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2 1 **Most likely causes of infection and risk factors for tuberculosis in Spanish cattle**
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5 2 **herds**

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48 21 **Keywords:** Bovine tuberculosis, epidemiological investigations, surveillance data, decision-tree
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50 22 models, case-control comparisons
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1
2 27 **Abstract**
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5 28 **Introduction:** This study aims to assess the most likely causes of bTB breakdowns in Spanish
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7 29 cattle herds and to identify the main risk factors at farm-level.
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10 30 **Methods:** Causes of bTB breakdowns were assessed through a qualitative risk-assessment based
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12 31 on decision-trees by analysing surveillance data from 3,819 bTB breakdowns detected during
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14 32 2014-2016. Results were compared to Veterinary Officers' opinions. Risk factors were identified
15
16 33 through a case-control study with data from 196 bTB cases and 160 controls collected during 2014-
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18 34 2018.
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22 35 **Results:** The decision tree analysis identified residual infections and interactions with wildlife as
23
24 36 the most frequent causes of breakdowns (36% each), followed by purchasing infected cattle (14%).
25
26 37 These results were not supported by Veterinary Officers' opinions. According to the regression
27
28 38 models, the risk of bTB increased by sharing pastures (OR=2.7;95%IC=1.6-4.4) and by increasing
29
30 39 inwards cattle movements. The presence of wildlife reservoirs represented a significant risk for
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32 40 extensively-managed farms if other cattle farms are situated within a one-kilometre radius
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34 41 (OR=2.3;95%IC=1.1-5.1).
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39 42 **Discussion:** To prevent bTB breakdowns, efforts should be devoted to decrease the likelihood of
40
41 43 residual infections and improve farm biosecurity. The adoption of biosecurity measures might be
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43 44 influenced by farmers' perceptions, which should be carefully evaluated to ensure the
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45 45 effectiveness of such strategies.
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51 INTRODUCTION

52 Bovine Tuberculosis (bTB) is a chronic disease of cattle caused by members of *the Mycobacterium*
53 *tuberculosis* Complex (MTC). *Mycobacterium bovis* and *M. caprae* are recognised as the causative
54 aetiological agents. However, it has been recommended to consider as a bTB case the infection in
55 cattle due to any disease-causing specie within the MTC[1-2]. Bovine Tuberculosis can be
56 transmitted from infected animals to humans (i.e. zoonotic TB)[3]. The transmission of bTB from
57 humans back to cattle or to other humans may also occur [4-5]. Although cattle are considered to
58 be the most important reservoir of bTB, the disease has been reported in a wide range of other
59 domestic and wild species that, in a diversity of situations, can act as reservoirs[2,6]. In Spain,
60 there is evidence that goats[7], extensively-farmed pigs[8], sheep[9], wild boar and red deer[10-
61 11] may play a role in the maintenance of the disease. Additionally, the environment itself might
62 contribute to maintaining viable MTC bacteria in water or soil[12-13].

63 The Spanish bTB eradication programme is based on a “test-and-slaughter” strategy as laid down
64 by the European Council Directive 64/432/EEC and the national Royal Decree 2611/1996. Within
65 the programme, cattle herds are routinely screened using the Single Intradermal Tuberculin Test
66 (SITT) as primary test. Herds are classified as bTB-free if no positive animals are detected for at
67 least two consecutive routine screenings. Animals that test positive (“reactors”) are culled and
68 subjected to *post-mortem* examination at the slaughterhouse. The routine screening is
69 complemented by the passive surveillance at the slaughterhouse (i.e. reporting of all suspicious
70 lesions for analysis and confirmation) and by the pre-movement testing of purchased cattle[14].
71 Confirmation of the infection is made by bacteriological culture and isolation of MTC bacteria.
72 The Oligonucleotide Typing (spoligotyping) method is also used to differentiate the isolated
73 strains and support epidemiological investigations[15]. In Spain there are about 117,000 cattle
74 farms of which more than 90% is annually tested for bTB.

75 The identification of both the origin of bTB outbreaks and the main factors that define the risk of
76 infection can give important indications for the design of effective prevention and control

1
2 77 strategies. According to the Spanish bTB eradication programme, for each newly infected herd
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4 78 confirmed by culture (i.e. bTB breakdown) a questionnaire-based epidemiological investigation is
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6 79 conducted by the Veterinary Officers (VOs) to identify the possible source of the breakdown. At
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9 80 the end of each questionnaire, the VOs should also indicate what in their opinion the most likely
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11 81 cause of the breakdown is. Since 2009, these data have been recorded in a national database named
12
13 82 BRUTUB which is held by the Spanish Ministry of Agriculture, Fisheries and Food (MAPA).
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15 83 Furthermore, from 2012 onwards, epidemiological questionnaires were also conducted on SITT-
16
17 84 negative farms (i.e. control farms) selected by the VOs in order to identify possible risk factors.
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20 85 These surveys are also recorded in the institutional BRUTUB database[14].
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23 86 Previously, Guta et al,[16] investigated the origin of the bTB breakdowns reported in Spain
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25 87 between 2009 and 2011 by analysing the data recorded in the BRUTUB database (22% of the total
26
27 88 breakdowns). The investigation showed that residual infection was the most frequent cause,
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29 89 followed by interaction with wildlife reservoirs. Since that study, the national eradication
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32 90 programme has been reinforced through the implementation of measures such as compulsory
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34 91 training courses for both private veterinarians conducting bTB testing and for VOs involved in
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37 92 management of the disease; and the strengthening of audits of field testing practices[14].
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39 93 Moreover, a surveillance programme for wildlife was implemented in order to further reduce the
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41 94 likelihood of bTB breakdowns[14]. Therefore, the present study aims to provide an update of the
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43 95 most likely causes of bTB breakdowns in Spain and to identify farm-level risk factors for bTB
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46 96 infection in cattle by comparing SITT-positive with selected SITT-negative herds.
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49 **MATERIAL & METHODS**

51 **Assessment of the most likely causes of bTB breakdowns**

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54 99 This study analysed surveillance data from bTB breakdowns (i.e. newly infected cattle herds
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56 100 confirmed by tissue culture) detected in Spain between January 1, 2014, and December 31, 2016.
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59 101 The data used were collected by the VOs through a standardized questionnaire and retrieved from
60
102 the BRUTUB database. The questionnaire (Spanish language version) is available on the web page

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2 103 of the Spanish Ministry of Agriculture, Fisheries and Food (MAPA)[17]. The information
3
4 104 collected included: i) Farm characteristics, management and practices: location, size, production
5
6 105 type, presence of domestic animals other than cattle, transhumance, use of shared pastures,
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8 106 implementation of quarantine, sharing fomites and machines with neighbouring farms, presence
9
10 107 of hunting residues within farm; ii) Cattle movements (previous 3 years): number of movements,
11
12 108 size of introduced batches, reactors among introduced animals; iii) Characteristics of the area:
13
14 109 presence of bTB-positive livestock farms within a one-kilometre radius, presence of natural parks
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16 110 and/or game farms within a one-kilometre radius, presence of wildlife reservoirs (bTB positive
17
18 111 wild boar, red deer, badgers, etc. by serology and/or MTC culture detected from the same
19
20 112 municipality in a 5-year period), access of wildlife to water points, feed or grazing areas; iv) Health
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22 113 information: results of SITT and laboratory testing (previous 3 years), presence of replacements
23
24 114 that were born from bTB-positive cow, presence of reactors among replacements from positive
25
26 115 mothers, history of TB in people linked to the farm. Additional epidemiological data (i.e. animal
27
28 116 movements, bTB status of herds and official testing results) were retrieved from other institutional
29
30 117 databases held by the MAPA; and, from the national database for animal tuberculosis
31
32 118 (mycoDB.es), held by the Center for Veterinary Health Surveillance (VISAVET), which contains
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34 119 spoligotype patterns of MTC isolates from domestic and wildlife animals[18].
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41 120 The most likely causes of breakdowns were identified by applying the qualitative approach based
42
43 121 on decision-trees which was developed by Guta et al,[16]. Hence, the same decision-tree models
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45 122 previously proposed[16] were used to estimate the likelihood of seven possible causes of
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47 123 breakdown:
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- 51 124 i) Reappearance of the infection in a bTB-free farm due to the presence of truly infected cattle
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53 125 which remained undetected during testing, resulting in an erroneous classification of the
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55 126 bTB status of herds (i.e. residual infections)
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57
58 127 ii) Purchase of undetected infected cattle.
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60 128 iii) Sharing of pastures with infected animals from other herds.

- 1
2 129 iv) Presence of infected goats on the farm.
3
4 130 v) Contiguous spread from infected neighbouring herds (i.e. farm-to-farm spread).
5
6 131 vi) Contact with infected wildlife reservoirs.
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8
9 132 vii) Contact with humans infected by *M. tuberculosis*.

10
11 133 For each bTB breakdown, separate models representing a series of related events were run for all
12
13 134 the causes considered. Tree diagrams started with a single key question (i.e. root node), which
14
15 135 branches into self-excluding occurrences (i.e. decision nodes); that, in turn, branch off into
16
17 136 different possible situations or events (i.e. end nodes). Nodes were weighted and categorized into
18
19 137 qualitative risk groups ranging between 0 (no risk) and 9 (extremely high risk) using the values
20
21 138 obtained by Guta et al.,[16] in the expert opinion workshop. The time window to consider an
22
23 139 epidemiological link was set at one year before the last routine screening with negative results. As
24
25 140 a result, each breakdown was associated to seven ordinal values which represented the likelihood
26
27 141 of occurrence of each of the seven considered causes. All causes with a value greater than or equal
28
29 142 to five were considered as probable origins of the breakdown in order to exclude only the pathways
30
31 143 associated with a negligible likelihood of occurrence. For one single cause to be considered as the
32
33 144 most likely origin of the breakdown, its associated value had to be at least one point higher than
34
35 145 the values of the other probable origins of the breakdown. When two or three different causes were
36
37 146 associated to a value greater than or equal to five, but the difference between them was less than
38
39 147 one point, all were considered as probable with an assigned value of 0.5 or 0.33, respectively.
40
41 148 Finally, when three or more causes showed the same likelihood, the cause of the breakdown was
42
43 149 considered as unknown. Further details of the methodology can be found in the work of Guta et
44
45 150 al.,[16].

46
47 151 Additionally, the most likely cause of the bTB breakdown identified by the risk assessment models
48
49 152 was compared with the most likely cause indicated by the VOs after conducting the
50
51 153 epidemiological investigation. In order to do so, the origin of the breakdown was considered
52
53 154 unknown when the Veterinary Officer reported three or more options; otherwise, a value of one

1
2 155 was assigned to the mentioned causes and zero to all the other considered causes. Similarly, each
3
4 156 of the most likely causes of breakdown that resulted from the risk assessment models was assigned
5
6 157 a value of one. Then, the degree of agreement was assessed through a concordance analysis, using
7
8 158 the Cohen's kappa measure[19]. Kappa values can range from -1 to $+1$, where negative and
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11 159 positive values can be interpreted as disagreement and agreement, respectively.
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14 160 **Identification of herd-level risk factors**

17 161 Recruitment and questionnaire

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19
20 162 The case-control study was performed using survey data collected by the Veterinary Officers in
21
22 163 the frame of the national bTB eradication programme, which were available in the BRUTUB
23
24 164 database. Farms surveyed as cases were cattle herds with a confirmed bTB breakdown (i.e. newly
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26 165 infected herds with presence of SITT-reactors animals and isolation of MTC species by
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28 166 bacteriological culture). Among those, all breakdowns detected between 2014 and 2018 which had
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31 167 available at least one control farm were considered for inclusion in the study. Control farms were
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33 168 SITT-negative cattle herds, which were selected by VOs at the time when the outbreak was
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35 169 confirmed, based on the herd size, production type (i.e. beef and dairy) and geographical location
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37
38 170 (i.e. same county or local veterinarian unit) of the related breakdown[14]. Among controls
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41 171 available in the BRUTUB database, only those that remained bTB-free at least for two consecutive
42
43 172 years prior to and within one year after the surveys, were recruited as controls for this study.
44
45 173 Accordingly, for each enrolled control farm, the associated bTB breakdown herd (i.e. case) was
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47 174 selected.
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49

50 175 The original language version of the standardized questionnaires can be found on the web page of
51
52 176 the MAPA[17]. Besides the outcome and the selection variables (i.e. variables on which the
53
54 177 selection of herds was based), 29 explanatory variables were derived from the survey answers and
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56
57 178 included: production type; farming system (i.e. Intensive: animals are kept indoor at high stocking
58
59 179 densities and fed on concentrated foodstuffs; Extensive: animals are kept mostly outdoor with total
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2 180 or partial provision of a territorial base and animal feeding is essentially based on grazing);
3
4 181 purchase of cattle (number and size of batches of cattle moved into the herd); herd management
5
6 182 and practices (i.e. drainage system and use of fertilizer, presence of replacements that were born
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8 183 from bTB positive cows, use of common grazing areas, etc.); health data (i.e. evidence of
9
10 184 Paratuberculosis, presence of SITT-reactor calves born from bTB positive cows, history of TB
11
12 185 cases among people, etc.); holding structures (i.e. drainage from or to other properties, hunting
13
14 186 waste within the farm, etc.); presence of sheep, goats or domestic carnivores; characteristics of the
15
16 187 neighbouring area (i.e. presence of other livestock farms or hunting game areas); and, presence of
17
18 188 wildlife reservoirs (i.e. red deer, wild boars, badgers etc. with positive serology and/or MTC
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20 189 culture-positive detected from the same municipality within a 5-year period).

25 190 Statistical analysis

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27
28 191 After excluding the explanatory variables with more than 10% of missing values, logistic
29
30 192 regression models were used to identify bTB herd-level risk factors. Data were analysed using
31
32 193 both conditional and unconditional methods; as the results were similar, for the final analysis
33
34 194 unconditional logistic regression was used on the complete dataset. To screen the putative factors,
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36 195 univariate analyses were conducted and variables associated with a p-value ≤ 0.25 were included
37
38 196 in the set of designated predictors. Correlation between variables was assessed by using either the
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40 197 Pearson or Spearman tests, depending on the nature of the studied variables; and, coefficient values
41
42 198 below -0.4 or above 0.4 were considered indicative of the existence of correlation. When
43
44 199 correlation was detected, the variable showing the largest impact on the Akaike Information
45
46 200 Criterion (AIC) of the univariate model was entered in the multiple regression models.

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48
49 201 An automated method based on an information-theoretical approach was used for the variable
50
51 202 selection process and to build a set of candidate models[20-21]. The basis for the selection of the
52
53 203 final model was the biological plausibility of interactions and the variables' impact on the model
54
55 204 performances. Model performances were evaluated looking at the discriminatory ability and
56
57 205 calibration of the final model. The discriminatory ability was assessed by computing the Area
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60

Under the Receiver Operating Characteristic curve (AUC)[22], where a value of 0.5 indicates no discrimination and values > 0.8 suggest excellent discrimination. The model calibration was assessed by applying the Hosmer-Lemeshow test[23] and the GiViTI calibration belt, which is a graphical approach[24]. *Post-hoc* comparisons were performed, and estimates were reported as odds ratios (ORs) and 95% Confidence Intervals (95% CIs). Variables and interactions associated with a p -value < 0.05 were considered significantly associated with the outcome (i.e. likelihood of being a bTB case). Statistical analyses were performed with Rstudio version 1.0.153[31], using packages “glmulti”[21], “Emmeans”[26], and “pROC”[27].

RESULTS:

Assessment of the most likely causes of bTB breakdowns

Descriptive results

Across the study period (2014-2016), 5,045 bTB breakdown cattle herds were detected in Spain of which 3,819 had the epidemiological questionnaire recorded in the BRUTUB database. Information on survey coverage is provided in Table 1 and Figure 1.

Year	bTB breakdowns	Surveys recorded (no.)	Survey Coverage
2014	1,148	645	56.2%
2015	2,204	1,536	69.7%
2016	1,693	1,638	96.8%
Total	5,045	3,819	75.7%

Table 1. Annual number of Bovine tuberculosis (bTB) breakdowns detected in Spain between 2014 and 2016 and number of surveys recorded in the BRUTUB database.

Out of 3,819 bTB breakdowns, 81.5% were detected through the routine screening of herds. Passive surveillance at the slaughterhouse contributed to the detection of 2.7% of the recorded breakdowns, and another 12.3% was detected due to an epidemiological link to a previously detected farm (i.e. farms with an epidemiological link to a confirmed or suspected bTB case are investigated). The information was not available for the remaining 3.5% of breakdowns. The vast majority of the surveyed farms were beef herds (98%), whereas dairy and bullfighting farms

228 represented 0.6% and 0.7%, respectively; the information was not available for 25 farms. The
 229 median size of the studied herds was 50 cattle (5th and 95th percentiles 15 and 212, respectively).
 230 With regard to the number of positive animals (i.e. reactors) detected in each breakdown, it ranged
 231 from one to three in 70% of the farms. However, in 1% of them the number of reactors was very
 232 high, reaching 30 or more detected animals.

233 Most likely causes of bTB breakdowns

234 The results of the qualitative assessment through decision-tree models highlighted that residual
 235 infections and interaction with infected wildlife reservoirs were the two most frequent causes of
 236 bTB breakdowns in Spain, followed by cattle movements (Table 2). Among farms which reported
 237 TB history in people (N=7), only for one farm was the transmission from humans to cattle one of
 238 two most likely causes of the breakdown, together with residual infection.

29 Most likely causes of infection	29 No. of herds	29 Proportion (%)	29 95% CIs
30 Residual infection	30 1,374	30 36.0%	30 34.4% - 37.5%
31 Contact with wildlife reservoirs	31 1,359	31 35.6%	31 34.1% - 37.1%
32 Purchase of cattle	32 526	32 13.8%	32 12.7% - 14.9%
33 Sharing of pastures	33 223	33 5.8%	33 5.1% - 6.6%
34 Neighbouring farms	34 116	34 3.0%	34 2.5% - 3.6%
35 Humans	35 0.5	35 0.01%	35 0.00001% - 0.1%
36 Presence of goats	36 0	36 0%	36 0.0% - 0.1%
37 Unknown	37 221	37 5.8%	37 5.1% - 6.6%
44 Total	44 3,819	44 100%	

239 Table 2. Most likely causes of Bovine tuberculosis (bTB) breakdowns in Spanish cattle herds assessed by using
 240 decision-tree models. Proportion of herds and 95% Confidence Intervals (95% CIs) are shown (decimals in the number
 241 of herds are due to the fact that when there were two or three possible causes, 0.5 and 0.3 have been accounted for
 242 respectively.)

243 In the opinion of the Veterinary Officers who carried out the epidemiological investigations, the
 244 two most frequent causes of the bTB breakdowns were the contact with wildlife reservoirs and the
 245 presence of infected neighbouring farms; whereas little importance was given to cattle movements
 246 (either cattle purchasing or movements to pastures) (Table 3).

Possible causes	Model results*	Veterinary Officers' opinion**	Kappa value (K)^	95% CIs	
				Lower	Upper
Residual infection	40.2%	17.8%	0.18	0.14	0.21
Contact with wildlife reservoirs	30.6%	68.4%	0.11	0.09	0.14
Purchase of cattle	32.6%	9.8%	-0.01	-0.05	0.04
Sharing of pastures	23.8%	10.5%	0.21	0.16	0.26
Neighbouring farms	11.8%	35.5%	-0.02	-0.06	0.02
Unknown	17.7%	9.4%	-0.08	-0.14	-0.03

247 Table 3. Cohen's kappa statistic results: level of agreement between decision-tree models and Veterinary Officers on
 248 the most likely causes of bTB breakdowns in Spain, 2014-2016. (*)Proportion of questionnaires in which a certain
 249 cause was identified by the model; (**)Proportion of questionnaires in which a certain cause was mentioned by the
 250 Veterinary Officer. (^)Kappa values (K= 1 full agreement; K= 0 Veterinary Officers' opinion and the models' results
 251 agreed as frequently as would have been expected by chance; K = -1 full disagreement) and 95% Confidence Intervals
 252 (95% CIs).

253 Identification of herd-level risk factors

254 A total of 356 farms (160 controls and 196 cases) were included in the risk factor analysis. The
 255 difference in number between cases and controls was due to the fact that 20 of the farms surveyed
 256 as controls were excluded because they later became infected (i.e. within one year after the survey).

257 Additionally, 16 control farms were removed because they were duplicated records (i.e. the same
 258 farm was selected as control for two or more different case farms). Farms included in this analysis
 259 were from two Autonomous Communities: Extremadura (southwest with bTB herd
 260 prevalence >10%, 209 herds) and Galicia (northwest with bTB herd prevalence <0.1%, 147 herds)
 261 (Figure 2); among them, 84% were small (0–50 heads) to medium (51–150 heads) size cattle herds,
 262 and the vast majority were beef farmed in extensive systems (306 out of 356).

263 Four out of 29 explanatory variables were excluded from further analysis because they had more
 264 than 10% of missing data: the presence of replacements that were born from bTB positive cows
 265 (68.5%); the presence of SITT-reactor calves born from positive cows (72.1%); the size of batches
 266 (i.e. number of cattle) moved into the herd within two years prior to the survey (24.3%); and, the
 267 practicing of transhumance (12%). Therefore, 25 explanatory variables were evaluated through

268 univariate logistic regression models to test their level of association with the bTB herd status, and
 269 results are shown in Table 4.

Predictors	Levels	Cases No. (%)	Controls No. (%)	ORs	95% CIs	p-value
<i>Farm management and practices</i>						
		Range: 0-33	Range: 0-16			
Number of inwards cattle movements [^]	discrete variable	Median (5th and 95th percentiles): 1 (0 - 9)	Median (5th and 95th percentiles): 0 (0 - 6)	1.08	1.01 - 1.17	0.03
Farming system	Intensive			baseline		
	Extensive	182 (92.9%)	141 (88.1%)	1.75	0.85 - 3.68	0.13
Sharing pastures*	No			baseline		
	Yes	106 (54.1%)	50 (31.2%)	2.59	1.68 - 4.03	<0.0001
Use of shared water points and/or common paths with other herds	No			baseline		
	Yes	87 (44.4%)	64 (40%)	1.2	0.78 - 1.83	0.41
Use of manure as fertilizer	No			baseline		
	Yes	114 (58.2%)	107 (66.9%)	0.69	0.44 - 1.06	0.09
Use of sewage water as fertilizer	No			baseline		
	Yes	66 (33.7%)	55 (34.4%)	0.97	0.62 - 1.51	0.89
Presence of hunting waste within the farm	No			baseline		
	Yes	6 (3.1%)	1 (0.6%)	5.02	0.85 - 95.35	0.14
<i>Holding structures</i>						
Presence of permanent water points	No			baseline		
	Yes	132 (67.3%)	101 (63.1%)	1.2	0.78 - 1.87	0.41
Receive drainage from other properties	No			baseline		
	Yes	52 (26.5%)	30 (18.8%)	1.56	0.95 - 2.62	0.08
Offer drainage to other properties	No			baseline		
	Yes	59 (30.1%)	44 (27.5%)	1.14	0.72 - 1.81	0.59
Presence of a perimeter fence	No			baseline		
	Yes	160 (81.6%)	131 (81.9%)	0.98	0.57 - 1.69	0.95
<i>Presence in the farm of domestic species other than cattle</i>						
Presence of sheep	No			baseline		
	Yes	29 (14.8%)	14 (8.8%)	1.81	0.94 - 3.65	0.08
Presence of goats	No			baseline		
	Yes	9 (4.6%)	2 (1.2%)	3.8	0.96 - 25.18	0.09
Presence of other domestic animals	No			baseline		
	Yes	37 (18.9%)	28 (17.5%)	1.1	0.64 - 1.9	0.74
Sharing water and feed with domestic species	No			baseline		
	Yes	52 (27.2%)	38 (24.8%)	1.13	0.7 - 1.85	0.62
Presence of companion animals with access to feed bins	No			baseline		
	Yes	55 (28.1%)	26 (16.2%)	2.01	1.2 - 3.43	0.01
<i>Herds' health information</i>						
Evidence of parasitisation	No			baseline		
	Yes	16 (8.2%)	9 (5.6%)	1.49	0.65 - 3.61	0.35
Evidence of paratuberculosis	No			baseline		
	Yes	15 (7.7%)	2 (1.2%)	6.55	1.81 - 41.95	0.01
Tuberculosis human cases	No			baseline		
	Yes	4 (2.1%)	1 (0.6%)	3.26	0.48 - 64.19	0.29
<i>Characteristics of the neighbouring area</i>						
Presence of cattle farms within a one-kilometre radius	No			baseline		
	Yes	157 (80.1%)	113 (70.6%)	1.67	1.03 - 2.74	0.04
Adjoining to natural reserve	No			baseline		
	Yes	183 (93.4%)	149 (93.1%)	1.04	0.44 - 2.39	0.93
Adjoining to hunting game area	No			baseline		
	Yes	129 (65.8%)	108 (67.5%)	0.93	0.59 - 1.44	0.74

<i>Wildlife and hunting</i>						
Presence of wild boar and/or red deer	No			baseline		
	Yes	172 (87.8%)	128 (80%)	1.79	1.01 - 3.22	0.05
Presence of wild carnivores (e.g., badger or foxes)	No			baseline		
	Yes	161 (82.1%)	121 (75.6%)	1.48	0.89 - 2.49	0.13
Access of wildlife to feed, water or pasture	No			baseline		
	Yes	177 (90.3%)	134 (83.8%)	1.81	0.96 - 3.44	0.07

270 Table 4. Descriptive and univariate logistic regression results (Odds Ratio (ORs) and 95% confidence intervals (CIs))
 271 for herd-level risk factors associated with Bovine tuberculosis (bTB) infection. The results are based on 356 surveys
 272 from Spanish cattle farms recorded in the BRUTUB database between January 1, 2014, and December 31, 2018. (^)
 273 Definition for “Number of inwards cattle movements”: number of batches of cattle moved into the herd within two
 274 year prior to the survey (*) Definition for “Sharing pastures”: use of community pastures and/or shared use of grazing
 275 areas and/or access to contaminated grazing areas.

276 Fourteen explanatory variables resulted associated with bTB with a p-value ≤ 0.25. Among these,
 277 the presence of wild carnivores and the access of wildlife to feed, water supply and/or to cattle
 278 grazing areas, were excluded from the multivariate model because they were positively correlated
 279 with each other and with the presence of deer and wild boar (coefficient values ≥ 0.5; p-values <
 280 0.001). The final model included six main effects and three interactions between predictors.
 281 Adjusted Odds Ratios (aORs) and respective 95% Confidence Intervals (95% CIs) are shown in
 282 Table 5.

Predictors	Levels	aORs	95% CIs	p-value
<i>Predictors without interactions</i>				
Sharing pastures* (LRT p-value < 0.001)	No	baseline		
	Yes	2.68	1.64 - 4.43	0.0001
Presence of companion animals with access to feed bins (LRT p-value < 0.01)	No	baseline		
	Yes	2.47	1.38 - 4.4	0.002
<i>Number of in-farm movements and interaction with Presence of cattle farms within a one-kilometre radius</i>				
Number of inwards cattle movements^ (LRT p-value < 0.001)	NO movements & NO neighbouring farms	baseline		
	Movements (per batch) & NO neighbouring farms	1.32	1.11 - 1.64	0.006
	Movements (per batch) & YES neighbouring farms	1.08	1.01 - 1.19	0.044
<i>Farming System and interaction with Presence of wild boars and red deer</i>				
Farming System (LRT p-value < 0.05)	INTENSIVE farming & NO wildlife reservoirs	baseline		
	EXTENSIVE farming & NO wildlife reservoirs	0.34	0.09 - 1.26	0.11
	EXTENSIVE farming & YES wildlife reservoirs	3.24	1.01 - 10.3	0.048
<i>Presence of neighbouring farms and interaction with Presence of wild boars and red deer</i>				
Presence of cattle farms (within a one-kilometre radius) (LRT p-value < 0.05)	NO neighbouring farms & NO wildlife reservoirs	baseline		
	YES neighbouring farms & NO wildlife reservoirs	0.52	0.12 - 2.35	0.4
	YES neighbouring farms & YES wildlife reservoirs	1.86	1.03 - 3.35	0.04
<i>Presence of wildlife reservoirs and interaction with Farming System and Presence of cattle farms within a one-kilometre radius</i>				

		NO wildlife & INTENSIVE farming & NO neighbouring farms	baseline		
		YES wildlife & INTENSIVE farming & NO neighbouring farms	0.07	0.01 - 0.45	0.005
	Presence of wild boars and red deer (LRT p-value<0.01)	YES wildlife & INTENSIVE farming & YES neighbouring farms	0.25	0.05 - 1.31	0.099
		YES wildlife & EXTENSIVE farming & NO neighbouring farms	0.65	0.14 - 3.15	0.6
		YES wildlife & EXTENSIVE farming & YES neighbouring farms	2.33	1.06 - 5.12	0.035

Table 5. Multivariable logistic regression model results (adjusted Odds Ratio (aORs) and 95% confidence intervals (CIs)) for herd-level risk factors associated with Bovine tuberculosis (bTB) breakdowns in Spanish cattle herds. The results are based on a case-control study conducted on 356 cattle farms surveyed between January 1, 2014, and December 31, 2018. LRT: Likelihood Ratio Test (*) Definition for “Sharing pastures”: use of community pastures and/or shared use of grazing areas and/or access to contaminated grazing areas. (^) Definition for “Number of inwards cattle movements”: number of batches of cattle moved into the herd within two year prior to the survey.

Results evidenced that the risk of bTB increased by using pastures and by the number of inwards cattle movements in the last two years. Moreover, the presence of red deer and/or wild boars resulted to be a significant risk factor for extensively-managed cattle farms, and when there were other cattle farms within a one-kilometre radius (Table 5). The ROC curve for the final model retrieved an AUC of 71% (95%CI: 66% to 77%) indicating an acceptable ability to discriminate between case and controls; the Hosmer–Lemeshow test was not significant (χ^2 test statistic 5.8, df = 8, p-value = 0.7) as well as the Calibration Belt test (p-value= 0.5), showing a reasonable fit between observed and predicted values.

DISCUSSION

The relative importance of bTB risk factors and sources of infection may change over time and upon local contextual conditions[28-29]; thus, their continuous re-evaluation is necessary to ensure the effectiveness of national bTB control strategies. By analysing surveillance data of over 3,800 SITT-positive cattle herds detected during 2014-2016, this study provides an update of the most likely causes of bTB breakdowns previously identified in Spain (2009-2011)[16]. Additionally, bTB herd-level risk factors were identified through a case-control study, using surveys of 356 farms recorded in the institutional database BRUTUB between 2014-2018.

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2 305 With regard to the most likely causes of bTB breakdowns assessed by using decision-trees,
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4 306 residual infections together with interaction with wildlife reservoirs remain the most important
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6 307 causes of bTB breakdowns in the country. Compared with the previous investigation[16], the
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8 308 current study has far greater survey coverage (76% versus 22% of detected breakdowns), a better
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10 309 geographical representativeness (16 versus 10 Autonomous Communities), and the proportion of
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12 310 breakdowns for which the origin of infection remained unknown was strongly reduced (from 42%
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14 311 to 5.8%), which reflects an improvement of both the quality of survey data and the availability of
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16 312 information on spoligotypes. Nevertheless, the most frequent causes of bTB breakdowns identified
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18 313 in both studies did not differ much.

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23 314 Similar to Guta et al.,[16], there was a low agreement between the results of the risk-assessment
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25 315 based on decision-trees and the opinion of the Veterinary Officers (VOs). Results indicate that
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27 316 VOs overestimated the importance of factors out of their own control, as for example the presence
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29 317 of wildlife reservoirs or the density of cattle farms in the neighbouring area. This could be related
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31 318 to personal perceptions about the risk that wild animals pose to cattle farms or about the perceived
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33 319 lack of control over the disease[30]. Another hypothesis for the observed low agreement might be
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35 320 due to time constraints for the VOs who might sometimes fail to make an exhaustive evaluation
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37 321 of the case before stating their opinion. Moreover, since VOs might not have received proper
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39 322 training (especially on administration of questionnaires and data collection) both assessments
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41 323 could be biased due to a lack of quality in collected data (i.e. the decision-tree results) or a lack of
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43 324 objectivity in the evaluation of data (i.e. VOs opinions). In order to better understand these
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45 325 incongruences, it would be interesting, and useful, to conduct further research to identify the
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47 326 underlying reasons.

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53 327 The high proportion of bTB breakdowns linked to residual infections observed in this study could
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55 328 be partially explained by some additional measures which were implemented to reinforce the
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57 329 Spanish bTB eradication programme. In particular, since 2012, compulsory training courses for
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59 330 veterinarians involved in the bTB programme were established, and audits on testing practices

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2 331 were intensified. It is, therefore, possible that the diagnostic sensitivity in the field has increased
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4 332 through the incorporation of such initiatives and that several of the previously undisclosed bTB
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6 333 cases have been detected. Taking this into account, for future studies it would be interesting to
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8 334 evaluate whether the importance of the residual infections as cause of bTB breakdown has been
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10 335 reduced. Besides, the strong presence of beef cattle farms (98%; 99%CI: 97% - 99%), mostly kept
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12 336 in extensive conditions, could also have slightly contributed to the observed proportion of
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14 337 breakdowns linked to residual infections. As a matter of fact, extensively-managed herds and
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16 338 reduced handling of animals have been linked to the lack of SITT performance[31-32]. Such
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18 339 conditions can be quite common in Spain, especially in the Central and South-Western areas (i.e.
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20 340 *dehesas* systems)[33], increasing the likelihood of bTB infected cattle being left undetected within
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22 341 a herd[34-35].
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28 342 Contact with wildlife reservoirs was the other most likely cause of bTB breakdowns identified in
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30 343 this study, showing a significant increase compared with findings of Guta et al.[16]. Their paper
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32 344 described some limitation in assessing the importance of wildlife as a source of bTB breakdown,
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34 345 such as insufficient information about the presence of bTB in wildlife and the spoligotype of
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36 346 isolates. Here, these constraints were overcome, also due to new data generated through the
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38 347 national wildlife surveillance programme introduced in 2012[36]. In Spain, the role of the wildlife
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40 348 component in the bTB maintenance system is well documented and heterogeneous across the
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42 349 country[10,35,37-38]. Interestingly, the current study pointed out that the presence of wildlife
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44 350 reservoirs increases the risk of bTB only for extensively-managed farms and if other cattle farms
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46 351 are present in the neighbouring area. This result might indicate that local transmission of bTB
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48 352 between wildlife reservoirs and cattle occurs especially on farms where the implementation of
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50 353 biosecurity measures is more challenging and that interactions with wildlife might become more
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52 354 relevant in areas with a high density of both cattle herds and wildlife reservoirs (i.e. red deer and
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54 355 wild boar)[39]. Therefore, to improve biosecurity in extensively-raised cattle farms is of
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56 356 paramount importance in order to reduce the likelihood of contact between domestic and wild
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2 357 animals and, thus, reduce the risk of transmission of bTB. A few initiatives, which investigate
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4 358 concrete biosecurity measures to be applied in this type of farms, have been undertaken[40-41]
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6 359 and further research in this line would be beneficial. Additionally, recommendations on biosecurity
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8 360 practices have also been integrated into an action plan on bTB in wild species which was launched
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10 361 at national level in 2017 (Royal Decree RD128/2020)[42]; and, its impact on the relative
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12 362 importance of wildlife as cause of breakdowns in cattle should be assessed in the future.
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14 363 Furthermore, it should be taken into account that farmers' decisions on the adoption of biosecurity
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16 364 can also be influenced by the perceived feasibility of the proposed measures and other
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18 365 psychosocial factors[43-44]. It is, therefore, necessary to achieve a better understanding of these
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20 366 aspects to ensure changes in farmers' practises and the implementation of biosecurity at farm level.
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25 367 The proportion of bTB breakdowns due to the introduction of purchased cattle was also high, and
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27 368 the number of inwards movements resulted to be a significant bTB risk factor, as also reported
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29 369 elsewhere[28,32,45]. The purchase of infected cattle was the third most frequent cause of bTB
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31 370 breakdowns in spite of the fact that, in Spain, movements of cattle are subjected to a compulsory
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33 371 pre-movement test since 2006 in order to reduce the risk of bTB spread by trade. The observed
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35 372 result might be linked to the lack of sensitivity of pre-movement surveillance; it is known that
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37 373 recently infected cattle may fail to respond to the SITT[46]; thus, especially in low bTB prevalence
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39 374 areas, it might occur that truly infected cattle are missed by the SITT. Besides, it has been
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41 375 highlighted that the sensitivity of this surveillance component can vary significantly among areas,
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43 376 being influenced also by the size of batches and of the herd of origin (i.e. higher sensitivities
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45 377 associated with larger sizes of the batches or with smaller sizes of the herds)[47]. Additionally,
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47 378 non-authorized/unrecorded movements of cattle might occur[48].
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53 379 In accordance with findings from other countries[28,32,48-49], other risk factors for bTB in cattle
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55 380 identified in this study were associated with wildlife/livestock densities and husbandry practices,
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57 381 such as the sharing of pastures and grazing areas which was linked to direct and indirect contacts
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59 382 between herds or with wildlife reservoirs[28,48-49]. Herd size and production type are also
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2 383 acknowledged factors increasing the bTB risk [28,32,45,49]; in this study, their effect was
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4 384 controlled in the selection process of surveyed farms.

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7 385 Some limitations have to be taken into account when interpreting the presented findings. With
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9 386 regard to the case-control study, assuming an exposure of 20% for the controls, the number of case
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11 387 and control farms surveyed allowed us to detect OR estimates equal or higher than 2.1 (smallest
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13 388 odds-ratio > 1) with a 95% level of confidence and a power of 90%. Therefore, it is possible that
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15 389 some non-significant effects were due to the sample size not being large enough if differences
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17 390 were smaller or rates of exposure were lower. For example, the presence of hunting waste was
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19 391 described in only 7 farms and it was not identified as a risk factor, whereas other authors found
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21 392 that eliminating this waste from the farms reduced the risk of bTB infection from wildlife[50]. The
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23 393 results of this study could have some underlying misclassification bias due to the lack of diagnostic
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25 394 sensitivity of the SITT in the field. To reduce such biases, cases were farms confirmed as infected
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27 395 by isolation of MTC species, and only control farms which remained SITT-negative for three
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29 396 consecutive annual testing (i.e. routine screenings) were selected for inclusion in our analysis.
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31 397 However, results might be affected by biases introduced during the collection of exposure and
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33 398 outcome data due to previous knowledge on the bTB status of farms which are intrinsic to the
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35 399 retrospective design of the study. With regard to the decision-trees analysis, results might be
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37 400 limited by the quality and completeness of data collected as part of the routine bTB surveillance.
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39 401 Moreover, some other aspects, such as exchanges and sharing practices between farmers, fomites
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41 402 and spread from the environment were not considered in this study; this could have led to some
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43 403 overestimation when quantifying the importance of the residual infections or interactions with
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45 404 wildlife. Finally, since VOs who conduct the surveys receive limited training specifically for
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47 405 interviewing, selection bias and bias specific to questionnaires (i.e. administration) might have
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49 406 also occurred; this limitation could potentially be overcome by improving interviewers' skills
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51 407 through a more specialised training. Despite limitations, the here presented study contributes to
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1
2 408 the knowledge about bTB infection risks, using data which were already available from
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4 409 institutional databases.
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7 410 In conclusion, this study highlighted that in Spain residual infections and the transmission of bTB
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9 411 at the cattle-wildlife interface are the most important causes of breakdowns, followed by inwards
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11 412 cattle movement. Improving the execution and interpretation of SITT is crucial for the correct
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13 413 classification of the herds' bTB status; substantial progress has been made and continuous
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15 414 communication and training on bTB is indispensable to maintain a high awareness level among
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17 415 farmers and veterinarians. However, it is unlikely that this alone will significantly reduce the risk
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19 416 of bTB infection all over the country; and, simultaneous interventions to address different bTB
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21 417 sources may be needed for an efficient control of the disease. The implementation of biosecurity
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23 418 represents an effective tool for the prevention of bTB, with the advantage of preventing several
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25 419 other infectious diseases at the same time. Nevertheless, especially for some production systems,
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27 420 it can be hindered by costs and holding structures, or farmers' perceptions; such factors need to be
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29 421 carefully considered to ensure the adoption of biosecurity measures or changes in management
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31 422 practices.
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1
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5
6 434 authors have no conflicts of interest to declare.
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9

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11
12 436 All the authors listed have contributed significantly to the work, took part in the interpretation of
13
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17
18 439 application; study design; data analysis; evaluation of findings; guarantor for the overall content
19
20 440 of the manuscript. JC: project funding application; study design; data analysis planning; evaluation
21
22 441 of findings. JLS: acquisition and validation of data; evaluation of findings. MLG: literature review;
23
24 442 analysis planning; preparation, cleaning and analysis of data. GC: literature review; analysis
25
26 443 planning; data analysis; evaluation and reporting of findings; manuscript writing, in consultation
27
28 444 the other co-authors, and editing.
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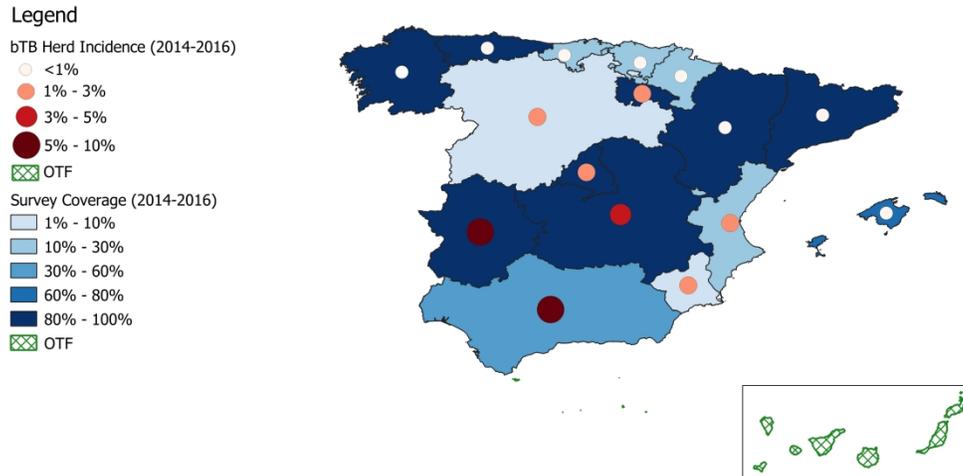


Figure 1. Geographical distribution of surveys recorded in the national BRUTUB database during the studied period (2014 - 2016). The proportion of investigated bovine tuberculosis (bTB) breakdowns by Autonomous Community (survey coverage) is represented in different gradient of blue. The bTB herd incidence is indicated with circles of different sizes and gradient of red. OTF: Officially Tuberculosis Free.

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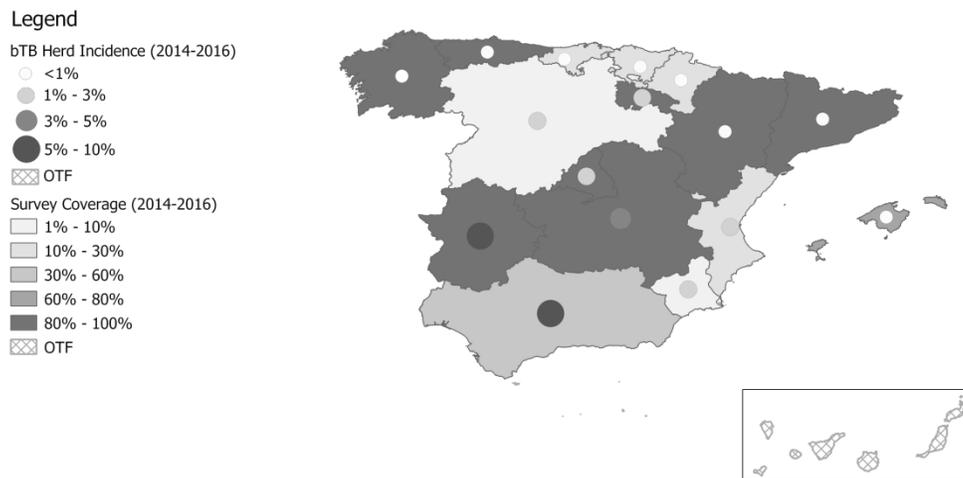


Figure 1. Geographical distribution of surveys recorded in the national BRUTUB database during the studied period (2014 - 2016). The proportion of investigated bovine tuberculosis (bTB) breakdowns by Autonomous Community (survey coverage) is represented in different gradient of grey. The bTB herd incidence is indicated with circles of different sizes and gradient of black. OTF: Officially Tuberculosis Free.

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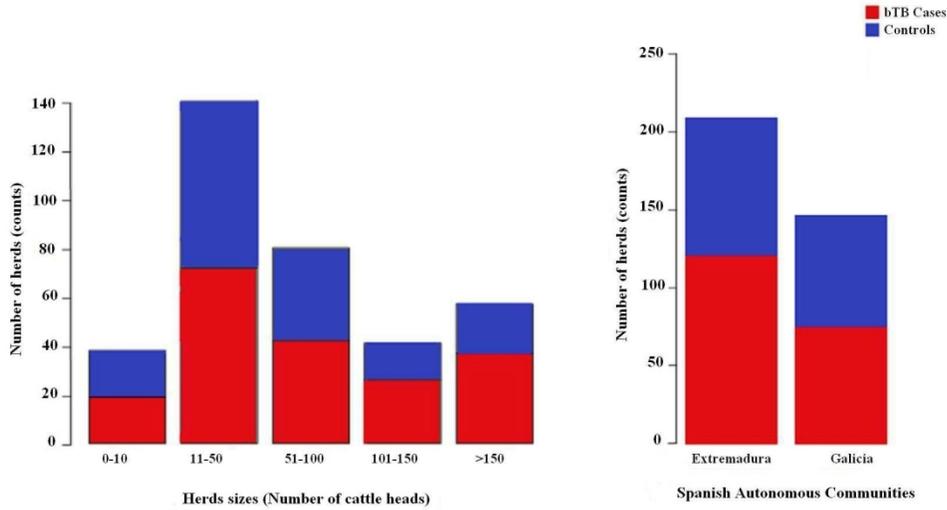


Figure 2. Characteristics of the analysed farms split by bTB status; case (No. 196) and control (No. 160) farms are coloured in red and blue, respectively. In (a) it is shown the distribution of case and control farms by herd size; and, in b) their distribution by geographical location (i.e., Autonomous Community).

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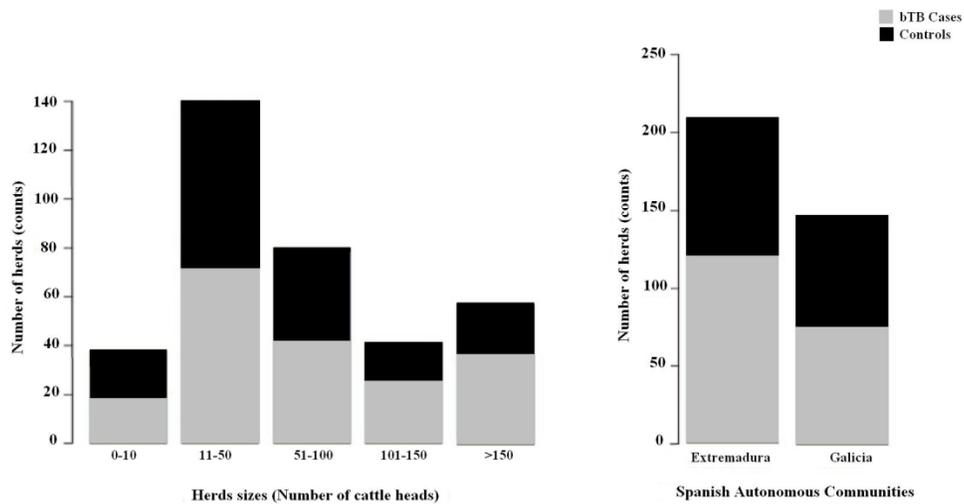


Figure 2. Characteristics of the analysed farms split by bTB status; case (No. 196) and control (No. 160) farms are coloured in grey and black, respectively. In (a) it is shown the distribution of case and control farms by herd size; and, in b) their distribution by geographical location (i.e., Autonomous Community).

72x38mm (600 x 600 DPI)