

RESEARCH ARTICLE

Risk factors associated to a high *Mycobacterium tuberculosis* complex seroprevalence in wild boar (*Sus scrofa*) from a low bovine tuberculosis prevalence area

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Abstract

Animal tuberculosis is a worldwide zoonotic disease caused principally by *Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis* complex (MTC). In southern Iberian Peninsula, wild reservoirs such as the wild boar, among other factors, have prevented the eradication of bovine tuberculosis. However, most of the studies have been focused on south-central Spain, where the prevalence of tuberculosis is high among wild ungulates and cattle herds. In northern regions, where wild boar density and bovine tuberculosis prevalence are lower, fewer studies have been carried out and the role of this species is still under debate. The aim of this study was to describe the temporal and spatial distribution of antibodies against MTC in wild boar from the Basque Country, northern Spain. Sera from 1902 animals were collected between 2010 and 2016. The seroprevalence was determined with an in house enzyme-linked immunosorbent assay and the search of risk factors was assessed by Generalized Linear Models. Overall, 17% of wild boars (326/1902; 95%CI, [15.5%–18.9%]) showed antibodies against MTC. Risk factors associated with seropositivity were the year and location of sampling, the number of MTC positive cattle, the distance to positive farms and the percentage of shrub cover. Younger age classes were associated with increased antibody titres among seropositive individuals. The seroprevalence detected was higher than those previously reported in neighbouring regions. Hence, further studies are needed to better understand the role of wild boar in the epidemiology of tuberculosis in low tuberculosis prevalence areas and consequently, its relevance when developing control strategies.

Introduction

Animal tuberculosis (TB) is a worldwide zoonotic disease caused principally by *Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis* complex (MTC) that infects a wide range of domestic and wildlife species [1]. Because of its impact on public health and economic

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losses in livestock industry, eradication programs in cattle have been implemented in Europe through the last decades [2]. Meanwhile, the increase of wild ungulates populations reported in Europe results in biodiversity reduction and the increment of competent hosts for many diseases, including animal TB [3–5]. This change comes partially from the absence of predators, which could potentially contribute to both wild ungulates populations and diseases control. The appearance of habitats suitable for wild ungulates due to increased food availability and rural abandonment may also favour this tendency [4–6]. Thus, the implication of wild reservoirs, among other factors, has prevented the complete eradication of bovine TB in many countries [7]. Some recognized examples are the Eurasian wild boar (*Sus scrofa*) and the red deer (*Cervus elaphus*) in the Iberian Peninsula [2]. Moreover, other ungulates and carnivores seem to play a role in the epidemiology of bovine TB in this territory, either as spillovers, such as the red fox (*Vulpes vulpes*), the roe deer (*Capreolus capreolus*) and the Iberian lynx (*Lynx pardinus*); or as potential reservoirs, such as the fallow deer (*Dama dama*) or the Eurasian badger (*Meles meles*) [8–12]. Together with the domestic hosts, including goats [13], sheep [14] and pigs [15], as well as the main and most well studied host, cattle [16], we are facing a multi-host pathogen system, where *M. bovis* persistence and transmission depends on several factors, such as the high resistance of this agent in the environment, the density of hosts and species interactions [17], a scenario most likely applicable to other members of the MTC like *M. caprae* and *M. microti*. Nevertheless, many evidences point to the wild boar as the most important wild reservoir within some Mediterranean epidemiological contexts [18], bearing in mind that domestic reservoirs (e.g. goats) might be even more relevant than this wild species [19]. Besides, its opportunistic omnivorous diet and its capacity of living in a huge variety of habitats [20] turn this ungulate into an obstacle for bovine TB control strategies when its population is infected. However, the role of this host in the epidemiology of animal TB can vary from one country to another, or even between regions of the same country, since it will not only depend on the species characteristics, but also on the environment and the probability of interacting with other susceptible individuals [21]. In the Iberian Peninsula, most of the studies performed on the epidemiology of animal TB in wild boar are focused on south-central Spain, where artificial management of game species has also increased their density and aggregation [22]. Moreover, the prevalence of TB is high among wild ungulates [11] and cattle herds [23] inhabiting this area. However, in northern Atlantic and Mediterranean regions, where wild boar density and aggregation are lower, as well as the TB prevalence among cattle herds (< 1%) [23], fewer studies have been carried out and the research related to the role of wild boar is currently ongoing [24–26]. So far, whether this wild ungulate may act as a spillover or a reservoir is still under debate in northern Spain [24,26]. Hence, an increase of research is required in order to obtain a bigger picture of the understudied low TB prevalence areas, since the relevance of wild boar may increase as the prevalence in livestock decreases [27].

Therefore, this study aimed to increase the body of knowledge on animal TB epidemiology by describing the temporal and spatial distribution of antibodies against MTC in wild boar from a low bovine TB prevalence area, as well as to identify risk factors associated to the likelihood of having contact with the bacterium.

Materials and methods

Ethics statement

Serum samples used in this study were obtained by competent local authorities from legally hunted wild boars or from wild boar carcasses found in the field, in complete agreement with Spanish and European regulations. No animals were killed specifically for this study. No ethical approval was deemed necessary.

Study area

This study was carried out in the Basque Country, northern Spain. This area covers 7234 km² and it is divided into three provinces (Araba, Bizkaia and Gipuzkoa), according to political and administrative criteria. In northern provinces an Atlantic climate predominates with mild winters and high precipitations. In the south, there is a Continental Mediterranean climate with hot summers and cold winters [28]. Habitats also differ, being pine forests (mainly *Pinus radiata*) more common in the north and deciduous forests (dominated by *Fagus sylvatica* and *Quercus faginea*) alternated with pastures and crops in the south. Scrublands represent almost the ten per cent of the surface of the Basque Country, being distributed throughout the whole territory [29]. The prevalence of bovine TB among cattle herds from the Basque Country was less than 0.1 per cent in 2017, remaining close to official eradication [23]. On the other hand, the management of wild boar populations in this area does not imply artificial interventions such as fencing or feeding, but mostly relies in hunting activities within certain game preserves.

Wild boar sampling

Serum samples from 1902 wild boars belonging to 185 out of 247 hunting areas were collected during 2010–2016 in the context of a wildlife health serological surveillance program in the Basque Country (Fig 1). Most of the animals (89.6%) were shot by authorized hunters during the regular hunting season (October to February) and sera were obtained in the field. Almost nine per cent of the serum samples were obtained from wild boar's population control programs where animals were trapped and put down by competent authorities. A smaller proportion of sera were collected from animals with not recorded cause of death (1.5%) or from carcasses of run over animals (0.05%). Serum samples were mainly obtained by intracardiac puncture or intracavernous venipuncture, individually identified and stored at -20°C until processing.

Serological assay

The presence of IgG antibodies against MTC was determined by using an in house enzyme-linked immunosorbent assay (ELISA) previously validated for wild boar, following the protocol previously described [30]. The control sera were the same used for the validation of this assay. All samples were analysed in duplicate. Optical densities (OD) were determined at 405 and 450 nm (MultiskanFC, ThermoScientific). OD_{450 nm} was subtracted from OD_{405 nm} and the results were expressed as an ELISA index (EI), calculating the ratio between the resulting mean sample OD and the mean OD of the positive control. Samples with an EI \geq 0.200 were considered positive.

Database

Wild boar data. Whenever it was possible, data of each wild boar such as sex (male; female), age (piglet < 1 year; yearling between 1 and 2 years; adult > 2 years), date and geographic location of collection (province, region and municipality) were recorded. Age of the animals was determined based on the sex, weight and tooth eruption patterns.

Livestock data. According to the last official 2009 census obtained from the Basque Statistics Institute [31], there are about 136246 cattle in 5930 farms, 272167 sheep in 4539 farms and 21547 goats in 1605 farms in the study area. Attending to these data, variables based on livestock density (number of cattle-sheep-goats/Km²) were calculated at region level. The whole livestock censuses of the Basque Country were taken into account for these estimations,

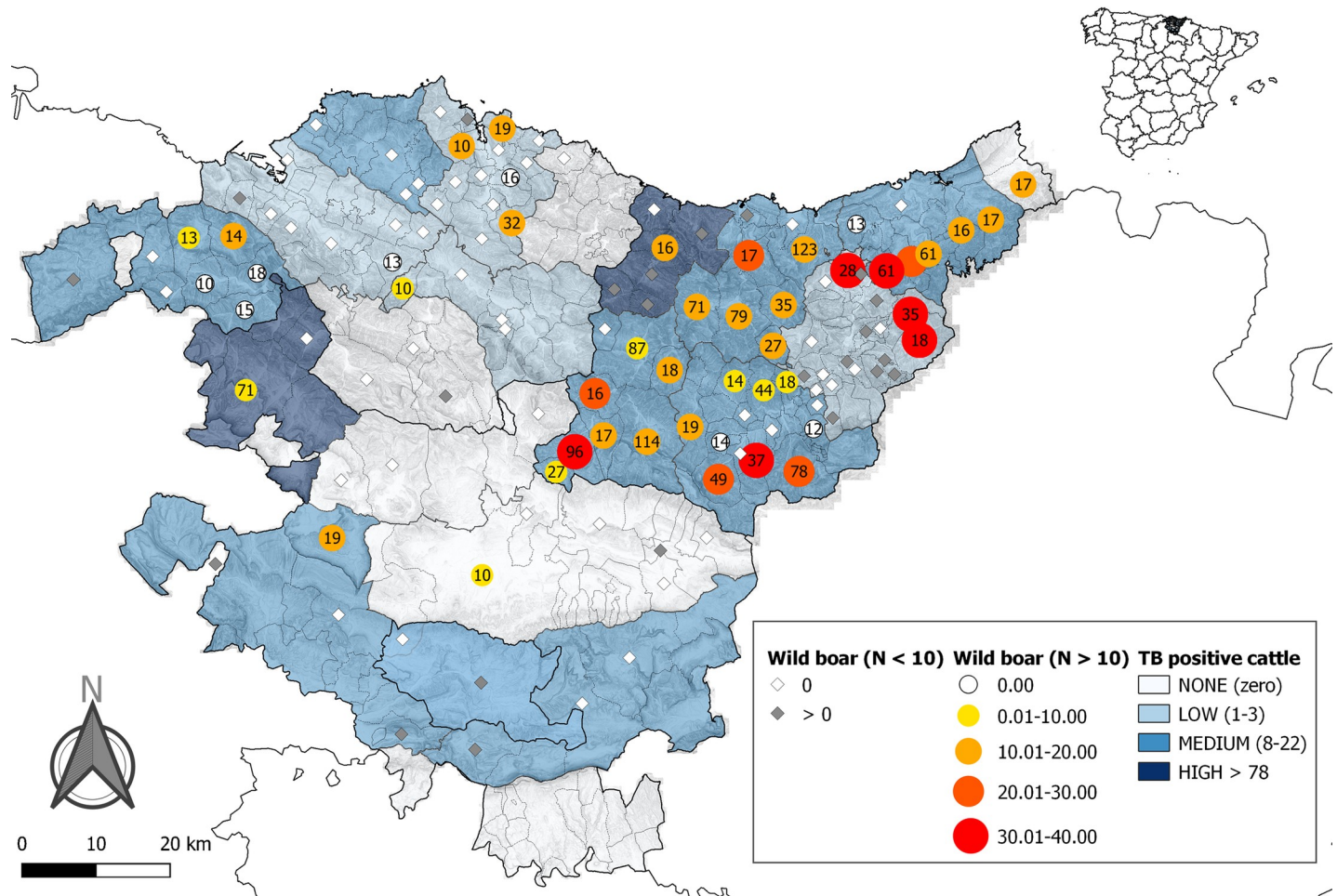


Fig 1. Spatial distribution of MTC seroprevalence (%) detected in wild boar and bovine TB positive cattle. Dot sizes and intensity of colour increase with the seroprevalence detected in municipalities where more than 10 wild boars were analysed. Labels inside these dots indicate the number (N) of animals analysed. Rhombuses indicate municipalities where less than 10 wild boars were analysed. Those in white mean they were negative and greys mean at least one animal was positive. Blue fill colour intensity increase with the number of TB positive cattle detected in each region: No (zero), low (1–3), medium (8–22) and high (> 78).

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because farms are not closed, biosafety measures are lacking and animals can remain in pastures regardless of the management system, allowing for potential direct or indirect contacts with animals outdoors, including wildlife.

MTC positive cattle. According to the information obtained from the Spanish Database of Animal Mycobacteriosis (mycoDB) [32], 304 MTC-infected cattle were detected by official diagnostic methods and/or inspection at slaughter and confirmed by culture in the Basque Country between September 2009 and July 2017. Herd prevalence and incidence of new positive herds during this period were highest in 2009 (0.57 and 0.55%, respectively) and lowest in 2017 (0.09 and 0.07%, respectively) according to the reports of the National Bovine TB Eradication Program [23]. Taking advantage of these data, the amount of positive cattle per region was calculated and classified according to the number of positive cows detected (zero, low, medium, high).

In addition, the Euclidean distance from each wild boar to the nearest positive cattle herd was calculated. Because of the lack of information on the exact location of each wild boar, the centroid of every municipality of sampling was used. As for the positive cows, the finest scale

available was also used, being the farm's UTM coordinates in 220 cases, the centroid of the village in 47 cases and the centroid of the municipality in 37 cases. The software QGIS Valmiera v2.2.0 [33] was used for this spatial analysis.

Hunted wild boar. Counts of hunted wild boar within each hunting season and game preserve were obtained from the Provincial Councils. These counts were transformed into a measure of relative abundance (hunted wild boar per km²) [34,35], only taking into account the habitable surface for this wild species within each game preserve, which was assessed with the software QGIS Valmiera v2.2.0 [33].

Vegetation cover. The vegetation cover was obtained from the 2016 Forest inventory map of the Spatial Data Infrastructure of the Basque Country [36] and from the 2006 Spanish forestry map of the Nature Databank [37]. The vegetation cover of interest was reclassified into six categories: "pine forest", "deciduous forest", excluding the beech forests from this category due to their lack of undergrowth; "oak forest", "beech forest", "scrubland" and "pastures and crops" [20,34,38]. An intersection between the surface of each municipality where every wild boar was hunted and the reclassified vegetation cover was created and the percentage of each vegetation category was calculated for every municipality. The software QGIS Valmiera v2.2.0 [33] was used for this spatial analysis.

Statistical analysis

Two Generalized Linear Models (GLM) were implemented. The first model included 1811 wild boars and was adjusted to a binomial distribution and a logit link function, using the ELISA results (binomial variable: positive or negative) as the response variable. Then, a second model was built with a subset of positive wild boars (N = 168), using the antibody titres (continuous variable) as the response variable. This model was adjusted to a gamma distribution and a log link function. Before the implementation of these models, the normality of data was checked with the Kolmogorov-Smirnov test and several univariate analyses were performed between the response and the explanatory variables (N = 17) in order to identify potential risk factors. Non-parametric Mann-Whitney tests were used between continuous and categorical variables with two levels, while non-parametric Kruskal-Wallis tests were used when categorical variables had more than two levels; Chi-Square Tests were used between categorical variables and GLM adjusted to a gamma distribution and a log link function were used between continuous variables. In all tests, significance was set at $p < 0.05$. Explanatory variables for which $p < 0.25$ at the univariate analysis and that were correlated by less than 0.7 were considered for inclusion in the models [39]. Finally, a manual bidirectional stepwise strategy was used to select the final models. First, the two models were built including all the selected predictors. Those predictors showing a non-significant association with the response variables were sequentially excluded from each model. Confounding variables were assessed by checking for changes in the regression coefficients when removing any variable. If changes were higher than 20%, the variable was included again in the model, otherwise it was definitely removed. The Akaike Information Criteria (AIC) and the percentage of explained deviance were taken into account when selecting the final models. All the statistical analyses were performed using the R Software 3.5.0 [40]. The data set employed for the statistical analyses is deposited in a public repository [41].

Results

Overall, 17% of wild boars (326/1902; 95%CI, [15.5%–18.9%]) showed antibodies against MTC. In Fig 1, the spatial distribution of the seroprevalence detected in wild boar among the municipalities of the Basque Country is shown, as well as the spatial distribution of the MTC-

positive cattle during the same period at a regional scale. The highest seroprevalences in wild boars were mainly observed in municipalities from the east of the study area, within the province of Gipuzkoa.

Results obtained after the univariate analysis are shown in Table 1 (categorical variables) and Table 2 (continuous variables) taking into account seroprevalence data (positive and negative wild boars). On the other hand, the distribution of ELISA index values among positive wild boars according to the categorical and continuous variables are described in Table 3 and Fig 2, respectively. In these four images, p-values of each univariate analysis are shown as a previous step for the selection of variables included in the binomial and gamma models.

The final (binomial) model explained 7% of the deviance (AIC = 1531.4). The results indicate that the probability of being positive for a wild boar changed over the sampling years, being significantly higher during the first years of the study period (2010–2013) when

Table 1. Seroprevalence of MTC detected in wild boars according to categorical variables.

Categorical variable	N. tested	% positives (95% CI)	p-value
Sex			0.513
Female	757	12.8 (10.6–15.4)	
Male	679	14.0 (11.6–16.8)	
Age			0.380
Piglet	217	15.7 (11.4–21.1)	
Yearling	438	12.1 (9.4–15.5)	
Adult	565	14.5 (11.8–17.7)	
Sampling year			< 0.001*
2010	138	23.2 (16.9–30.9)	
2011	190	23.2 (17.7–29.7)	
2012	128	22.7 (16.3–30.6)	
2013	320	25.0 (20.6–30.0)	
2014	571	13.3 (10.8–16.3)	
2015	323	13.0 (9.8–17.1)	
2016	232	9.9 (6.7–14.4)	
Season			0.330
Spring	72	13.9 (15.0–19.4)	
Summer	108	23.1 (7.7–23.7)	
Autumn	1124	17.1 (16.2–31.9)	
Winter	598	16.6 (13.8–19.7)	
Positive cattle/region			0.002*
Zero (0)	66	9.1 (4.2–18.4)	
Low (1–3)	392	23.2 (19.3–27.6)	
Medium (8–22)	1354	15.7 (13.8–17.7)	
High (> 78)	58	17.2 (9.6–28.9)	
Province			< 0.001*
Araba	94	11.7 (6.7–19.8)	
Bizkaia	297	6.7 (4.4–10.2)	
Gipuzkoa	1511	19.5 (17.6–21.6)	

“*” indicates a significant association between the response and the explanatory variable at the univariate analysis ($p < 0.05$). P-value in bold type indicates variables included in the binomial model (after excluding correlated variables). The number of positive cattle per region was categorized as follows: zero (0), low (1–3), medium (8–22) and high (> 78).

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Table 2. Descriptive statistics of the continuous variables values among positive and negative wild boars.

Continuous variable	ELISA POSITIVE		ELISA NEGATIVE		p-value
	N	Median (IQR)	N	Median (IQR)	
Hunted wild boar/km ²	295	0.7 (0.5–1)	1503	0.8 (0.4–1)	0.105
Distance to positive cattle (km)	296	7.5 (3.3–9.4)	1515	6.1 (2.2–9.4)	0.090
Pine forest (%)	296	36.0 (27.8–45.7)	1515	36.0 (27.3–48.0)	0.597
Pastures & crops (%)	296	18.6 (14.6–22.2)	1515	18.6 (14.5–24.4)	0.686
Oak forest (%)	296	7.7 (2.9–12.2)	1515	5.6 (2.9–11.5)	0.789
Deciduous forest (%)	296	9.7 (3.8–16.5)	1515	7.6 (4.2–15.8)	0.676
Beech forest (%)	296	6.2 (2.5–16.9)	1515	5.0 (1.3–13.2)	0.001*
Shrubs (%)	296	5.6 (4.7–8.9)	1515	4.8 (3.8–7.7)	0.003*
Cattle/km ²	318	22.9 (16.0–32.1)	1545	18.0 (13.8–42.6)	0.551
Sheep/km ²	318	75.9 (56.8–90.8)	1545	64.3 (36.8–90.8)	<0.001*
Goats/km ²	318	2.7 (2.1–2.7)	1545	2.7 (2.1–3.5)	0.051

“*” indicates a significant association between the response and the explanatory variable at the univariate analysis ($p < 0.05$). P-value in bold type indicates variables included in the binomial model (after excluding correlated variables). IQR = Interquartile Range.

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comparing with 2014 (Table 4). Hereafter, this probability began to decrease until the end of the study period, even though this change was not significant (2015–2016). As for the positive cattle, wild boars had a higher probability of being positive in regions where MTC-positive cattle were detected, compared to those where cattle were negative. Nevertheless, this increase was only significant in those regions with a low number of cattle outbreaks (Table 4). Moreover, a higher probability of being seropositive was associated with the increase of the distance to MTC positive farms (Table 4). The sampling province was also associated with the probability of being positive. This probability was higher in Araba and Gipuzkoa, compared to Bizkaia (Table 4). Lastly, a higher probability of being positive was also observed with the increase of the percentage of shrub (Table 4). With regard to the analysis of the continuous variable, the gamma model (11% of explained deviance and AIC = -176.32) showed that piglets and yearlings were significantly associated with an increase of the ELISA index, when comparing with adults (Table 4).

Discussion

The research on the epidemiology of animal TB in wild boar populations is quite scarce in the north of the Iberian Peninsula when comparing to the south. For this reason, this study was necessary to obtain a wider perspective of the epidemiology of TB in wild boars from low bovine TB prevalence Atlantic areas. The ELISA test is considered a useful tool when developing a first screening in wildlife, because of its speed, ease of use and relatively low cost [42,43]. The application of this method to the 1092 wild boar sera collected in this area revealed an overall seroprevalence (17%) unexpectedly higher than that detected in neighboring regions from northern Atlantic Spain (<5%) [24,44]. This suggests that the role of wild boar in the epidemiology of TB in northern Spain may be more relevant than it was expected. Despite this, the tendency observed throughout the study period points to a general drop of the seroprevalence, even though the lowest one detected in this survey (9.9% in 2016) is still high compared to data from the aforementioned studies. Several factors may have triggered this decreasing trend in TB seroprevalence in wild boar, but it could be related to the general drop of TB herd prevalence seen in cattle during the same period (from 0.37% in 2010 to 0.17% in 2016) [23]. On the other hand, considering that in a previous study from northern Spain *M. avium*

Table 3. Descriptive statistics of the ELISA index of the positive wild boars according to categorical variables.

Categorical variable	ELISA index ≥ 0.200		p-value
	N	Median (IQR)	
Sex			0.897
Female	97	0.267 (0.234–0.389)	
Male	95	0.274 (0.229–0.380)	
Age			0.001*
Piglet	34	0.284 (0.249–0.523)	
Yearling	53	0.289 (0.241–0.500)	
Adult	82	0.244 (0.217–0.313)	
Sampling year			0.241
2010	32	0.257 (0.223–0.354)	
2011	44	0.274 (0.228–0.318)	
2012	29	0.279 (0.255–0.385)	
2013	80	0.271 (0.236–0.425)	
2014	76	0.283 (0.238–0.514)	
2015	42	0.272 (0.227–0.334)	
2016	23	0.261 (0.228–0.302)	
Season			0.505
Spring	10	0.274 (0.229–0.291)	
Summer	25	0.271 (0.237–0.369)	
Autumn	192	0.270 (0.228–0.379)	
Winter	99	0.284 (0.239–0.395)	
Positive cattle/region			0.009*
Zero (0)	6	0.240 (0.230–0.254)	
Low (1–3)	91	0.376 (0.240–0.436)	
Medium (8–22)	212	0.274 (0.227–0.367)	
High (> 78)	10	0.229 (0.210–0.253)	
Province			0.009*
Araba	11	0.228 (0.218–0.239)	
Bizkaia	20	0.263 (0.226–0.297)	
Gipuzkoa	295	0.277 (0.234–0.400)	

“*” indicates a significant association between the response and the explanatory variable at the univariate analysis ($p < 0.05$). P-value in bold type indicates variables included in the gamma model (after excluding correlated variables). The number of positive cattle per region was categorized as follows: zero (0), low (1–3), medium (8–22) and high (> 78).

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complex (MAC) isolates were recovered from wild boar tissues in a higher proportion than MTC isolates [24] and being aware of the antigenic repertoire similarities found between different species of this genus, some cross-reactivity with other non-tuberculous mycobacteria cannot be completely excluded. Infection with members of the MTC other than *M. bovis* like *M. caprae* or *M. microti* is also detectable using bPPD-based ELISAs [45]. For these reasons, further research including not only serology, but also confirmatory microbiological culture and species identification are needed to better assess the significance of different mycobacterial infections in wild boar from this region. In any case, given the high specificity attributed to this ELISA test in its validation with field samples [30], we think that the involvement of false positive results would minimally change these figures.

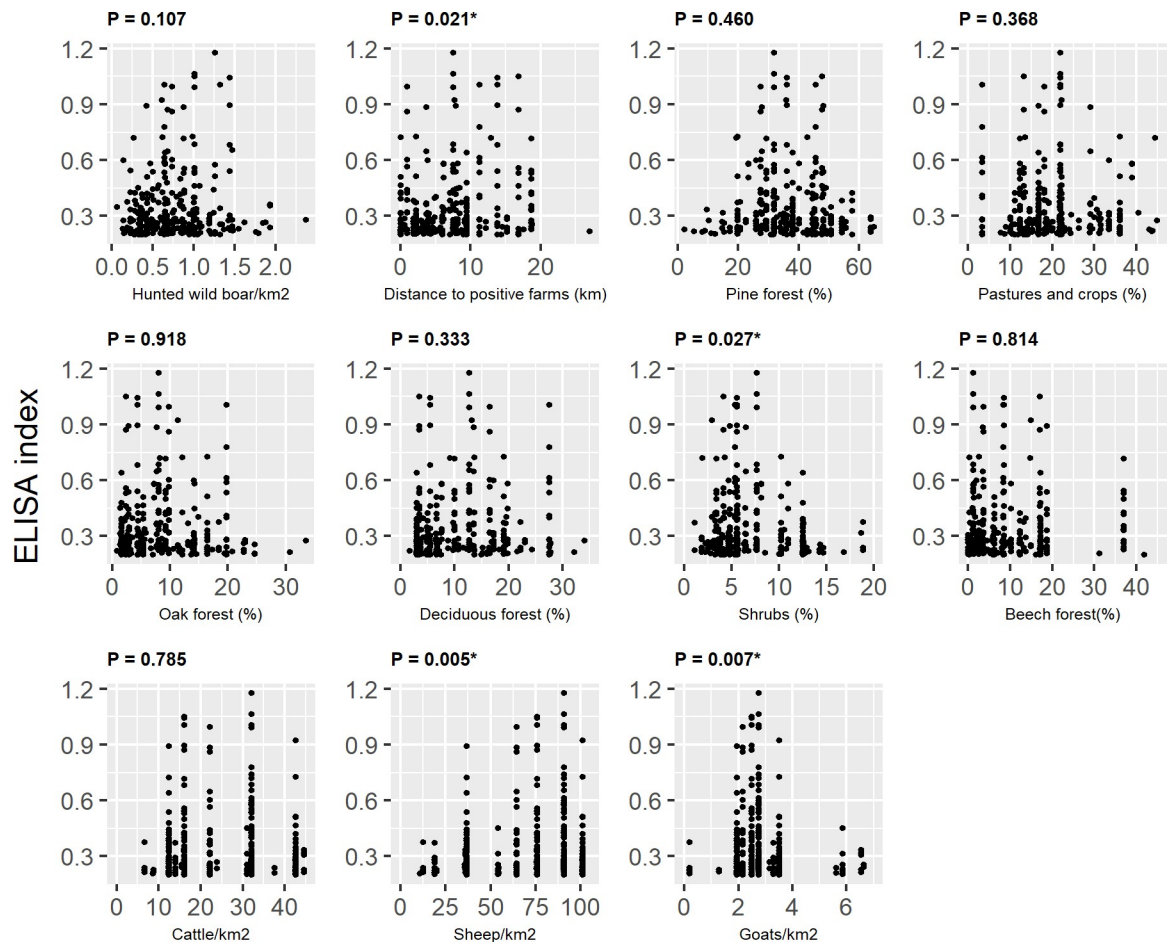


Fig 2. Descriptive statistics of the ELISA index of positive boars according to continuous variables. “*” indicates a significant association between the response and the explanatory variable at the univariate analysis ($p < 0.05$). Variables with a p-value lower than 0.25 were included in the gamma model.

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In the binomial model, a higher seroprevalence was found in regions where bovine outbreaks were detected, suggesting a potential risk of transmission at the wild-domestic interface. However, this increase was only significant when the amount of positive cattle was low. This could be due to the fact that interspecies interactions are not the only factor involved in the circulation and/or transmission of the bacterium. Actually, intraspecies interactions are often more common [46,47], but this is influenced by each epidemiological scenario. In our study area, most of the seropositive animals were detected in Gipuzkoa, a province where wild boars showed also the highest antibody titres. This could be due to a higher dissemination of bacteria among wild boar. Therefore, despite a bacterial circulation between cattle and wild boars cannot be dismissed, wild boar intraspecies transmission might have a more relevant role in our study area and period. However, the seroprevalences observed in some municipalities suggest that wild populations could still represent a threat in terms of TB transmission and maintenance. Thus, more studies are needed to determine the mycobacteria species and spoligotypes circulating in wild boar from this area.

Another factor significantly related to the increase of the seroprevalence was the distance between wild boars and TB positive farms. However, this association showed just the opposite effect of what was expected, since the probability of wild boars being positive increased with

Table 4. Results of the generalized linear models.

Response variable	Predictor	Level	OR(95%CI)	Estimate	P-value
ELISA results (binomial. N = 1811)	Intercept	-	0.01 (0.00–0.03)	-4.68	< 0.001
	Positive cattle/region	Zero ^a	1	NA	NA
		Low	3.28 (1.19–9.02)	1.19	0.021
		Medium	1.44 (0.54–3.85)	0.36	0.468
		High	1.64 (0.51–5.26)	0.49	0.409
	Distance to TB positive farms (km)	-	1.04 (1.02–1.07)	0.04	0.002
	Percentage of shrubs	-	1.05 (1.01–1.09)	0.05	0.011
	Year of sampling	2010	1.79 (1.08–2.98)	0.58	0.025
		2011	2.43 (1.54–3.85)	0.89	< 0.001
		2012	2.41 (1.44–4.05)	0.88	< 0.001
2013		2.12 (1.44–3.13)	0.75	< 0.001	
2014 ^a		1	NA	NA	
2015		1.09 (0.71–1.67)	0.09	0.690	
Provinces	Bizkaia ^a	1	NA	NA	
	Araba	4.30 (1.66–11.12)	1.46	0.003	
	Gipuzkoa	5.70 (3.28–9.93)	1.74	< 0.001	
ELISA index (gamma. N = 168)	Intercept	-	-	-1.21	< 0.001
	Age	Adult ^a	-	NA	NA
		Yearling	-	0.33	< 0.001
		Piglet	-	0.27	0.012

Significant values are written in bold letters. NA = Not applicable.

^a indicates the reference level selected for each categorical variable to run the model.

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longer distances to the farms. Looking for a pattern at such a fine scale without the exact location of hunted animals could have led to an inaccuracy of the distance data and, consequently, to distort the statistics. Moreover, dichotomizing the ELISA index into a binomial variable results in information loss, due to the inclusion of individuals displaying an index around 0.2 (probably exposure) with those displaying an index around 1 (probably infection) in the same level. This can result in a reduced precision of the OR [48]. Despite this assumptions, a previous work found that exposure to MTC in wild boar was related to shorter distances between them and TB outbreaks in cattle, using the centroid of the commune of sampling as it was also the finest scale of spatial position available [27]. Nevertheless, the statistical approach was different, since it was carried out using a bootstrap method.

Lastly, the percentage of shrub was positively associated with the seroprevalence. Although wild boars can live in different kind of habitats, the shrub cover may be especially attractive from a survival perspective, because it can provide them a good shelter. In northern areas, unlike south-central areas in Spain [22], spatial aggregation of wildlife seems less likely to occur, since wild boar densities are lower and humid habitats prevent wild species overcrowding [24]. Thus, shrub cover may not produce a clear aggregation of wild boars, but it may hinder their movements, forcing them to use the same paths and limiting the excretion of and exposure to the bacterium to their own routes. Hence, it may not be about wild boar aggregation in northern bushy areas, but instead, we could think about a restricted movement capacity along this kind of vegetation as an enhancer of bacterial accumulation in their passages. In addition to this, shrub cover might also provide a moist microhabitat protected from the sun radiation that prompts mycobacteria survival and persistence in the environment. In the

Basque Country, the shrub cover has been gradually increasing through the years, ranging from six per cent of the surface in 1986 to almost 10% during our study period [29,49]. This change in the vegetation cover seems to be linked with the abandonment of rural areas and thus, with an insufficient maintenance of forests and lands. If this rural abandonment phenomenon does not cease, other measures should be implemented to prevent the numerous problems that can derive from shrub progression, including the formation of potential hot spots for bacterial persistence.

In the gamma model, it is remarkable that, among positive wild boars, increased antibody titres were mainly observed in yearlings and piglets, compared to adult individuals. It is generally considered that increased antibody titres are associated with more severe forms of TB in many wild species, including the wild boar [50,51]. Previous studies have found evidences of severe illness in young animals, rather than in adults, as animals with large lesions in more than one anatomical region were more frequently detected among juveniles (12 to 24 months) [52], but these findings belong to a different epidemiological context (southern Spain). In another study, there was a decrease in the proportion of lesions from which mycobacteria could be isolated with increasing age [53]. The social behaviour of this wild species might also explain this difference among age classes. Adult females and their young live in groups and maintain close contact, favouring exposure by different routes. Piglets may not only suckle from their own mother, if other sows have given birth at the same time [20], increasing their chances of exposure or even of acquiring an infection. Adult males, conversely, have a solitary lifestyle, reducing their chance of contact with other wild boars out of the mating season [20] and, consequently, their risk of exposure to MTC. On the other hand, piglets and yearlings have had less time in their life to get in contact with the bacterium than the adults, and a detectable immune response needs time to develop after bacterial exposure [43]. We expected to have higher antibody titers amongst adults than amongst younger boars, as it has been suggested that recent infections in younger age-class might cause lower antibody levels and lower ELISA sensitivity [42]. In spite of this, the same study reported a seroprevalence of 29.3% (95% CI 21.3–37.2) amongst 2–6 month-old piglets with or without visible lesions and, interestingly, the antibody levels detected by the bPPD ELISAs did not correlate with the lesion score [42]. Based on the aforementioned studies, one hypothesis could be that part of adult individuals were exposed to the bacterium when they were younger but managed to control or even to clear the infection, and at the moment of hunting their immune response to an old contact or infection was less intense. Or it could be simply that reaching adulthood with progressive disease is less probable under the conditions of this area. Nevertheless, considering that the detection of higher antibody titres could be related to more extended lesions and, consequently, to higher excretion of mycobacteria [50], the dispersal behaviour of the yearlings [54] might be considered a factor that could easily contribute to the geographical spread of MTC.

The seroprevalence observed in our survey was higher than that reported earlier in other northern areas, suggesting that the spillover role of wild boar in these regions might change at any time and become more relevant, if the appropriate factors are given [26]. Hence, in areas such as the Basque Country where TB prevalence among cattle herds is minimal, a possible spillback transmission from this ungulate to cattle should not be neglected [21]. We suggest a potential risk of transmission at the wildlife-livestock interface of the study area, even though it might not be as important as the risk of wild boar or cattle intraspecies transmission. Measures to reduce the surface of shrub cover should be considered, since in addition to other risks, such as bushfires, it could be related to the exposure of wild boars to MTC. Hunting strategies should keep in mind those individuals that can have an effect on bacterial circulation or spread, such as the piglets and yearlings. The role of other domestic animals should be deeply studied, in order to gather more information of this multi-host pathogen system.

Considering that the general expansion of wild boar populations in Europe through the last decades is a widely recognized problem [34,38,54], we highlight the necessity of better understanding the relevance of wild boar in the epidemiology of animal TB in northern Spain, in order to develop appropriate surveillance and control strategies, if needed, able to prevent the dissemination of the disease within wild populations and transmission to livestock.

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