were persistently infected herds, which means that the disease was still present within the herd after two years of routinely IDT screening (Allepuz et al., 2011; Garcia-Saenz et al., 2014). The process of elimination of bTB from infected herds can be very complicated

and may take even several years to eliminate the disease from those herds (Guta et al., 2014b), with severe economic losses for the farmer, due to movement restrictions, and to the government for surveillance and control strategies (Riviere et al., 2014).

Understanding the dynamics of bTB spread within Spanish herds would be helpful for the design of new strategies that allowed the reduction of the time needed for both the detection of infected herds and also for the elimination of the disease from affected herds.

Dynamic modelling of bTB has been widely applied because studying bTB spread in infected herds is hindered by the long incubation periods, while models offer the opportunity to assess bTB transmission in a more cost-effective way (Perez et al., 2002; Conlan et al., 2012; Brooks-Pollock et al., 2014).

Different mathematical models have been used to evaluate the dynamics of bTB infection with the purpose of estimating bTB within-herd transmission rates and evaluating the effectiveness of control measures and new strategies of surveillance (Barlow et al., 1997; Perez et al., 2002; Griffin et al., 2000; Alvarez et al., 2012; Brooks-Pollock et al., 2014; Bekara et al., 2014). However, there is a wide variation in the cattle-to-cattle transmission rates reported on those studies, probably associated with different types of production or intrinsic variability in the transmission process (Alvarez et al., 2014), but also depending on the methodology used for the estimation. This variation makes it difficult to extrapolate results from studies carried out in other countries.

It is often easy to construct models to plausibly describe our observations or to simulate artificial data sets for given parameters in the model. In contrast, simulating

the parameter values that could have given rise to a given data set is often complicated (Beaumont, 2010).

In the present work we estimated parameters related to bTB transmission in Spanish herds. In order to do that, we selected 33 herds in which bTB was considered to have been introduced by the purchase of infected animals, out of the 764 bTB outbreaks investigated and recorded in the BRUTUB system between 2010 and 2013. On those selected herds information to estimate bTB transmission (i.e. number of infected animals introduced, time for bTB transmission and final number of infected animals), was available. On the other hand, we developed a stochastic continuous-time compartmental model to allow us to simulate bTB within-herd transmission. And by feeding the data from the 33 herds to the transmission model, bTB transmission parameters in Spanish herds could be inferred using a Markov Chain Monte Carlo (MCMC) algorithm within an Approximate Bayesian Computation (ABC) framework.

In this work, we present a method for the estimation of the within-herd transmission parameters in Spanish herds that will allow the surveillance strategies for both the detection of bTB in infected herds and the elimination of bTB from affected herds to be adapted depending on the situation, so that their cost-effectiveness can be maximized (Van Asseldonk et al., 2005).

Materials and Methods

1. Sources of data

The Spanish national bTB eradication program specifies that when a newly infected herd is confirmed by bacteriological culture, an epidemiological questionnaire is carried out by a veterinary officer and the data is stored in a national database called

BRUTUB, which is maintained by the Spanish Ministry of Agriculture, Food and Environment (MAGRAMA) (Anonymous, 2014a).

Guta et al. (2014a), developed a methodology to analyze the most likely cause of the 687 bTB outbreaks recorded in Spain between 2009 and 2011. In order to assess the likelihood of occurrence of the different routes of infection, a qualitative risk assessment approach was used. Decision trees for each of the seven possible causes: i) residual infection; ii) purchase of infected cattle; iii) sharing of pastures with infected herds; iv) infected neighbours; v) infected goats; vi) interaction with wildlife reservoirs and vii) contact with an infected human, were developed. Decision trees allowed the different routes of infection to be evaluated through weights based on expert opinion.

In the present study, farms newly infected in Spain between 2010 and 2013 (n=764) for which epidemiological data had been recorded through the BRUTUB system, were analyzed by implementing the decision trees developed by Guta et al. (2014a). That allowed the probability of introduction of bTB by different routes to be evaluated, and those herds in which both introduction through purchase of animals was likely, and the remaining routes could be discarded, were selected for the estimation of parameters related to within-herd transmission. On those selected herds, data available included:

a) The date of purchase of animals (i.e. likely date of introduction of bTB into the herd) and the date of detection. And the difference between those two gave us the time for disease spread. We assumed that the disease started in the herd once the purchased (infected) animals enter the herd and finished after the bTB was detected by SIT testing.

b) Number of infected among purchased animals: maximum number of infected animals introduced into the herd and the number of test positive animals together

with the total number of animals tested when infection of the herd was detected. We assumed a constant population size between infection and detection.

2. Model specification

Bovine tuberculosis within-herd transmission was modelled using a continuous time-compartmental stochastic SEI (Susceptible, Exposed (Latent), Infectious) model (Blower et al., 1995; Brooks-Pollock et al., 2014). We defined a density-dependent model were, the transmission term (dI/dt) defined as the rate of change in the number of infected individuals with respect to time, was equal to dI/dt = βSI, assuming that the farm size remains constant during time. The equations of the transmission compartments are defined as:

$$S_{t+1} = S_t - \beta S_t I_t$$

$$E_{t+1} = E_t + \beta S_t I_t - \alpha E_t$$

$$I_{t+1} = I_t + \alpha E_t$$

Where, β is the transmission coefficient, defined as the average number of individuals that are newly infected from an infectious individual per unit of time. And the rate of infectiousness (α), which is defined as the rate at which infected (E) cattle become infectious (I), where 1/ α is the duration of the latent period in days. Moreover, the proportion of infected individuals which are infectious (ϵ) was also calculated.

However, the only way of measuring the progress of the infection within the farm is through the detection of infected animals by means of the diagnostic tests (mainly SIT), which are not perfect and therefore, some infected animals may be missed. There is a great deal of uncertainty about the true sensitivity of this test applied in the field. Consequently, the number of animals detected at any point in time will depend on the

number of exposed and infectious animals, and the sensitivities of the SIT test for each of these categories: φ and ρ , respectively (Brooks-Pollock et al., 2014):

$$D_{e_t} \sim Binomial(E_t, \phi)$$

$$D_{i_t} \sim Binomial(I_t, \rho)$$

Where D_{et} and D_{it} are defined as the number of exposed and infectious cattle detected per unit of time, respectively. Transitions between compartments were modeled in continuous time using the Gillespie's direct algorithm (Keeling and Rohani, 2008; Vynnycky & White, 2010).

3. Parameter estimation

A Markov Chain Monte Carlo-Approximate Bayesian Computation (MCMC-ABC) method was used to generate posterior distributions of bTB transmission parameters and the sensitivity of the SIT in Spanish cattle farms. ABC-MCMC simulations, with 10.000 iterations, were performed using the "coda" package (Plummer et al., 2006). Parameter estimates were summarized with their mean and their 95% Highest Posterior Density (HPD) interval. The model was built in R version 3.2.1 (R Development Core Team., 2013).

Steps in parameter estimation:

a) Prior distributions: The uncertainty of parameters (β, α, φ, ρ and ε) was described by using prior distributions. To avoid constraining the values of β within the limits established by previous studies, its values were allowed to vary between one tenth of the minimum value reported (Kao et al, 1997) and ten times the maximum value reported (Barlow et al, 1997). Similarly, the values of α were allowed to vary between a minimum which corresponded to a latency of 1 month (half the minimum

value of 2 months reported by Neill et al, 1991) and a maximum which corresponded to latency of 50 months (above the maximum latency of 34 months reported by Perez et al, 2002). Therefore, β was described by a uniform distribution [0.00000002–0.0002712] day^{-1}, and α by a uniform distribution [0.000667–0.03333] day^{-1}.

Prior distribution for SIT sensitivity for detection of exposed (φ) and infectious animals (ρ) was uniform [0.4–1] below the minimum value obtained from Alvarez et al. (2011); and above the maximum value from De la Rua-Domenech et al. (2006). Finally, the ε parameter was fixed to 0.5 to reduce the uncertainty of the model.

b) Selection of candidate parameters: Because of the high uncertainty of the prior distributions, the candidate parameters sampled from the prior distribution may be potentially very far from the posterior distribution, and the MCMC-ABC may result in very long chains or chains may get stuck in regions of low probabilities for long periods of time (Toni et al., 2009). In order to avoid those problems, we developed an algorithm that drew samples from the prior distributions until values complied with the condition that the distance (difference) between the summary measures of observed and simulated was within the tolerance limit. And those were used as the initial values of the chains from which the posterior distributions were derived.

c) Summary measures: The most obvious approach for comparing the bTB within-herd spread observed in the herds with the values simulated using the within-herd spread model would be to use the difference in the absolute number of cases detected. However, while a difference of a few infected animals may be considered as acceptable in a large herd, the same difference may not be acceptable in a small herd. On the other hand, if we used prevalence to account for the size of the herd, while a relatively small difference in prevalence may be considered as acceptable in a small

herd, the same difference may not be acceptable in a large herd (as it would represent a huge difference in the number of infected animals). Because of that, we chose a combination of absolute number of infected animals and prevalence (i.e. number of infected animals times prevalence) as the summary measure.

d) The tolerance limit of that SM was set at 0.25 which corresponded to a difference (between values observed and simulated) of 1 infected animal in herd of up to 15 animals, 2 infected animals in a herds of between 16 and 35 animals, 3 infected animals in herds between 36 and 63 animals and so on.

Results

1. Data description

After analyzing all possible routes of infection, 33 selected herds were included into the model (Table 1). The selected herds belonged to Autonomous Communities of Spain with high bTB herd prevalence: Andalusia (19 herds), Extremadura (10) and Castilla La Mancha (3), with an average of prevalence in 2014, for each Autonomous Community, of 11.51%, 4.62% and 7.21%, respectively. And finally, only 1 farm belonged to Navarra, a low bTB prevalence area (0.67% in 2014). All selected herds were beef cattle.

Table 1. Descriptive values of the included variables from the 33 selected herds.

	Mean	*Min*	*Max*	*sd*
Positive animals introduced	1.72	1	8	1.37
Time spread (days)	277.5	34	832	210.16
Positive animals tested	4.24	1	13	3.34
Herd size	98.72	26	430	86.90

1. Model results

In table 2 are shown the descriptive values of the posterior distributions of the estimated transmission parameters. The average of β estimated from the 33 farms was 1.36E-04 (Figure 1) newly infected cows per day (min: 1.08e-4; max: 2.07e-4; sd: 3.02e-5) which corresponds to 4.70 e-2 infected cows per year. The average of α estimated from the 33 farms was 9.07e-3 (min: 7.17e-3; max: 1.10e-2; sd: 1.06e-3), which corresponds to an incubation period of 111.1 days (1/α).

Figure 1. Values of 33 transmission parameters with their correspondent upper and lower 95% Highest Posterior Density (HPD) interval.

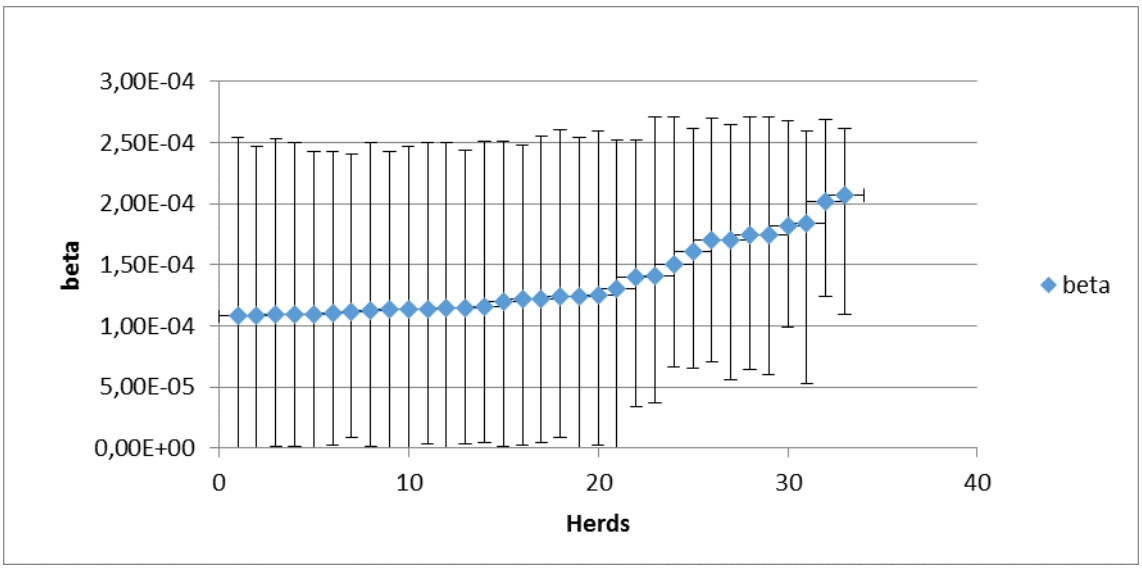


Table 2. Descriptive values of the posterior distributions of the estimated transmission parameters.

	Beta (β)	*Alpha (α)*	*Phi (φ)*	*Rho (ρ)*
prom	1.36E-04	9.07E-03	7.14E-01	7.22E-01
min	1.08E-04	7.17E-03	5.49E-01	5.24E-01
max	2.07E-04	1.10E-02	9.44E-01	9.26E-01
sd	3.02E-05	1.06E-03	9.28E-02	9.69E-02

Discussion

Mathematical models are increasingly being used to help taking decisions in relation to animal diseases. In the case of bTB, several models have been used to evaluate the dynamics of bTB infection by estimating bTB within-herd transmission rates, and then evaluate the effectiveness of control measures and new strategies of surveillance (Barlow et al., 1997; Perez et al., 2002; Griffin et al., 2000; Alvarez et al., 2012; Brooks-Pollock et al., 2014; Bekara et al., 2014). A good design of the screening-test policy is essential to ensure the earliest detection of infected herds, reducing the risk that the herd remains infected, after the test with the potential spread of the disease to other farms (Barlow et al., 1997). However, there is a wide variation in the cattle-to-cattle transmission rates reported in those studies, probably associated with different types of production or intrinsic variability in the transmission process (Alvarez et al., 2014), but also depending on the methodology used for the estimation. This variation makes it difficult to extrapolate results from studies carried out in other countries.

For the estimation of parameters related to bTB transmission in Spanish herds, we used a previously developed methodology to select herds in which bTB was likely to have been introduced by the purchase of infected animals and therefore get the data needed for the estimation of bTB transmission (i.e. the number of infected animals introduced, time for bTB transmission and final number of infected animals).

A common problem of all bTB transmission models is the availability of reliable data on for example the approximated date of infection or the initial number of animals infected, from which the transmission parameters can be inferred. Therefore, in the present work, we present a novel basis for estimate the transmission parameters from a data base where infected animals (purchased) were introduced into a free herd,

without the need to assume and simulate the moment of infection from the last negative IDT test. Besides, by using the method developed by Guta et al. (2014a), we can infer the parameters from a relative large number of farms, which allow us to assess the variation in transmission parameters among Spanish herds.

For the simulation of bTB spread, we developed a stochastic continuous-time SEI compartmental model. The disease state transitions used in the present model (Susceptible, Exposed (latent) and Infectious) have been described in other works (Rossi et al., 2015; Bekara et al., 2014; Brooks-Pollock et al., 2014; Conlan et al., 2013, Blower et al., 1995) and they are consistent with the pathology of bTB (Bekara et al., 2014). However, we are aware that other works (Kao et al., 1997; Barlow et al., 1997; Perez et al., 2002; Alvarez et al., 2012) have described two subcategories within the exposed period: first, the occult period for animals recently infected but that not respond to in vivo diagnostic tests; and secondly, a reactive period in which the animal does respond to in vivo diagnostic tests, but it is not yet infectious.

Continuous time (time to next event) models have the advantage that they allow the size of the time step to vary, allowing a better description of the transmission process (Vynnycky and White., 2010). Previous studies have used either discrete-time models (Bekara et al., 2014) or Reed-Frost models, which rely on several strict assumptions that in the case of bTB transmission cannot be met. In this study parameters related to bTB transmission in Spanish herds were inferred using a Markov Chain Monte Carlo (MCMC) algorithm within an Approximate Bayesian Computation (ABC) framework. Traditionally, parameter estimation has been performed by maximum-likelihood estimation (Toni et al., 2009). However, in complex ecological or biological systems, with different sources of heterogeneity, likelihoods cannot be calculated directly, and

we need to rely on methods such as Approximate Bayesian Computing to overcome that limitation (Hartig et al., 2011). They are particularly useful in the case of bTB, as the infection process is only partially observed (only a fraction of infectious animals is detected depending on the sensitivity of the diagnostic test).

For instance, Bekara et al. (2014), developed a compartmental stochastic model, using the ABC-rejection algorithm-method, which additionally allowed to simulate different herd management practices using data from cattle herds in France. The disadvantage of the ABC rejection sampler is that the acceptance rate is low when the prior distribution is very different from the posterior distribution. To avoid this problem, Bekara and colleagues used an additional step of local linear regression to reduce the variance of the posterior distribution, as proposed by Beaumont et al., (2002). To avoid limitations of rejection sampler, other algorithms have been developed, such as the ABC method based on Sequential Monte Carlo (SMC) (Brooks-Pollock et al., 2014) or Markov Chain Monte Carlo (Toni et al 2009). We used an MCMC-ABC method, but to facilitate deriving the posterior distribution, we developed an algorithm that drew samples from the prior distributions until values complied with the tolerance limit, and which were then used as the initial values of the chains from which the posterior distributions were derived.

The average of the transmission coefficient obtained in this study (β) was 1.30 e^{-4} newly infected cows per day. Even though those are preliminary results, they are consistent with other studies (Griffin et al., 2002; Kean et al., 1999; Barlow et al., 1997; Kao et al., 1997) in which a density-dependent model has been developed. It is important to highlight that some differences on the outputs of different reported

transmission parameters might be explained by the choice of using a density or frequency-dependent model (Alvarez et al., 2014).

On the other hand the present results have been obtained under extensive management conditions from areas of high prevalence in Spain, where beef cattle has the most important contribution to the total number of bTB cases each year (Allepuz et al., 2011; Garcia-Saenz et al., 2014). Other studies, carried out with similar methodology, obtained values in accordance with their local epidemiological context.

For instance, Bekara et al. (2014) evaluated the differences between intensively and extensively husbandry practices, in a low prevalence area of France, obtaining average values of 2.66 e^{-3} and 1.43 e^{-2} for β (daily units) in extensive and intensive production systems, respectively. In the UK, transmission values reported by Brooks-Pollock et al. (2014) ranged from 1.36 e^{-5} to 4.26 e^{-3} newly infected cows per day.

The mean IDT sensitivities obtained in the simulation for detecting exposed and infectious animals, 71.4% and 72.2% respectively, are consistent with the IDT sensitivities reported in other works (Alvarez et al., 2011; De la Rua et al., 2006). And also, the transition rate between exposed and infectious has been reported with similar values in other studies reported in the review by Alvarez et al. (2014).

Finally, the transmission parameters obtained in the present study are a first step to develop a within herd transmission model in Spanish herds with the final objective to evaluate, in future works, the different surveillance strategies in order to find the most cost-effective options. The determination of transmission parameters is essential to explore the effectiveness of different strategies for both detection of infected herds and elimination of bTB from affected herds, allowing the strategies to be adapted depending on the context.

Chapter 7: GENERAL DISCUSSION

In terms of animal disease surveillance, some countries have been recently evaluating and implementing a surveillance approach based on risk (Bessell et al., 2012b; Ribero-Lima et al., 2015; Rossi et al., 2015). The essence of a risk-based strategy relies on implementing a higher priority of surveillance resources in those herds that present higher risk to get the infection, with the final objective to reduce the cost of the system and increasing the likelihood of identifying outbreaks (Stärk et al., 2006). This concept also includes the use of information about the probability of occurrence and consequences in order to plan and interpret the results (Hoinville et al., 2013). Regarding surveillance for bTB, this approach has been evaluated in different studies. For instance, in Scotland which was designated as an officially bTB free region in 2009, the risk of infection for each herd (Bessell et al., 2012a) was combined with the probability of detection, in order to develop a model that enabled to maximized sensitivity while reducing the cost of the surveillance system Bessell et al. (2012b). In north-western Minnesota (US), Ribero-Lima et al. (2015) developed a surveillance strategy based on cattle movements and network analysis parameters, given that the pattern of animal movements was identified as an important risk factor of bTB infection in the country. In order to consider the application of risk-based surveillance approaches, a comprehensive understanding of the epidemiology of the disease in the country in question, for instance, the geographical distribution, the influence of different risk factors or the sensitivity of the different surveillance components, is essential (Stärk et al., 2006).

In Spain, the bTB eradication campaign started in 1993 and this allowed an important progress on the reduction of the prevalence. However, in the last 10 years herd prevalence has only declined from 1.80% in 2004 to 1.72%, in 2014. Every year a considerable number of new positive or re-infected herds still appear, despite the implemented measures of control and surveillance, and the high economic investment on resources. Therefore, there is still a need to investigate which are the factors that difficult the eradication in Spain and to evaluate new strategies of control and surveillance.

In the first study of this PhD Thesis, we developed a space-time model which allowed us to evaluate the risk of bTB infection in Spain at county level and to obtain information about the evolution of the disease eradication over the five years of study (2006-2011). Moreover, we identified several areas of Spain (Northern-Spain) where the risk of bTB was low during the whole period of study, which could be targeted for a first scenario of risk-based surveillance in future works. In addition, some factors (i.e. animal movements and bullfighting type of production) were identified as important determinants for the risk of infection. However, an important handicap for the applicability of these results in a risk-based context is that they were obtained using data aggregated at county level, and relations found at the aggregated level do not necessarily exist at individual/herd level (i.e. ecological fallacy). Therefore, the utility of these results would be dependent of the geographical level of implementation of the risk-based surveillance system (i.e. herd *versus* county level).

At herd level, some factors have already been identified as important determinants of the risk of infection in Spain. In fact, Guta et al. (2014b) carried out a study to assess risk factors related to bTB persistence within farms by means of a case–control study.

Persistent versus transient bTB infected beef farms from Central and Southern Spain were compared, and according to the results, farms with large pasture areas and bTB infected neighbours had more difficulties in eradicating the disease. In another study Guta et al. (2014a), the most likely routes of infection in herd breakdowns in Spain were investigated. Results from the second study identified residual infection as the most frequent cause of bTB breakdowns, followed by interaction with wildlife reservoirs. Herd size and type of production have also been identified as relevant risk factors in other studies conducted in central Spain (Alvarez et al., 2012).

These previous studies have identified several factors with potential to categorize herds according to their risk of infection and therefore to be used in a risk-based system. However, those studies have not analysed other herd-level factors such as the risk by movement of animals, the influence of the frequency of tuberculin testing or previous bTB history. This data could be relevant to be included in such a system as they are routinely data collected and therefore would be possible to have updated information. Therefore, before considering which factors should be included as important determinants of the risk of infection in a risk-based system, further studies at herd level should be developed.

In the second study of the PhD Thesis, the steps needed for the development of a risk-based surveillance system were evaluated by exploring the approach for bTB that is under development in New Zealand. A risk-based scoring based on four determinants of the disease (previous infection history, the amount of testing carried out on individual herds, geographic location and herd movement behaviour) was proposed in order to decide whether to conduct a preferential sampling of the herds (targeting those at higher risk). Although this was a pilot study, the preliminary results allowed us

to prove that a considerable number of herds at risk of infection where located in areas outside from the Movement Control Areas and the potential of routinely collected data to categorize herds according to risk.

Based on this experience, we decided to conduct further studies in Spain in order to evaluate the sensitivity of the current surveillance components which are: slaughterhouse surveillance, routinely IDT testing and pre-movement testing.

In the third study of this PhD thesis, we evaluated the sensitivity of the slaughterhouse surveillance for bTB in Catalonia (North-East of Spain). The results showed a low average of individual slaughterhouse sensitivity (31.4%) and that there was heterogeneity among slaughterhouses (CI 95%: 28.6-36.2).

In countries where the eradication programs have been implemented for many years, infected animals are usually detected by IDT testing in the early stages of infection. For that reason, the probability of an animal showing visible lesions during the meat inspection is very low and so it is the probability of detection (Domingo et al., 2014). Besides, under such circumstances, improving the sensitivity of post mortem detection of bTB at slaughterhouses would be really difficult; however, continuous education and training of slaughter inspectors are certainly very important to maintain the quality of inspection (Schiller et al., 2011).

Moreover, the sensitivity could be further reduced if the new European legislation of "visual only" was implemented, where meat inspection would be carried out without palpation and incision of the different parts of the carcass, with the objective to deal with biological hazards and occupational health risks. With that method, the sensitivity of slaughterhouse surveillance would be dramatically decreased (EFSA, 2013a).

The next surveillance component to be evaluated was the frequency of the routinely IDT testing. In order to perform so, the first step, which has been done in the fourth study of the present PhD Thesis, was to elaborate a within-herd disease transmission model in order to estimate the parameters related with the disease spread inside a herd.

Given that the variation on the accuracy of routinely IDT testing is related with the timescales of transmission and the frequency of testing (Conlan et al., 2012), a good design of the screening-test policy is essential to ensure the earliest detection of infected herds, reducing the risk that the herd remains infected after the test, with the potential spread of the disease to other farms (Barlow et al., 1997).

In further studies, those results will be applied to evaluate different diagnostic strategies and to find an optimal frequency of IDT testing. Additionally, bTB spread within a farm will be modelled with the final objective to estimate how much time would take an infected herd to be detected by the slaughterhouse under different levels of infection scenarios.

Chapter 8: CONCLUSIONS

1. There were no significant changes in the annual risk of bTB in Spain between 2006 and 2011, although the risk was higher in central and southern areas. Space–time interactions highlighted a heterogeneous temporal pattern of bTB at county level. In some counties, between some years, the increase or decrease of the incidence and prevalence was higher as compared to the mean rate for the whole Spain.

2. Animal movements and the bullfighting type of production were identified as factors related to a higher prevalence and incidence of bTB at county level.

3. Routinely recorded data from a bTB eradication program is potentially useful to rank herds according to risk of infection within a risk-based surveillance framework.

4. The probability of detecting cattle infected with bTB by the slaughterhouse surveillance varies among the Catalonian slaughterhouses from 28.6% to 36.2%.

5. The low bTB slaughterhouse surveillance sensitivity in Catalonia was mainly related to the low probability that a bTB-infected animal arrived at the slaughterhouse presenting bTB visible lesions. Nevertheless, factors such as increasing the number of meat technicians, the attendance to training courses and having an optimal speed of the slaughter line would help to improve the probability of detecting bTB visible lesions.

6. The mean within herd transmission rate (β) estimated in 33 Spanish herds varied between 0.0001 and 0.0002 per day, and the mean rate at which infected cattle become infectious (α) varied between 0.011 and 0.0001.

Chapter 9: REFERENCES

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Chapter 10: APPENDIXES

Appendix 1. Questionnaire used in study III.

Enquesta a veterinaris d'escorxador

Objectiu: càlcul de la sensibilitat de la inspecció a escorxadors per a la detecció de LCT (lesions compatibles amb tuberculosi) en vaques procedents de granges T3 (lliures).

1) Dades generals de l'escorxador:

Establiment:

Servei Regional:

En l'actual escorxador es sacrifiquen animals provinents del sanejament de Tuberculosi (granges T2)?

a. Si
b. No

Nombre de veterinaris a la plantilla actual:

Nombre d'auxiliars a la plantilla actual:

Nombre de punts d'inspecció actualment:

2) Formació:

Plantilla de veterinaris:

Anys treballant a l'actual escorxador	Anys d'experiència treballant en altres escorxadors on es sacrifiquin bovins

Els veterinaris de la plantilla actual han rebut alguna formació específica per al reconeixement de LCT en el moment d'incorporar-se a la feina?

	Escorxador actual	General
Sí		
No		
No sap		

Existeix un pla de formació continuat per a la identificació de LCT?

	Escorxador actual	General
Sí		
No		
No sap		

En cas afirmatiu, en que consisteix aquest programa:

Considera que aquesta formació és suficient per al reconeixement de LCT?

	Escorxador actual	General
Sí		
No		
No sap		

AOIV (Auxiliar Oficial d'Inspecció veterinària):

Els auxiliars han rebut alguna formació específica per al reconeixement de lesions en el moment d'incorporar-se a la feina?

	Escorxador actual	General
Sí		
No		
No sap		

Existeix un pla de formació continuat per a la identificació de lesions?

	Escorxador actual	General
Sí		
No		
No sap		

En cas afirmatiu, en que consisteix aquest programa:

Considera que el nombre d'auxiliars és suficient per al reconeixement de lesions?

a. Si
b. No

Alguna vegada ha estat insuficient?

3) Instal·lacions:

En l'escala del 0 al 10 (0: totalment inadequat - 10: adequada), com valoraria la velocitat de la línea per a què el tècnic pugui identificar lesions?

Escorxador actual

En l'escala del 0 al 10 (0: totalment inadequat - 10: adequada), com valoraria l'estat de les instal·lacions (llum, espai, etc) en quant a si és adequat per a què el tècnic realitzi la inspecció amb detall?

Escorxador actual

4) Procediment d'inspecció

BOE	Visual	Palpació	Incisió
Cap i coll:			
In submax			
In retrofarin			
In parotidis			
musc masseter			
Traquea y esófago:			
tràquea			
pulmons			
In bronquials			
In mediast			
Corazón:			
pericardi i cor			
diafragma			
Hígado y bazo			
Fetge			
In hepàtics			
In pancreàtics			
melsa			
Gastrointestinal			
gastrointestinal			
In gàstrics			
In mesentèrics			
Urinario			
ronyons			
In renals			
Otros			
pleura			
peritoneu			
mamelles			
In supramamaris			

En el cas de què hagi treballat en un altre escorxador, troba que el procediment d'inspecció era similar a l'actual?

a. Si
b. No
c. No sap

Fa quant de temps va treballar en aquest altre escorxador?

5) Enviament de mostres sospitoses:

En cas de sospita de LCT existeix un protocol escrit d'enviament de mostres per a la confirmació laboratorial?

	Escorxador actual	General
Sí		
No		
No sap		

Coneix el SESC?

a. Si
b. No

6) Opinió sobre la sensibilitat (en l'actual escorxador)

En la seva opinió:

En el cas de que arribi un animal amb LCT visibles (provinent de una granja T3) quina seria la probabilitat de NO detectar aquestes lesions (en l'escala del 0 al 10 (0: molt baixa - 10: molt alta):

En cas es detectin aquestes lesions, en l' escala del 0 al 10 (0: molt baixa - 10: molt alta) quina seria la probabilitat de què el veterinari no enviï aquesta mostra sospitosa al laboratori per confirmar la presencia de TBb:

En cas de no haver enviat alguna vegada lesions sospitoses por TBb, a que s'ha degut?

En la seva opinió, quins aspectes es podrien millorar per tal d'incrementar la capacitat de l'escorxador de detectar LCT?

- Comentaris:

Appendix 2. Additional papers coauthored during the PhD Thesis related with the bTB project.

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PLOS ONE

Epidemiological Investigation of Bovine Tuberculosis Herd Breakdowns in Spain 2009/2011

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Abstract

We analyzed the most likely cause of 687 bovine tuberculosis (bTB) breakdowns detected in Spain between 2009 and 2011 (i.e., 22% of the total number of breakdowns detected during this period). Seven possible causes were considered: i) residual infection; ii) introduction of infected cattle from other herds; iii) sharing of pastures with infected herds; iv) contiguous spread from infected neighbor herds; v) presence of infected goats in the farm; vi) interaction with wildlife reservoirs and vii) contact with an infected human. For each possible cause a decision tree was developed and key questions were included in each of them. Answers to these key questions lead to different events within each decision tree. In order to assess the likelihood of occurrence of the different events a qualitative risk assessment approach was used. For this purpose, an expert opinion workshop was organized and ordinal values, ranging from 0 to 9 (i.e., null to very high likelihood of occurrence) were assigned. The analysis identified residual infection as the most frequent cause of bTB breakdowns (22.3%; 95%CI: 19.4–25.6), followed by interaction with wildlife reservoirs (13.1%; 95%CI: 10.8–15.8). The introduction of infected cattle, sharing of pastures and contiguous spread from infected neighbour herds were also identified as relevant causes. In 41.6% (95%CI: 38.0–45.4) of the breakdowns the origin of infection remained unknown. Veterinary officers conducting bTB breakdown investigations have to state their opinion about the possible cause of each breakdown. Comparison between the results of our analysis and the opinion from veterinary officers revealed a slight concordance. This slight agreement might reflect a lack of harmonized criteria to assess the most likely cause of bTB breakdowns as well as different perceptions about the importance of the possible causes. This is especially relevant in the case of the role of wildlife reservoirs.

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Introduction

Bovine tuberculosis (bTB) is a chronic infectious disease of cattle (including all *Bos* species); buffaloes (*Bubalus bubalis*) and bison (*Bison bison*) caused by any of the disease-causing mycobacterial species within the *Mycobacterium tuberculosis*-complex [1]. In industrialized countries, bTB control programs are mainly based on routine intra-dermal skin tests and removal of positive reactors supplemented by slaughterhouse surveillance [2]. In Europe, some countries have achieved the officially tuberculosis free (OTF) status, which implies reporting 99.9% of bTB-free herds during 6 consecutive years (Council Directive 64/432/EC). However and despite intensive eradication efforts applied over the years, bTB continues to be present in some other European countries [3]. In Spain, the bTB eradication program has been progressively reinforced through the years (e.g., pre-movement testing, inspections of the field teams, etc), but the OTF status has not yet been achieved. Herd prevalence in 2012 was around 1.3%, but in the last years there has been only a moderate decline [4]. This stagnation is related to the high number of new infected herds detected each year. Between 2006 and 2011, approximately 50% of the positive herds were new infected herds [5], and that poses a serious challenge towards the eradication of the disease.

A bTB herd breakdown may occur due to the persistence of the mycobacteria within the herd (i.e. residual infection), or because of its introduction in a previously free herd. Residual infection could be due to the presence of false negatives to the skin test, reviewed by De la Rua-Domenech [6], or be the consequence of the incorrect application of the test [7]. Also, indirect transmission due to the persistence of the microorganism in the environment could result in residual infections [8]. The presence of infected goats in the farm could also contribute to the recirculation of bTB within the cattle herd [9–12].

As external sources of bTB infection, the purchase of infected animals and the interaction with infected cattle or goats at common pastures could be the origin of bTB breakdowns [13–16].

The presence of neighboring bTB positive herds may also result in the introduction of the mycobacteria into a herd, via direct contact with infected animals over farm boundaries, or by drainage of contaminated sewage [17–20]. In many countries, the presence of wildlife reservoirs endemically infected poses a challenge to bTB eradication schemes. Examples of such reservoirs include the European badger (*Meles meles*) in Great Britain and Ireland [21,22] or the brushtail possum (*Trichosurus vulpecula*) in New Zealand [19]. In Spain, the Eurasian wild boar (*Sus scrofa*), the red deer (*Cervus elaphus*) and the fallow deer (*Dama dama*) have been identified as bTB maintenance hosts [23,24,25]. Finally, humans infected with tuberculosis could also act as a source of infection for cattle [26–29].

The determination of the mechanisms by which herds get infected, and the quantification of their relative importance, could be useful information to determine what would be the most appropriate and cost effective preventive measures. Therefore, the main objective of this study was to identify the most likely causes of the bTB herd breakdowns detected in Spain between 2009 and 2011.

Materials and Methods

Data

The Spanish national bTB eradication program, according to Council Directive 64/432/EEC, is based on periodical testing of cattle and culling of positive cattle. In each herd test, all animals older than 6 weeks of age are tested annually with the single intradermal test (SIT). Herds are classified as bTB-free if no positive animals are detected in at least two consecutive follow-up herd tests, and as non-bTB free if at least one positive animal is detected. In newly infected herds, based on animal field testing, confirmation of infection is performed by tissue culture for isolation of the causative agent. If the herd is confirmed as infected an epidemiological questionnaire is carried out by a veterinary officer and data is stored in a national database called BRUTUB, which is maintained by the Spanish Ministry of Agriculture, Food and Environment (MAGRAMA) [4]. The questionnaire registers data about management of the herd, history of bTB testing results, animal movements, bTB status of neighbor herds, and interaction with other domestic and wild animals. Besides, the most likely cause of the breakdown in the opinion of the veterinary officer conducting the survey is also recorded. This questionnaire can be accessed in [30]. Data recorded in BRUTUB between 2009 and 2011 were obtained from MAGRAMA.

Additional data about animal movements and bTB status of herds with epidemiological links (i.e., related due to animal movements, neighborhood or pastures) with the studied herds were obtained also from MAGRAMA. For Catalonia (north-eastern Spain), we had access to the ear tag number of all the reactor animals detected in the breakdown, which allowed us to trace individual animal movements. Those data were obtained from the Department of Agriculture, Food and Environment of the Autonomous Government of Catalonia (DAAM).

Also, within the Spanish national bTB eradication program a molecular technique called spoligotyping is applied to strains isolated from the breakdowns. By this technique strains are classified in different groups called spoligotypes as a function of the polymorphism detected within a region in the bacterial genome [31]. The spoligotype patterns of the different isolates of *M. bovis* and *M. caprae* from domestic animals and wildlife (aggregated at municipality level) related with the breakdowns under study were obtained from the mycoDB.es database [32]. The spoligotype patterns of the isolates from the studied herds were provided by the VISAVET Health Surveillance Center located at the Complutense University of Madrid. Additional molecular data from wildlife isolates at county level were provided by the Research Center for Hunting Studies (IREC) and the regional governments of Andalusia and Galicia. Data about bTB testing results in goats were also provided by regional governments.

Statistical analysis

Descriptive statistics of the number of reactors and within herd incidence by type of production (i.e., beef, dairy or bullfighting) and method of detection (i.e., slaughterhouse, epidemiological link or routine testing) of those breakdowns recorded in the BRUTUB database between 2009 and 2011 were calculated. Differences between groups were assessed by an analysis of variance model and Tukey's test. Due to their highly right skewed distribution the variables were log transformed. The level of significance for the analyses was set to $p<0.05$. These analyses were performed by using the free software R version 3.0.2.

Investigation of the most likely cause of bTB herd breakdowns

In order to assess the most likely cause of bTB breakdowns we followed these steps:

1) Determination of the possible causes of a bTB herd breakdown.
   Based on bTB epidemiology we considered seven possible causes of herd breakdowns: i) residual infection; ii) introduction of infected cattle from other herds; iii) sharing of pastures with infected herds; iv) contiguous spread from infected neighbor herds; v) presence of infected goats in the farm; vi) interaction with wildlife reservoirs; and vii) contact with an infected human. If the origin of the breakdown could not be attributed to any of the previous causes, it was considered as unknown.
2) Determination of the different events within each possible cause.
   For each possible cause a decision tree was developed and key questions where included in each of them. Answers to these key questions lead to different events within each decision tree. In figure 1, the decision tree for the introduction of infected animals is shown. The rest of the decision trees are included in the supplementary material (figure S1 in File S1). For example, event E3 in figure 1 would correspond to a herd that had introduced cattle into the herd one year before their last negative herd test. At least one animal came from a herd that had been confirmed as bTB-infected in the herd test after the movement occurred (note that bTB-infected herds are not allowed to move cattle to other herds). Moreover, the same spoligotype was isolated in the herds of origin and destination.
3) Assessment of the likelihood of occurrence of the different events.
   In order to assess the likelihood of occurrence of the different events a qualitative risk assessment approach was used. For this purpose, an expert opinion workshop was organized following recommendations included in the Handbook on Import Risk Analysis for Animals and Animal Products [33]:

   i) We selected experts on the basis of their knowledge, and from a variety of disciplines concerned with the subject. The participants in our Workshop included experts with different backgrounds (i.e., researchers

working on domestic and wildlife bTB epidemiology, veterinarians working at regional and central administrations), and came from different regions of Spain (with different epidemiological situations). In order to facilitate the discussion among experts a "manageable number" of experts are recommended. For this workshop nine national experts where contacted. In table S2 in File S1 in the supplementary material a table with the background and expertise of the different national experts that participated in the workshop can be found.

ii) Once they agreed to participate, an introduction about expert opinion methodology together with the decision trees was sent to the experts by email, so that they had time to think about it before the meeting. Following recommendations by Dufour et al., [34] ordinal values on the scale of 0 to 9 (table 1) were used.

iii) A one day workshop was held in June 2012 in the Veterinary Faculty of the Autonomous University of Barcelona (UAB). In order to solve doubts and avoid misunderstandings, a brief introduction about expert opinion was given together with the instructions on how to assign the values.

iv) Time was given to the experts to, individually, assign the considered ordinal values described in table 1 to the different events included in each decision tree.

v) After that, break time was given to the experts, and during that time all results were compiled. Histograms showing the distribution of the ordinal values assigned by experts to each event were prepared.

vi) These histograms were discussed with the entire group. During this discussion, experts had the chance to change their ordinal values if they considered that they had overestimated or underestimated any of the events.

vii) Finally, descriptive statistics of the nine values provided by the experts in this second questionnaire to each of the 56 events across all decision trees were calculated. The mean value of each of the events was assumed to be the likelihood of occurrence of each event and the mean value of the standard deviations associated with each of them was considered as the overall variability of the experts' opinion. In table S3 in File S1 included in the supplementary material a table with the descriptive statistics of each of the events, a histogram of the standard deviations associated with each of them and a table with the raw values given by the 9 experts in the second questionnaire can be found.
Further details related with the "Workshop Method" can be found in the Handbook on Import Risk Analysis for Animals and Animal products [33].

4) Data management and determination of the different events that had occurred in each herd breakdown.
Based on available data for each breakdown, we extracted the events, within each possible cause of infection, that had happened following the criteria described in the decision trees (e.g., did cattle enter the herd one year before the last negative herd test?; If yes, has the herd (where these cattle came from) been confirmed as bTB-infected in the herd test after the movement occurred? and so on). Therefore, each herd finished with seven ordinal values (i.e., the likelihood of occurrence of each possible cause of breakdown). In order to perform this task automatically we developed a visual basic macro in Excel. Thanks to this macro, relevant data in the different data files was searched and a new file was generated containing the seven ordinal values by breakdown.

5) Determination of the most likely cause of each bTB herd breakdown.
In order to determine the most likely cause of the breakdown for each infected herd, the values of the seven different causes (i.e., the mean ordinal value of each event obtained in the expert opinion workshop) were compared following this criterion:

i) When the seven possible causes of breakdown had values less than 5, the cause of infection of the herd was considered as unknown.

ii) In each breakdown, causes for which a value of 5 or more had been assigned were compared among them following these steps:

a) First, we ranked the values from the highest to the lowest value.

b) Then, the cause with the maximum value was considered as the most likely if the difference with the second one was higher than the mean value of the standard deviations of the different events (i.e., one point).

c) In those breakdowns in which three or more causes were within this interval (i.e., three or more values within the highest value minus one point) the cause of infection was considered as unknown.

d) In those breakdowns in which only two causes were within that interval, we considered both options as equally likely, and we assigned 0.5 points to each cause.

e) The 95% confidence intervals of the proportion of each of the most likely causes of breakdown were calculated with the free software R version 3.0.2 using the epiR library [35].

Most likely causes of breakdown attributed by veterinary officers versus causes obtained in our study

The last question that the veterinary officers had to complete in the epidemiological questionnaire [30] was their opinion about the possible cause of the breakdown. They had the option to provide more than one possible cause. In those breakdowns in which two options had been provided, we assigned a value of 0.5 to each of the causes. When the veterinary officers had selected more than two options we considered the cause of breakdown as unknown. In order to calculate the concordance between the opinion of veterinary officers and our results, we made the comparison only for those herds in which a single cause of infection had been obtained by both methods. The agreement between both results was assessed by the Kappa value [36], and calculated with the free software R version 3.0.2 using the epiR library [35]. Kappa values less than 0.2 were considered as indicative of slight agreement, whereas greater than 0.8 would indicate an almost perfect agreement.

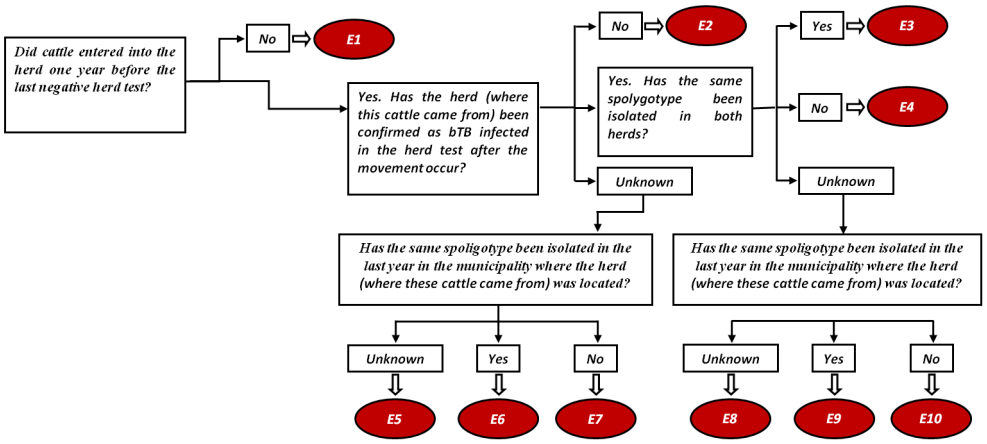

Figure 1. Introduction of infected cattle from other herds decision tree.
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Results

Descriptive results

On 30th May 2012, date when we stopped collecting data, information from 687 breakdowns had been recorded in the BRUTUB system. In figure 2 the geographical distribution of the recorded surveys is represented.

These 687 breakdowns represented the 22% of the breakdowns detected between 2009 and 2011 in Spain. However, the coverage (i.e., percentage of breakdowns recorded in BRUTUB) by regions was variable. There were data of 139 breakdowns from regions with low prevalence (i.e., north and eastern parts of Spain) and of 548 breakdowns from high prevalence regions (i.e., center and south).

Descriptive statistics on the number of reactors and within-herd incidence by type of production (i.e., beef, dairy or bullfighting) and method of detection (i.e., slaughterhouse, epidemiological link or routine testing) are presented in table 2. Bovine TB herd breakdowns were detected mostly by routine herd tests. However, 14% and 22% of breakdowns were detected by slaughterhouse surveillance and epidemiological links (i.e., related by movements, pastures, etc) with infected herds, being an important complement for the detection of the infection.

The number of reactors was 4 or lower in half of the breakdowns. Median number of reactors or within herd incidence in herds detected by slaughterhouse surveillance, epidemiological link or routine testing was very similar and no statistically significant differences were identified between them. However, the median within herd incidence was significantly lower on breakdowns detected in dairy ($p = 0.007$) and bullfighting herds ($p = 0.04$) compared to beef herds.

Most likely cause of breakdown based on the decision trees

The most likely causes of herd breakdowns in Spain are shown in table 3. Residual infection was identified as the most important cause (22.3%; 95%CI: 19.4–25.6), followed by interaction with wildlife reservoirs (13.1%; 95%CI: 10.8–15.8). The introduction of infected cattle, sharing of pastures and contiguous spread from infected neighbor herds were also identified as relevant causes. The presence of infected goats and the contact with infected humans seemed to have lower relevance. In 286 herds (41.7%; 95%CI: 38.0–45.4) the origin of infection remained unknown. In 185 of them (64.7%) the likelihood of all the causes was below 5 and in 101 (35.3%) there were more than three plausible causes.

Table 1. Ordinal values and categories used for the qualitative risk assessment [34].

Ordinal scaling	Categories
0	Null
1	Nearly null
2	Minute
3	Extremely low
4	Very low
5	Low
6	Not very high
7	Quite high
8	High
9	Very high

doi:10.1371/journal.pone.0104383.t001

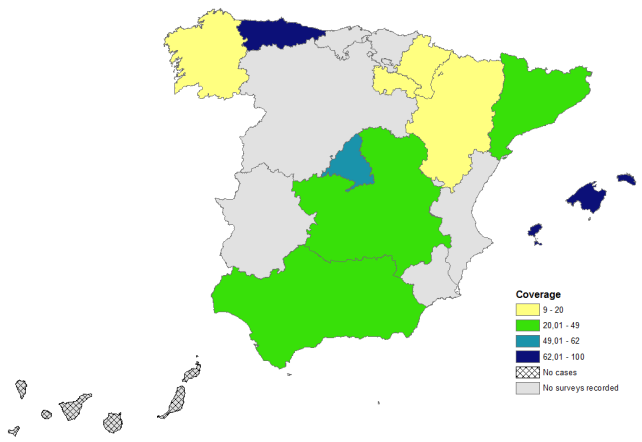

Figure 2. Percentage of breakdowns with a recorded survey (i.e., coverage) between 2009 and 2011.
doi:10.1371/journal.pone.0104383.g002

Table 2. Median number of reactors and within herd incidence (in brackets and expressed as a proportion) by detection method (i.e., slaughterhouse surveillance, epidemiological link or routine testing) and herd type (i.e., beef, dairy or bullfighting).

	Beef				Dairy				Bullfighting				Total			
Detection method	**Obs**	**Median**	**Q3**	**Max**	**Obs**	**Median**	**Q3**	**Max**	**Obs**	**Median**	**Q3**	**Max**	**Obs**	**Median**	**Q3**	**Max**
Slaughterhouse	73	3.0 (5.3)	9.5 (12.5)	128.0 (81.5)	18	5.0 (3.2)	20.5 (15.5)	59.0 (63.6)	3	1.0 (0.8)	4.0 (3.5)	4.0 (3.5)	94	3.0 (4.3)	9.5 (12.3)	128.0 (81.5)
Epidemiological link	135	4.0 (6.5)	7.5 (15.9)	65.0 (57.4)	6	4.5 (6.5)	17.0 (26.6)	37.0 (86.1)	8	5.0 (3.5)	16.0 (10.3)	32.0 (22.8)	149	4.0 (6.4)	7.5 (15.6)	65.0 (86.1)
Routine testing	345	5.0 (7.7)	10.0 (15.0)	83.0 (70.9)	59	2.0 (3.2)	5.5 (10.0)	110.0 (70.9)	26	9.0 (6.1)	24.5 (10.7)	91.0 (49.5)	430	4.0 (5.3)	10.0 (11.9)	110.0 (82.9)
Total*	553	4.0 (7.1)	9.5 (15.5)	128.0 (82.9)	83	2.5 (3.4)	6.5 (10.0)	110.0 (86.0)	37	8.0 (5.1)	24.0 (10.5)	91.0 (49.4)	673	4.0 (6.6)	9.5 (14.5)	128.0 (86.0)

*14 farms not included because no data on method of detection recorded.
Obs: number of observed breakdowns; Q3: third quartile; Max: maximum value.
doi:10.1371/journal.pone.0104383.t002

If only those herds with a single cause were considered (table 4), residual infection was also the most likely cause followed by interaction with wildlife, contiguous spread and introduction of infected cattle. In this case the importance of sharing of pastures was much lower. In 168 herds, the difference between the first and the second cause with the greater ordinal values was less than one point, for these herds, two possible causes of infection were considered. Within this group, the most frequent first option was residual infection (66.1%), while the most frequent second option was sharing pastures with other herds (48.8%).

There were some differences in the causes of bTB herd breakdown according to the type of herd (table 5). In dairy herds, 65% of the herd breakdowns remained unknown, while wildlife, movements to pastures or contiguous spread seemed to have very little importance. Residual infection was more relevant in bullfighting herds as compared to beef or dairy herds.

There were also some differences in the cause of bTB herd breakdowns according to the location of the herd (table 6). In areas of low prevalence such as the north and eastern part of the country, there were a greater percentage of herds with an unknown cause. Contiguous spread and interaction with wildlife reservoirs seemed to have a higher importance in the center and south of the country as compared to the north and eastern areas.

The mean ordinal values associated with the most likely cause for each breakdown where we could determine a possible cause of the breakdown (i.e., 401 herds) is represented in figure 3. Only in a small proportion of the breakdowns the cause of the breakdown was attributed with a "high" or "very high" likelihood of occurrence. In 29 out of 401 (7%) and in 8.5 out of 401 (2%) of the studied breakdowns the likelihood of occurrence was "high" or "very high" respectively. For the majority of the breakdowns (i.e., 330.5 out of 401 (82%)), the values were between 5.6 and 7.5, which corresponded to qualitative categories of "not very high" and "quite high". These low values were primarily due to the absence of molecular data, which were lacking for 364 of the 687 studied herds.

In table 7, the most likely events for each cause of infection are represented. Most of the residual infections were attributed to herds that had reactors in the previous 3 years, but for which we did not have enough data to assess whether the isolates had similar molecular characteristics and to herds where the incidence of reactors was not compatible with a recent infection. With regard to the introduction of infected cattle, only a small proportion of the breakdowns (3 out of 35) were associated with a "high" or "very high" likelihood of occurrence. All the breakdowns associated with goats except 1 were due to the presence of goats in the farm, but without data regarding their bTB status. Around 42% of the herds infected by contiguous spread had an infected neighbor herd, but without enough data to assess if they had the same spoligotype. From the breakdowns attributed to wildlife, only in 9.4% the likelihood of occurrence was "high", and corresponded to herds located near areas of hunting activity and where the spoligotype had been also isolated in wildlife animals of the area.

Results of our study versus conclusions from veterinary officers

In 190 breakdowns one single cause was identified as the most likely by both the qualitative assessment and the veterinary officers. Within these herds the agreement between the identified causes of the breakdowns was in general slight (Table 8). The higher disagreement was in the case of introduction of infected cattle and wildlife. Veterinary officers considered that wildlife was the most likely cause for 59 herds, while by applying the decision

Table 3. Most likely causes of bTB breakdowns.

	Most likely		
Causes of breakdown	**Herds**	**Proportion**	**95% CI**
Residual infection	153.5	22.3	19.4–25.6
Introduction of infected cattle	35	5.1	3.7–7.0
Presence of infected goats	17	2.5	1.6–3.9
Contiguous spread	55	8	6.2–10.3
Sharing of pastures	48.5	7.1	5.4–9.2
Interaction with wildlife	90	13.1	10.8–15.8
Contact with infected humans	2	0.3	0.1–1.1
Unknown (a)	286	41.6	38.0–45.4
Total	**687**		

(a) In 185 herds the likelihood of all the causes was below 5 and in 101 there were more than three plausible causes.
95% CI: 95% confidence interval.
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trees wildlife was linked to only 26 farms, moreover, we just agreed on 12 herds.

Discussion

According to the results of our study, residual infection was identified as the most important cause of bTB breakdowns. This result is in accordance with studies conducted in other European countries where bTB is endemic. In Great Britain, Conlan et al. [37] suggested that up to 21% of herds could harbor the infection after the herd had been classified as bTB free. Moreover, historical bTB incidence has been evidenced as a robust predictor of the rate of future breakdowns in United Kingdom and Ireland [38,39,40,41]. The presence of false negatives animals due to failure of the skin test to detect all the infected animals could be regarded as an important reason to explain the large number of breakdowns attributed to residual infection. However, other factors might be also implicated. In Spain, beef and bullfighting herds are usually kept under extensive conditions in large pasture areas, particularly in Southern and Central regions of the country, which might hinder the testing of all animals [42]. On the other hand, in some breakdowns the incidence found when bTB was first detected at the farm was high (i.e., greater than 25%) which is unusual after a recent infection as bTB is believed to have a low transmission rate within a herd [43,44,45]. This could be suggestive of lack of good veterinary practice; however, the presence of other factors that could accelerate bTB transmission, such as the presence of infected males (i.e., could interact with a greater number of cattle and therefore infect a greater number of animals), should not be discarded. The infection appears to be poorly transmitted between cattle in most, but not all circumstances [40]. If this is the case, some of the breakdowns attributed to residual infection could have been misclassified. In addition, the association between previous infection and a breakdown could be not only due to persistence of infected cattle but also to exposure to other risk factors not reflected in the survey (related with lack of biosecurity in high incidence areas), what could induce a certain degree of overestimation of the importance of residual infection.

Herds might also get infected due to an external source. The second most frequent cause of breakdown was the interaction with bTB wildlife reservoirs. In central and southern Spain, high bTB prevalence has been detected in wild boar, red deer and fallow deer, and therefore they could constitute an important source of infection to cattle [23,24,25,46,47]. In the north of the country the prevalence of infected wildlife reservoirs seems to be lower and therefore their role as bTB reservoirs has been suggested to be of

Table 4. Most likely causes of bTB breakdowns with a single cause (i.e., those breakdowns where the difference between the first and second cause was greater than one point) and with two plausible causes (i.e., herds where the difference between the first and the second cause was less than one point); for these breakdowns we assigned 0.5 points to each cause.

	Most likely		1st most likely		2nd most likely	
Causes of infection	**Herds**	**Proportion**	**Herds**	**Proportion**	**Herds**	**Proportion**
Residual infection	83	35.6	111	66.1	30	17.9
Introduction of infected cattle	28	12	7	4.2	7	4.2
Presence of infected goats	10	4.3	6	3.6	8	4.8
Contiguous spread	36	15.5	20	11.9	18	10.7
Sharing of pastures	7	3	1	0.6	82	48.8
Interaction with wildlife	67	28.8	23	13.7	23	13.7
Contact with infected humans	2	0.9	0	0	0	0
Total	233		168		168	

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Table 5. Most likely causes of bTB breakdowns by type of herd.

	Beef	%	Dairy	%	Bullfighting	%
Residual infection	126	22.6	15	17.9	11.5	30.3
Introduction of infected cattle	22.5	4.0	7	8.3	5	13.2
Presence of infected goats	13.5	2.4	2.5	3.0	0	0.0
Contiguous spread	49.5	8.9	0.5	0.6	4.5	11.8
Sharing of pastures	42	7.5	1.5	1.8	5	13.2
Interaction with wildlife	85.5	15.3	0.5	0.6	3	7.9
Contact with infected humans	0	0.0	2	2.4	0	0.0
Unknown	219	39.2	55	65.5	9	23.7
*Total**	558		84		38	

** 7 farms not included (other types).*

doi:10.1371/journal.pone.0104383.t005

low importance [48]. Moreover, in the northern area there are a higher number of dairy herds with an intensive production system as compared to the central and southern areas of the country. This is to some extent in accordance with the results of our study where wildlife had a higher importance in the central and southern regions of the country. Nevertheless, the evaluation of the role of wildlife was limited by the fact that we did not have data about the presence of bTB in wildlife in the corresponding county for 211 out of 687 studied herds, and for those for which we had data, the molecular identification data were lacking from 260 herds. In 2012, a national surveillance program on bTB in wildlife was launched, and therefore, with the generation of new data, some uncertainty regarding the role of wildlife in different areas of Spain might be clarified.

The importance attributed to the introduction of infected cattle in this study has been lower than that reported in previous ones. In north-east England, Gopal et al. [14] identified the purchase of infected cattle as the most likely source of the infection in 30 of 31 bTB breakdowns. Wilesmith et al. [49] linked the 25% of the breakdowns detected in the period 1972–1978 in Great Britain to animal movements. In Northern Ireland, Denny and Wilesmith [13] based on bTB epidemiological investigations performed by veterinarians from the Department of Agriculture, reported that in 23% of the breakdowns detected in 1996 the source was the purchase of infected cattle. In our opinion, our result is influenced by the quality of the data: in the epidemiological questionnaire only those animal movements considered to pose a risk (i.e. from herds not qualified as officially free for the whole of the last three years) were recorded, and therefore, we did not have data from all the movements. More detailed tracing of animal movements, plus molecular data, would be needed to assess the role of animal movements in bTB breakdowns.

We decided to consider a cause of a herd breakdown only if the likelihood of occurrence was at least "low" (i.e. with a value of 5 in the ordinal scale). This was based on the rationale that those events with a value under 5 corresponded to situations with a negligible biological likelihood of being the cause of the breakdown (e.g. the herd did not have bTB reactors in the previous 3 years together with annual tests conducted each year and an incidence compatible with a recent infection; no cattle have entered into the herd within the date of infection and 1 year before the last negative test, etc). On the basis of this threshold, 27% of the studied herds (i.e. 185 out of 687) were classified as having an unknown cause of breakdown. The rest of "unknown" (i.e. 101 out of 687) corresponded to breakdowns with more than three plausible causes. A 42% of breakdowns with an "unknown" cause of infection are a high number. However, this percentage is in accordance with that reported in other studies from Ireland and Great Britain, where in 32% and 40% of the breakdowns, an infection source could not be established [13,49]. The determination of the origin of infections, especially in chronic diseases is a difficult task. Moreover, there is not a standard methodology to investigate the cause of a breakdown. Different approaches have been applied in order to determine the possible origin of different

Table 6. Most likely causes of bTB breakdowns by area.

	NORTH AND EASTERN	%	CENTER AND SOUTH	%
Residual infection	24	17.3	129.5	23.6
Introduction of infected cattle	8	5.8	27	4.9
Presence of infected goats	2.5	1.8	14.5	2.6
Contiguous spread	2	1.4	53	9.7
Sharing of pastures	13.5	9.7	35	6.4
Interaction with wildlife	11	7.9	79	14.4
Contact with infected humans	2	1.4	0	0.0
Unknown	76	54.7	210	38.3
Total	*139*		*548*	

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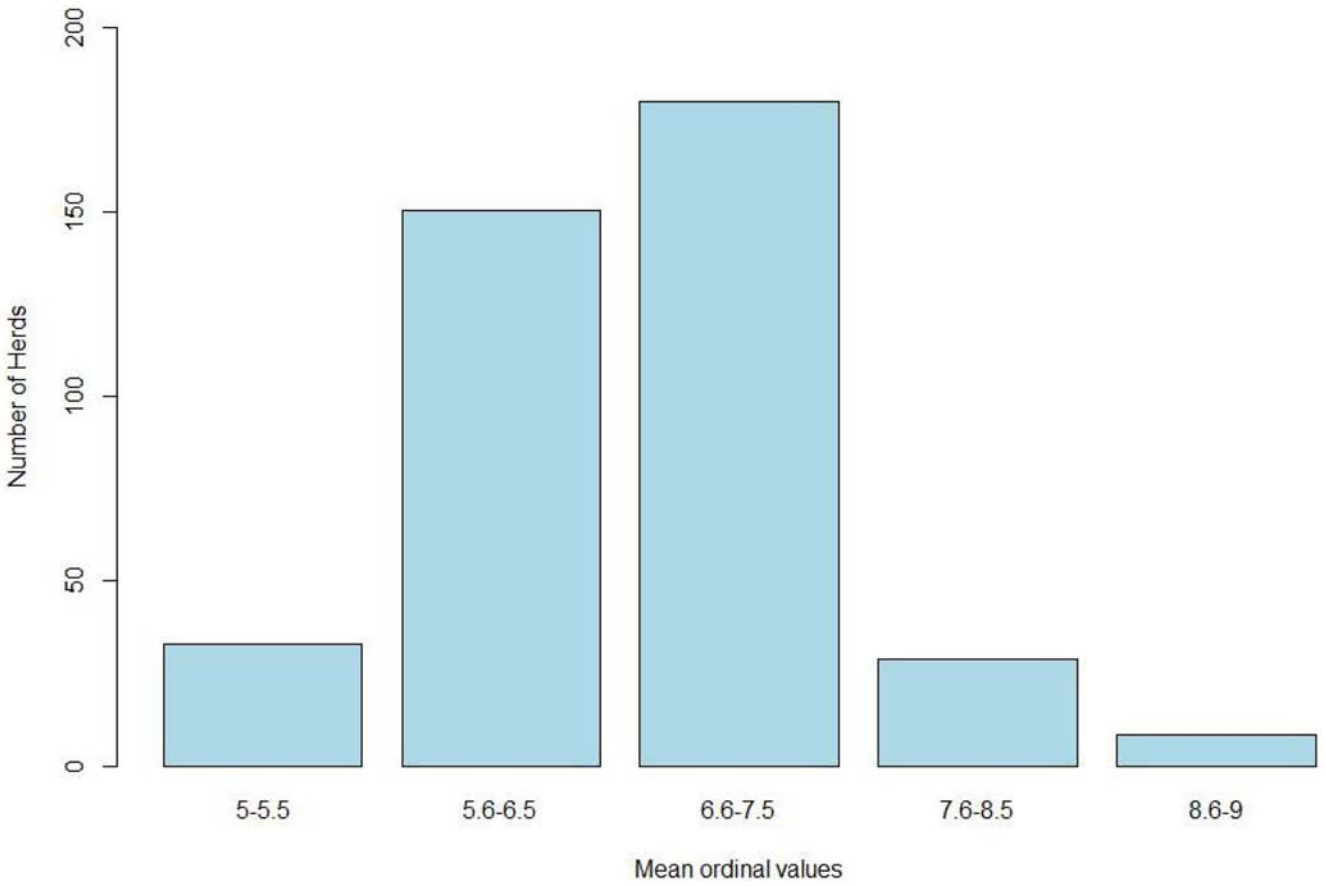

Figure 3. Distribution of the mean ordinal values associated with the most likely cause for each breakdown: ''5-.5.5'' corresponds to ''Low likelihood of occurrence''; ''5.6–6.5'' to ''Not very high likelihood of occurrence''; ''6.6–7.5'' to ''Quite high likelihood of occurrence''; ''7.6–8.5'' to ''High likelihood of occurrence'', and ''8.6–9'' to a ''Very high likelihood of occurrence''.
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diseases; Elbers et al. [50] used key questions to investigate the causes of infection of classical swine fever breakdowns in The Netherlands; the European Food Safety Authority [51] attributed different values to risk factors for bovine cysticercosis by using expert opinion. This methodology was adapted by Allepuz et al. [52] to investigate the most likely causes of infection of bovine cysticercosis in northeastern Spain. The decision trees developed in this study were designed and adapted to get the key information from each possible cause of breakdown. In our view, a key aspect of these decision trees is the assignment of a likelihood of occurrence to each possible event. In order to get estimates as objective as possible we decided to conduct an expert opinion workshop. We tried to reduce the possible bias associated with these estimates by including experts with different backgrounds (i.e. researchers working on domestic and wildlife bTB epidemiology, veterinarians working at regional and central administrations). However, there are inherent limitations derived from obtaining estimates from expert opinion workshops and it would be desirable to repeat this exercise in the future in order to update these values in the light of new scientific evidence about bTB epidemiology and including experts from other regions of Spain.

Moreover, in this study we did not consider some potential causes of infection as the interaction with other potential domestic reservoirs (such as pig or sheep). The role of pigs on bTB epidemiology has been traditionally considered of low importance as they are mainly kept in intensive systems and slaughtered at young ages [53]. However, in the western and southern Spanish regions there is an important population of Iberian breed pigs raised in a free-range system sharing natural resources with other wild and domestic animals. Moreover, in these areas there are reports of Iberian pigs infected with *M. bovis* with generalized lesions [54]. Reports of tuberculosis in sheep have been described in Italy [55] United Kingdom [56] and Spain [57] suggesting their potential to act as a reservoir for tuberculosis. The lack of data from these domestic species, together with the uncertainty regarding their role in bTB epidemiology in Spain made not possible to include them in the analysis. On the other hand, goats were not identified as a relevant cause of bTB breakdowns, which is not in accordance with their potential role in bTB epidemiology [11,12]. However, it has to be taken into account that just 52 out of the 687 herds reported to have goats in their herd, and only 9 of them had recorded the bTB test results on the survey.

By the development and application of this decision trees, we evaluated different possible causes of bTB breakdowns in the light of available data, and ideally, we should have had enough data in order to discriminate between them. However, for 53% of the breakdowns we did not have molecular data of the mycobacteria isolated in the herd, which limited the evaluation of the different causes, and especially the likelihood to a given cause. Molecular data missing could be due to no collection of the tissue samples at the abattoir, lack of recovery of mycobacteria by culture, typing in progress during the preparation of the manuscript or non-typable

Table 7. The most likely events within each cause of breakdown (see decision trees in figure S1 in File S1 for further clarifications).

Cause of breakdown	Event (value)	Herds	Percentage	Event
Residual infection	**E1 (6.1)**	7	4.6	Less than one annual test
	E2 (7.3)	56	36.5	Incidence not compatible with a recent infection
	E4 (5.6)	2.5	1.6	Reactors in the previous 3 years, but different spoligotype
	E5 (8.6)	5.5	3.6	Reactors in previous 3 years and the same spoligotype
	E6 (6.7)	82.5	53.7	Reactors in previous 3 years but spoligotype data lacking
	Total	153.5		
Introduction of infected cattle	**E3 (8.7)**	3	8.6	Herd of origin with the same spoligotype
	E5 (5.1)	14.5	41.4	Not known if the herd of origin was positive or if the same spoligotype was present in area of origin
	E6 (6.4)	2.5	7.1	Not known if the herd of origin was positive, but the same spoligotype was present in area of origin
	E8 (6.3)	12	34.3	Herd of origin was positive, but not known if the same spoligotype was present in area of origin
	E9 (7.7)	3	8.6	Herd of origin was positive, and a similar spoligotype was present in area of origin
	Total	35		
Presence of infected goats	**E4 (6.4)**	16	94.1	Goats present, but bTB status unknown
	E6 (7.3)	1	5.9	Positive goats, but spoligotype unknown
	Total	17		
Contiguous spread	**E2 (7.9)**	16.5	30.0	Positive neighbors and the same spoligotype
	E3 (5.1)	1	1.8	Positive neighbors but different spoligotype
	E4 (5.9)	23	41.8	Positive neighbors but unknown spoligotype
	E5 (7.1)	14.5	26.4	Positive neighbors (with unknown spoligotype) but same spoligotype in the area
	Total	55		
Sharing of pastures	**E4 (6.3)**	10.5	21.6	With positive herds, but spoligotype unknown
	E11 (6.0)	38	78.4	With other herds with unknown bTB status
	Total	48.5		
Interaction with wildlife	**E2 (5.3)**	4.5	5.0	Unknown if positive wildlife in the area
	E4 (7.6)	8.5	9.4	Positive wildlife in the area with the same spoligotype
	E5 (5.3)	12	13.3	Positive wildlife in the area, but different spoligotypes
	E6 (6.2)	39	43.3	Positive wildlife in the area, but spoligotype unknown
	E9 (6.4)	26	28.9	Positive wildlife in the area, with the same spoligotype (but not in hunting area)
	Total	90		
Contact with infected Human	**E1 (8.4)**	1	50.0	*M.tuberculosis* isolated in the herd, and history of cases in people
	E3 (5.1)	1	50.0	*M.tuberculosis* not isolated in the herd, but with history of cases in people
	Total	2		

Half values are due to those herds were the difference between the first and the second cause was less than one point. In these breakdowns two possible causes of infection were considered and we assigned 0.5 points to each cause.

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collected DNA. The molecular characterization of the different isolates in the breakdowns is essential to provide stronger evidence about the origin of the breakdown.

The comparison carried out between our results and those of the veterinary officers showed a poor agreement. Both methods (decision trees and the opinion of veterinary officers) have weak and strong points, and the reality could be somewhere between the results of both methods. The decision trees are an objective procedure based on expert opinion, group discussion and literature review. Besides, we were able to gather the information later, including some laboratory data that veterinarians might not have had when performing the survey. However, we did not know the particularities of the management, and facilities of each herd and the idiosyncrasy of the area. Besides, the veterinary officers had direct contact with the farm owners to get first hand information. Another likely source of discrepancy between our results and the ones of the veterinary officers is the importance attributed to the different epidemiological contacts. In our study the same criteria was applied to all the herds, while in the case of the veterinary officers there might be a higher heterogeneity due to

Table 8. Agreement between causes of breakdown determined by our study and those ones identified by official veterinarians in those herds where we both concluded one option.

	Our study	Veterinary Officer	Agreement	Kappa	IC 95%
Residual infection	38	35	12	0.16	0.03–0.31
Introduction of infected cattle	13	32	8	0.03	0.00–0.17
Presence of infected goats	4	8	0	0	
Contiguous spread	9	5	3	0.40	0.27–0.54
Sharing of pastures	2	3	2	0.79	0.65–0.93
Interaction with wildlife	26	59	12	0.11	0.00–0.23
Contact with infected humans	2	1	1	0.39	0.25–0.53
Unknown	96	47	38	0.30	0.17–0.42
Total	190	190	76		

IC95%: 95% confidence interval for the Kappa statistic.
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different regional or individual perceptions about the risk posed by the different epidemiological scenarios. It is remarkable the difference found in the importance attributed to the interaction with wildlife reservoirs. It would be desirable to harmonize the criteria used in the epidemiological investigations conducted by veterinary officers in order to get comparable results between and within the different regions of Spain.

In this study we have analyzed the most likely causes of breakdowns of the 22% of breakdowns detected on different regions of Spain between 2009 and 2011 which corresponds to all the data recorded in the BRUTUB system by 30th May 2011. The unavailability of data from the remaining breakdowns was due to the fact that BRUTUB system was first implemented in 2009 and has been gradually implemented in the different Spanish regions. When interpreting the results, it has to be taken into account that some regions are clearly under-represented and from some regions we did not have data from any breakdown. If there were differences in the causes of breakdowns among regions this would not be reflected in the results of our study. We believe that our results could give a good picture about the most likely causes of bTB herd breakdowns in Spain as we had data from different regions. Nevertheless, it would be desirable to update these analyses in the future when new breakdown data come available.

Conclusion

Residual infection seems to have an important role as a cause of bTB breakdowns in Spain. This result suggests that focusing efforts in the routine testing procedures in the bTB-positive and recently negative farms should result in an improvement of the eradication program. Nevertheless, it has been evidenced that external sources of bTB had also a relevant role as causes of breakdowns, and therefore measures directed at controlling these factors would be desirable. Interaction with wildlife reservoirs was especially important in the southern parts of the country evidencing that measures to minimize the interaction between infected wildlife reservoirs and domestic animals should contribute to the progress on the eradication of bTB. The high percentage of herds with an "unknown" cause of infection, especially high in areas of low prevalence (i.e., north and eastern parts of Spain), and in dairy herds, reflects the lack of relevant data to infer the most likely cause of breakdown. Gathering more detailed epidemiological information on bTB breakdown investigations together with molecular data would be desirable. The low agreement between the veterinary officer opinion and the results of our study might reflect a lack of harmonized criteria to assess the most likely cause of bTB breakdowns as well as different perceptions about the importance of the possible causes. This is especially relevant in the case of the role of wildlife reservoirs. When interpreting the result it has to be taken into account that a small percentage (i.e. 22%) of the total number of breakdowns detected in Spain between 2009 and 2011 were analyzed in this study, and therefore results have to be interpreted with caution. It would be desirable to update these analyses in the future when new breakdown data become available.

Supporting Information

File S1 Includes Figures S1–S2 and Tables S1–S4. **Figure S1**. Decision tree diagrams used for the bTB herd breakdowns investigation. **Figure S2.** Histogram of the standard deviations of the different events. **Table S1.** Main data contained in the epidemiological questionnaire carried out by veterinary officers in bTB herd breakdowns. **Table S2.** Background and expertise of the different national experts that participated in the workshop. **Table S3.** Mean ordinal values for each event together with the standard deviation (sd), minimum (min), median and maximum values (max). **Table S4.** Values given by the 9 experts in the expert opinion workshop.
(DOC)

Acknowledgments

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Author Contributions

Conceived the study: AA. Fulfilled the databases: SG. Developed the decision trees: SG JLS SN AGS JC AA. Assisted with the expert opinion workshop: SN. Performed the analysis with the help of JC and AA: SG. Provided data from different databases: JLS BPV BR JA. Wrote the first draft: SG. Reviewed the paper: All authors.

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Risk factors for bovine tuberculosis persistence in beef herds of Southern and Central Spain

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ABSTRACT

In order to assess risk factors related to bovine tuberculosis (bTB) persistence, a case–control study, comparing persistent versus transient bTB infected beef farms from Central and Southern Spain, was conducted. Farms were matched by herd size and geographical location (county). A questionnaire administered by personal interview was conducted on 150 herds (80 controls and 70 cases) from Andalucia and Castilla La Mancha regions. The questionnaire included questions related to the personnel involved in routine diagnostics, structure of the farm and of the herd, management, presence of other domestic species and of wildlife reservoirs.

According to the results of our study, farms with large pasture areas and bTB infected neighbors had more difficulties in eradicating the disease, and therefore, were more likely to suffer a persistent bTB infection. The odds of bTB persistence were between 1.2 and 5.1 (i.e., 95% confidence interval of the OR) times higher in those herds that had a neighbor infected herd. Farms with large pasture areas had odds between 1.2 and 12.7 (i.e., 95% confidence interval of the OR) times higher of having a persistent bTB episode than farms with small pasture areas.

1. Introduction

Bovine tuberculosis (bTB) is a chronic infectious disease of cattle (including all *Bos* species, and *Bubalus bubalus*) and bison (*Bison bison*) caused by any of the disease-causing mycobacterial species within the *Mycobacterium tuberculosis*-complex (Anon., 2013a).

The eradication of bTB has been an important issue over years due to its public health relevance and high economic impact in livestock production. Control programs, mainly based on the slaughter of animals positive to the tuberculin skin test (Reviriego-Gordejo and Vermeersch, 2006), have substantially reduced or nearly eradicated the disease from farm animals in many industrialized countries (EFSA, 2012). However, bTB is still widespread in Africa, Central and South America, parts of Asia and some Middle

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East countries (OIE, 2009). In Europe, despite the intensive eradication efforts applied over years, bTB continues to be present in countries such as the United Kingdom, Ireland, Spain, Greece, Portugal or Italy (EFSA, 2012). In Spain, the herd prevalence has been substantially reduced: from 11.1% in 1986 to 1.3% in 2012 (Anon., 2013b). However, in the last years, the decline has only been moderate: from 1.6% in 2007 to 1.3% in 2012. This slow progress poses a serious challenge for the achievement of a national official tuberculosis free (OTF) status.

In Spain, just recently, different studies have attempted to study bTB epidemiology in domestic animals (Alvarez et al., 2012; Allepuz et al., 2011; Rodríguez-Prieto et al., 2012; Martínez-López et al., 2013), but just one of them evaluated factors related to bTB persistence at farm level (Martínez-López et al., 2013). These authors found that previous bTB history, herd size, extensive production systems and a high number of fenced big game estates in the neighborhood of the farm were related to bTB persistence in farms from an area of South-Central Spain. The persistence of bTB in some cattle herds poses an important challenge to the eradication program, and therefore, improving the body of knowledge regarding those factors related to bTB persistence at herd level should be useful for disease management activities.

In addition to the limited knowledge about factors related to bTB persistence in Spanish cattle herds, most of the published bTB risk factors studies conducted in different countries did not discriminate between transient and persistent infections. The analysis of the causes of persistent infections has received little attention (Brooks-Pollock and Keeling, 2009; Wolfe et al., 2010), and differences on the factors that determine both situations might exist (Reilly and Courtenay, 2007). Persistent infections might be the result of the presence of infected but undetected cattle as a consequence of the lack of sensitivity of the test (De la Rua-Domenech et al., 2006) or due to the lack of good veterinary practice (Humblet et al., 2011). Moreover, *Mycobacterium bovis* could persist in the farm environment (Courtenay et al., 2006; Fine et al., 2011) or in other domestic reservoirs such as goats (Napp et al., 2013; Zanardi et al., 2013). Also, persistence of bTB within a farm may be the result of re-infections due to repeated contact with local wildlife or domestic reservoirs.

The aim of this study was to improve the understanding of bTB epidemiology in Spain by assessing which herd factors could be related to bTB persistent infections.

2. Materials and methods

2.1. Area of study

The study was conducted on farms from Southern and Central Spain (Andalucia and Castilla La Mancha). These areas were selected because of their higher risk of persistence as compared to the rest of the country (Allepuz et al., 2011).

2.2. Study design

A case–control study on beef farms matched by herd size and geographical location (at county level) was designed to detect Odds Ratio differences of 2.5, with a 95% level of confidence, 80% of power, and assuming exposure of 20% for the controls. A sample size of 200 (100 controls and 100 cases) was calculated.

2.3. Bovine TB eradication program in Spain

According to Council Directive 64/432/EEC, the Spanish bTB eradication program is based on testing of cattle and culling of positive animals. Moreover, cattle movement restrictions are implemented on infected herds in order to prevent the introduction of infected animals into free herds. Herds are classified as bTB free if no positive animals are detected within the herd in at least two consecutive follow-up herd tests, and as non-bTB free if at least one positive animal is detected. In each herd test, all animals older than 6 weeks of age are tested with the single intradermal test (SIT). In particular cases, where cross-reactions with other mycobacteria are suspected, single intradermal comparative cervical test (SICCT) may be used. In bTB free herds where positive animals are detected for the first time, the confirmation of bTB infection is carried out by tissue culture. Non-bTB free herds are tested at least 3 times per year. bTB free herds located in local veterinary units (i.e., counties) with prevalence higher than 3% are tested twice a year, otherwise they are tested once a year (Anon., 2013b).

2.4. Case–control definition and selection of farms

Case farms were defined as those farms in which bTB persisted for at least 5 consecutive years between 2002 and 2011, and control farms consisted of farms that achieved the elimination of the infection within a period of 1–2 years also between 2002 and 2011. Case farms were randomly selected among those that met the inclusion criteria, and control farms were matched to case farms based on herd size and location. For each case farm, given the herd size, and its location, provided by the regional governments of Andalucia and Castilla La Mancha, we first selected all the possible control farms for each case farm (i.e., same county and difference on herd size lower than 100 animals) and among them, we randomly selected one control for each case.

2.5. Questionnaire survey

An epidemiological questionnaire including potential risk factors for bTB persistence, based on existing literature, was designed. We included questions related to routine diagnosis (such as changes in personnel in charge of testing), structure of the farm (pasture area, number of holdings, etc.), structure of the herd (number of animals by age, breed, etc.), presence of other domestic species in the farm (goats, pigs, etc.), management (origin of purchased animals, feeding practices, etc.), wildlife reservoirs, health status of the herd and history of cases in people (full questionnaire available upon request). The

description of the questions included in the questionnaire can be found in the supplementary material. Questionnaires were administered by personal interviews with farm owners or veterinarians. Extra data (i.e., not obtained during the survey) was computed in order to include it in the analysis. The abundance of red deer in Spain was obtained at UTM 10 km × 10 km grid cells from Acevedo et al. (2010). The location of cattle farms and their bTB status between 2005 and 2010 were provided by the regional governments of Andalucia and Castilla La Mancha. From these data, and by using Quantum GIS software Version 1.8.0 (http://www.qgis.org/), the number of cattle farms and number of infected farms within a 5 km radius around each case and control farm were calculated.

2.6. Statistical analysis

The model was built following a series of steps (Dohoo et al., 2003):

(a) Bivariate analysis between the outcome (i.e., bTB persistent infection versus transient infection) and different predictor variables using a liberal p-value (we used $p < 0.30$). Categorical variables were screened using χ^2 test, and continuous variables with ANOVA or Kruskal–Wallis test. Bartlett's test for inequality of variances was applied to choose between both methods. In the case of non-homogeneity of variances the Kruskal–Wallis test was used.
(b) Evaluation of correlations among predictor variables: Spearman correlation coefficients were calculated among all those variables associated with persistence with a p-value lower than 0.30. In case of correlation (i.e., coefficient higher than 0.5), the variable with higher biological signification was retained.
(c) Before building the multivariable logistic model, and in order to avoid problems derived from non-linear relationships, quantitative variables were reclassified into four categories following their quartile distribution.
(d) A manual model-building selection was conducted for the development of the multivariable logistic regression model: as a first step we compared all the possible models with just one variable by the Akaike Information Criteria (AIC) value. To the model with the lowest AIC value and one predictor we included all the remaining covariates and compared them based on the AIC value. This process was repeated until the model with lowest AIC was obtained. This was considered as the most plausible one, and selected as the final model.
(e) Confounding was assessed by monitoring the changes in the model parameters when adding new variables. If substantial changes (i.e., higher than 20%) were observed in the regression coefficients, this was considered as indicative of confusion.
(f) Biologically meaningful interactions were tested and retained in the final model if the AIC value was reduced.
(g) To test the ability of the model to discriminate between cases and controls, we calculated a Receiver Operating Characteristic (ROC) curve, and the area under the curve (AUC). An AUC value greater than 0.8 and between 0.7 and 0.8 were considered as good and moderate discriminative capacities, respectively.

Epi Info 7 software (http://wwwn.cdc.gov/epiinfo/7/) was used for the bivariate analysis. The logistic multivariable analysis was conducted with SPPS software version 20.0.

3. Results

From the 200 farms initially selected for the study, some could not be surveyed for different reasons (refusal to be interviewed, farms belonging to same epidemiological unit, etc.), so the survey was carried out in 150 herds (80 controls and 70 cases). Categorical and quantitative variables included in the bivariate analysis are presented in Table 1 and Table 2, respectively. From the set of variables associated with the outcome, three variables were not considered for model building due to the presence of correlation with other predictor variables. Contact with pigs was correlated with presence of pigs (Rho = 0.73; $p < 0.05$). Contact with neighbors and contact with neighbor cattle herds were correlated with the variable contact with infected neighbor cattle herds (Rho = 0.60 and 0.55 respectively, both $p < 0.05$). The variables presence of pigs within the farm and contact with infected neighbor cattle herds were retained due to their higher biological significance.

The final multivariable logistic model included the presence of goats (OR = 3.7; 95% CI: 0.8–16.4; p-value = 0.08) and presence of pigs (OR = 0.4; 95% CI: 0.1–0.9; p-value = 0.03), replacement from positive mothers (OR = 2.2; 95% CI: 1.0–5.1; p-value = 0.06), contact with infected neighbor cattle herd (OR = 2.4; 95% CI: 1.2–5.1; p-value = 0.02), no isolation of test-positive cattle (OR = 2.1; 95% CI: 0.8–5.4; p-value = 0.14), and the size of the pasture area: not very large area (OR = 3.4; 95% CI = 1.0–11.6; p-value = 0.05); medium area (OR = 1.6; 95% CI = 0.6–4.7; p-value = 0.38) and large area (OR = 3.9; 95% CI: 1.2–12.7; p-value = 0.02). The different parameters estimated by the final multivariable model are represented in Table 3. The area under the ROC curve (AUC) calculated for this model was 0.75 (95% CI: 0.7–0.8), which means that the model had a moderate discrimination capacity.

4. Discussion

In this study we evaluated the association between different herd level factors and the success of bTB elimination from the herd by comparing transient and persistent bTB infected herds. We found that factors related to the structure of the farm, management and the presence of other domestic species could have an influence on the time needed to eliminate bTB from a herd.

Regarding associations related to the structure of the farm, we found that the odds of bTB persistence was between 1.2 and 5.1 times higher (i.e., 95% confidence interval of the OR) in herds in which contact with cattle from a neighbor infected herd was possible. This result is in accordance with previous studies. In Northern Ireland, Denny and Wilesmith (1999) reported that approximately 40% of breakdowns were attributed to the presence of a

Table 1
Categorical variables included in the bivariate analysis.

Categorical variables	Percentage		OR	95% CI		*p*-Value
	Case	Control		Lower	Upper	
Routine diagnostic tests						
Different company	48.6	50.0	0.9	0.5	1.8	0.861
Different veterinarian	34.3	36.3	0.9	0.5	1.8	0.801
Presence of other domestic species						
Presence of other species	62.9	65.0	0.9	0.5	1.8	0.785
Presence of sheep	25.7	20.0	1.4	0.6	3.0	0.404
Presence of goats	10.0	5.0	2.1	0.6	7.5	0.241[a]
Presence of pigs	17.1	32.5	0.4	0.2	0.9	0.030[a]
Presence of equines	31.4	30.0	1.1	0.5	2.1	0.849
Contact with sheep	15.7	11.3	1.5	0.6	3.8	0.422
Contact with goats	5.7	2.5	2.4	0.4	13.3	0.316
Contact with pigs	10.0	22.5	0.4	0.1	1.0	0.040[b]
Contact with equines	30.0	26.3	1.2	0.6	2.5	0.609
Structure of the farm						
More than one holding	34.3	27.5	1.4	0.7	2.8	0.368
Contact with neighbors	75.7	60.0	2.1	1.0	4.2	0.040[b]
Contact with neighbor goats	15.7	15.0	1.1	0.4	2.6	0.903
Contact with neighbor cattle	71.4	52.5	2.3	1.1	4.5	0.017[b]
Contact with neighbor pigs	2.9	2.5	1.1	0.2	8.4	0.892
Contact with infected neighbor cattle herd	55.7	35.0	2.3	1.2	4.5	0.010[a]
Drainage from infected farm	40.0	26.3	1.9	0.9	3.7	0.073[a]
Not fenced farm	10.0	8.8	1.2	0.4	3.5	0.792
Forest present within farm	71.4	73.8	0.9	0.4	1.8	0.75
Management						
Ecological farm	17.1	13.8	1.3	0.5	3.2	0.565
External origin of heifers	91.4	93.8	0.7	0.2	2.4	0.586
External origin of bulls	28.6	35.0	0.7	0.4	1.5	0.399
Replacement from positive mothers	38.6	18.8	2.7	1.3	5.7	0.006[a]
Transhumance (i)	2.9	2.5	1.1	0.2	8.4	0.892
Pasture sharing	8.6	10.0	0.8	0.3	2.6	0.764
Vitamin supplementation	44.3	43.8	1.0	0.5	2.0	0.947
Silage supplementation	91.4	93.8	0.7	0.2	2.4	0.586
Machinery sharing	2.9	3.8	0.8	0.1	4.7	0.761
Straw from other farm	10.0	15.0	0.6	0.2	1.7	0.358
No isolation of positive cattle	21.4	13.8	1.7	0.7	4.0	0.215[a]
Not all cattle tested	12.9	3.8	3.8	1.0	14.6	0.040[a]
Wildlife						
Neighbor game farm	41.4	40.0	1.1	0.6	2.0	0.858
Hunting residues within farm (ii)	12.9	8.8	1.5	0.5	4.4	0.416
Presence of wild boar	88.6	90.0	0.9	0.3	2.4	0.777
Presence of red deer	85.7	78.8	1.6	0.7	3.8	0.268[a]
Presence of fallow deer	35.7	35.0	1.0	0.5	2.0	0.927
Presence of roe deer	28.6	31.3	0.9	0.4	1.8	0.72
Presence of fox	94.3	90.0	1.8	0.5	6.4	0.334
Presence of badger	45.7	46.3	1.0	0.5	1.9	0.947
Presence of chamois	41.4	45.0	0.9	0.5	1.7	0.659

OR: odds ratio; 95% CI: 95% confidence interval of the OR; lower: lower limit of the 95% CI; upper: upper limit of the 95% CI.

[a] Variables considered for the multivariable analysis.

[b] Variables excluded from the multivariable analysis due to the collinearity.

(i) The term 'Transhumance' refers to the practice of seasonal movements of cattle related to feed availability. (ii) The term 'Hunting residues within farm' refers to the presence of offal from hunted and killed animals in the farm land which have not been eliminated after the animal has been hunted.

contiguous herd that had a confirmed breakdown. Local persistence of bTB infection has also been described as a key feature leading to recurrent episodes in United Kingdom and Ireland (Karolemeas et al., 2011; Kelly and More, 2010). This local persistence could be due to local movements (Green et al., 2008), contiguous spread by cattle-to-cattle transmission over farm boundaries (Denny and Wilesmith, 1999) or a consequence of the presence of a wildlife reservoir (Szmaragd et al., 2013; White et al., 2013). Another possibility could be persistence within the farm rather than re-infection from the outside, as it is difficult to differentiate which of these 2 mechanisms is responsible for the persistence of bTB within the farm (Szmaragd et al., 2013). Moreover, we did not get the whole bTB history results from neighboring herds, and therefore it was not possible to assess if the reported infected herds in the neighborhood were persistent or transient.

The exact transmission mechanisms involved in local bTB persistence are not clear. In the study conducted in Ireland by White et al. (2013), they concluded that an infected wildlife source was the best explanation for the existence of a neighboring herd risk at distances

Table 2
Quantitative variables included in the bivariate analysis.

Quantitative variables	Mean (sd)	25%	Median	75%	Max	*p*-Value
Structure of the farm						
Cattle farms within 5 km						
Control	19.1 (20.3)	6.5	14.5	25	138	0.41
Case	16.1 (15.3)	7	10.5	19	71	
Infected cattle farms within 5 km (2005–2010)						
Control	6.5 (5.5)	2	5	9	22	0.662
Case	6.9 (6.3)	3.5	5	9	30	
Farm pasture area (ha)						
Control	544.3 (880.7)	175	300	450	6000	0.0618[a]
Case	1029.4 (2539.8)	200	345	700	18,000	
Wildlife						
Deer density						
Control	11.3 (2.0)	10.7	12	12.5	14.8	0.061[a]
Case	10.6 (2.4)	9.7	11.6	12.1	14.2	
Management						
Mean time to slaughter of positive animals (days)						
Control	13.5 (6.3)	8.5	15	17	30	0.566
Case	15.4 (9.7)	7.5	15	20	50	
Structure of the herd						
Percentage of animals from 2 to 5 years						
Control	35.6 (14.7)	25.8	35.4	42.7	83.3	0.44
Case	32.7 (13.2)	24.5	32.1	41.4	89.3	
Percentage of animals from 5 to 10 years						
Control	35.1 (13.6)	26.5	34.2	43.6	73.6	0.76
Case	34.4 (12.1)	27.9	33.7	42.8	66.6	
Percentage of animals from 10 to 15 years						
Control	17.2 (10.5)	9.5	15.6	23.6	47.5	0.52
Case	16.1 (10.3)	9.7	14.2	21.2	63.8	
Percentage of animals older than 15 years						
Control	4.7 (3.3)	2.3	4.2	6.3	13.8	0.8
Case	5.2 (5.1)	2.1	3.8	6.6	28.5	

sd: standard deviation; 25%: first quartile; 75%: third quartile; Max: maximum value.
[a] Variables considered for the multivariable analysis.

greater than 25 m, and just explained some of the risk at distances lower than 25 m. These authors suggested that contiguous spread among cattle from neighboring farms had lower importance than infected wildlife reservoirs as a transmission mechanism to explain neighborhood persistence. However, these results might not be directly extrapolated to the situation in Spain, as the wildlife reservoirs are different. In Spain, wild boar and red deer are considered to be the main bTB reservoirs (Gortazar et al., 2011). The role of badgers in Ireland could be different of the role of wild boars or red deer in Spain.

As far as we know, in Spain, no studies have been conducted to assess the role of the different mechanisms in local persistence (local movements, contact with infected neighboring cattle, common wildlife reservoirs or residual infection within the herd). Further studies, attempting to gather more detailed data about the different mechanisms of local transmission would be desirable.

In relation to the structure of the farm, we found an association between persistence of bTB and the size of the pasture area: farms with large pasture areas had odds of having persistent bTB infection between 1.2 and 12.7 (i.e., 95% confidence interval of the OR) times higher than farms

Table 3
Results of the multivariate logistic regression model.

Variables	*B*	SE	Wald	df	*p*-Value	OR	95% CI	
							Lower	Upper
Presence of goats	1.32	0.76	3.02	1	0.08	3.7	0.8	16.4
Presence of pigs	−1.02	0.45	5.04	1	0.03	0.4	0.1	0.9
Replacement from positive mothers	0.80	0.42	3.58	1	0.06	2.2	1.0	5.1
Contact with infected neighbor cattle herd	0.89	0.37	5.69	1	0.02	2.4	1.2	5.1
No isolation of test-positive cattle	0.72	0.49	2.17	1	0.14	2.1	0.8	5.4
Area1								
Area2	1.23	0.62	3.87	1	0.05	3.4	1.0	11.6
Area3	0.48	0.54	0.78	1	0.38	1.6	0.6	4.7
Area4	1.37	0.60	5.22	1	0.02	3.9	1.2	12.7

B: coefficient estimated by the model; SE: standard error; Wald: Wald statistic; df: degrees of freedom; OR: odds ratio (i.e., exponential of *B*); 95% CI: 95% confidence interval of the OR; lower: lower limit of the 95% CI; upper: upper limit of the 95% CI. Area, 1, 2, 3 and 4 indicate the first, second, third and fourth quartiles of the farm pasture area. Area1 was used as the reference category for the pasture area variable.

with small pasture areas. In a review conducted in the United Kingdom, the independent Scientific Group (DEFRA, 2007) reported that an increase in farm land area was an important risk factor for bTB herd breakdowns in some regions of the country. The size of the pasture area could be related to different factors potentially linked to bTB persistence. It is reasonable to speculate that in farms with larger pasture areas, finding and testing all the animals may be more difficult, and also that there is an increased probability of interaction with neighboring infected herds or with infected wildlife reservoirs.

The mixed farm management system, characterized by handling of multiple species besides cattle, especially in the case of goats, which are reservoirs of *M. bovis* and *M. caprae*, could also contribute to the re-circulation of bTB in the herd (Zanardi et al., 2013). However, in our study, the presence of goats in the farm was not identified as a significant predictor of bTB persistence in the final model (OR = 3.7; 95% CI = 0.8–16.4). This could be related with the small number of farms that had goats. Only 11 farms (7 cases and 4 controls) had goats, which makes it difficult to draw conclusions. Nevertheless, the variable was kept in the final model, indicating that despite the lack of statistical significance, it was considered as a relevant piece of information to get the most parsimonious model (i.e., the model that explained the higher quantity of variation with the lower number of variables). Moreover, in the last years there have been some reports in Spain suggesting a role of goats in bTB epidemiology, and the need for further assessing their role (Napp et al., 2013). Testing of goats is only compulsory in farms with cattle and goats (Anon., 2013b). However, from the 11 herds which reported to have goats, just four had tested them (none with test-positive results).

Surprisingly, the farms where bTB had been eliminated in less than two years had pigs more frequently than farms where the infection remained for 5 or more years (OR = 0.4; 95% CI = 0.1–0.9). In the area of study (Southern and Central Spain) there is an important population of Iberian breed pigs, raised in a free-range system, sharing natural resources with other wild and domestic animals. The Iberian pigs infected with *M. bovis* have been reported to develop severe generalized infections with open lesions (Parra et al., 2003). Also, Di Marco et al. (2012) have reported that the Sicilian black pigs might act as a reservoir of bTB infection on the basis of the location of lesions, and the genetic profiles of *M. bovis* isolated during their study. Therefore, our results are in contradiction with previous studies, and the most likely explanation would be the presence of some confounding variable (such as different husbandry and management systems in farms with and without pigs), which was not measured during the survey. In view of these contradictory results, further studies are needed to elucidate the role of pigs in the epidemiology of bTB in Spain.

With regard to management-related factors, the replacement from test-positive mothers, and the failure to isolate test-positive animals were retained in the final model despite not having a statistical significant effect (p-value higher than 0.05). As in the case of the presence of goats within the farm, the fact that these variables reduced the AIC, and were retained during the variables selection procedure suggests that they could have a role in the probability of bTB persistence. Transmission from cow to calf by ingestion of infected colostrum or milk has been reported to have a very limited importance in countries where regular testing programs are implemented (Humblet et al., 2009). However, close contact between the calf and the infected mother could increase the likelihood of transmission via the inhalation of infected droplets released by the cow. Because of that, replacement from infected cows could contribute to recirculation of *M. bovis* within the farm, and therefore to a higher likelihood of persistence. Also, not isolating test-positive animals until slaughter could increase the risk of bTB persistence due to the increase of the time of contact with susceptible animals.

Not testing all the cattle was a variable with a significant effect in the bivariate analysis, but was not kept in the final model after the model selection procedure. Having troubles to test all the animals' increases the risk of leaving infected animals within the farm, and therefore increase the likelihood of bTB persistence. The limited number of farms that admitted that some animals were not tested (9 cases and 3 controls) make our results inconclusive. It has to be taken into account that obtaining reliable information on incorrect management practices from farm owners by ordinary interview is a very difficult task. Incorrect management practices could be related to the little importance attributed to bTB by some farmers. Therefore, multi-disciplinary approaches, including sociological studies, would be needed to quantify the importance of those factors on bTB persistence.

It is also important to take into account that the study was designed as a case–control study matched by type of herd (we just included beef herds), herd size and geographical location (i.e., county). By the application of this design we aimed to improve the power of our study (i.e., reduce the type II error) by comparing farms as similar as possible based on known risk factors, such as herd size (i.e., number of cattle within the farm) or type of herd (Reilly and Courtenay, 2007; Brooks-Pollock and Keeling, 2009; Karolemeas et al., 2011; Alvarez et al., 2012; Skuce et al., 2012; Martínez-López et al., 2013). Matching by county also had practical and logistical benefits, and also could control the possible presence of confounding factors such as management of bTB infected herds, etc. On the other hand, we might have hampered the analysis of the effect of those variables related to the area, such as the presence of bTB wildlife reservoirs. In fact, in a recent study carried out in one province from southern central Spain (Ciudad Real), Martínez-López et al. (2013) found that the presence of fenced hunting estates in the neighborhood of cattle farms increased the likelihood of suffering a bTB persistent breakdown.

Regarding variables related to the structure of the herd, age has been identified as a risk factor in different studies due to the fact that exposure to the disease increases with age, and the possible reactivation of the mycobacteria in older animals after a long period of time (Humblet et al., 2009). Due to the fact that our case–control study was matched by number of animals (i.e., herd size) we analyzed this variable as a percentage, i.e., the proportion of animals within a given age category. No association

between the age structure and the risk of persistence was found in the bivariate analysis. Nevertheless, as the age data was obtained during the personal interview, some lack of accuracy should be expected. Also, the current age structure may not be an accurate reflection of the age structure throughout the whole period of study. Therefore, this result should be interpreted with caution, and more accurate data would be desirable to assess the effect of the age structure of the herd on the probability of bTB persistence.

Most of the questions included in the epidemiological questionnaire such as if the veterinarian responsible for bTB testing had been the same, the presence of infected herds in the neighborhood or the presence of other domestic animals in the herd, referred to the situation in the last 10 years. However, collected data relied completely upon the answers given by the interviewed person, and some lack of accuracy in the responses might have occurred. If this is the case some associations found by this study could be biased. This possible lack of accuracy in some of the answers could also be responsible for the moderate discrimination capacity of the model as evidenced by the AUC of the ROC curve. Other approaches such as survival analysis with the inclusion of time-dependent variables may help to sort out those difficulties.

Also, it has to be taken into account that the interview could not be performed in some farms so the initial sample size of 200 farms was reduced to 150 (80 controls and 70 cases). This clearly limited the power of the study (i.e., the probability of finding statistically significant associations when they really exist), and therefore factors associated with the probability of bTB persistence may have been missed.

5. Conclusions

Farms with large pasture areas and bTB infected neighbors have more difficulties in eradicating the disease and therefore, are more likely to suffer a persistent bTB outbreak. Local spread seems to play a role in the maintenance of bTB within herds. However, the transmission mechanisms involved in this local persistence are not clear. Contiguous spread among neighbor herds, local movements, wildlife reservoirs and recirculation within the herd could be involved. Efforts should be made to ensure the compliance of the bTB eradication program. Further studies focused on the role of goats or pigs on bTB transmission and local bTB transmission mechanisms would be needed.

Conflict of interest

The authors declare that they are not in a situation of conflicting interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.prevetmed.2014.04.007.

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