Transboundary and Emerging Diseases

ORIGINAL ARTICLE

High-Density Dependence But Low Impact on Selected Reproduction Parameters of *Brucella suis* Biovar 2 in Wild Boar Hunting Estates from South-Western Spain

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Summary

Porcine brucellosis is a disease caused by *Brucella suis*, which is characterized by reproductive disorders in pigs. The number of cases of swine brucellosis has risen in many European countries, likely because of the presence of a wild reservoir of *B. suis* in wild boar. This study aimed at evaluating factors that may influence the probability of infection with *Brucella* spp. in wild boar and at assessing the impact of a previous contact with *Brucella* spp. on reproductive parameters of wild boar. Two hundred and four wild boar living in Extremadura (south-western Spain) were studied. The presence of anti-Brucella antibodies was determined using an indirect ELISA, while the presence of living bacteria in genital organs was evaluated through microbiological cultures. Sex, age, density of wild boar in summer and presence of outdoor pigs were selected as possible risk factors for being seropositive for *Brucella* spp. in wild boar. In addition, reproductive parameters such as breeding status or potential fertility in females and testis weight in males were estimated and related to the presence of anti-Brucella antibodies. A total of 121 animals were seropositive, resulting in a prevalence of 59.3% (95% CI). In addition, seven isolates of *B. suis* biovar 2 were obtained. Wild boar density in summer, as well as age and sex, was proposed as factors to explain the probability of *Brucella* seroconversion, although wild boar density in summer was the key factor. Current measures of reproductive parameters were not influenced by a previous contact with *Brucella* spp. Isolation of *B. suis* confirms that wild boar could represent a risk to domestic pig health in the study area. Wild boar density seems to have a great influence in the probability of infections with *B. suis* and suggests that density management could be useful to control *Brucella* infection in wild boar.

Introduction

Porcine brucellosis is an infection of pigs caused by biovar (bv) 1, 2 or 3 of *Brucella suis* (*B. suis*) that results in infertility or abortion at any stage of gestation in females or in orchitis in male boars (MacMillan et al., 2006). This infection shows a worldwide distribution and rarely becomes endemic but the occurring sporadic outbreaks can lead to serious (OIE, 2009) economic losses on intensive pig farms (Algers et al., 2009).

Although no cases of porcine brucellosis have ever been declared in most northern European countries (e.g. Norway, Finland, Sweden or the United Kingdom), the number of cases of infection in France, Denmark and Germany in

domestic pigs has shown a sharp increase since the 1990s (Godfroid and Kahlsbohrer, 2002). It has been suggested that transmission from wild reservoirs (especially wild boar and hares) could play an important role in explaining these differences in the occurrence of *B. suis* (Cvetnic et al., 2009). In fact, *B. suis* spillover from wild boar (*Sus scrofa*) to livestock has been reported several times (Godfroid et al., 1994; Andersen and Pedersen, 1995) and thus provides a link between the presence of wild boar and the latest outbreaks of swine brucellosis in Europe (Cvetnic et al., 2009).

However, despite the importance of wild boar as a reservoir of *B. suis* in the European Community, few efforts have ever been made to identify the principal environmental-, population- or human-related risk factors in *B. suis* infection in the wild boar. Currently, aside from a clear seasonal-, gender- and age-dependent prevalence of *B. suis* (Bergagna et al., 2009; Muñoz et al., 2010), the density of outdoor domestic pigs (measured at regional scale) is the sole extrinsic factor that has been linked to *B. suis* occurrence in wild boar (e.g. Spain, Muñoz et al., 2010). Surprisingly, to date, no work has detected density dependence of *B. suis* infection in wild boar, a risk factor typically linked to common pathogens of this species such as porcine circovirus type 2 (Vicente et al., 2004) or *Mycobacterium bovis* (Vicente et al., 2007). According to an extensive epidemiological study of brucellosis in Spanish wildlife (Muñoz et al., 2010), this apparent lack of density dependence warrants further research. Wild boar density can be influenced by management measures (e.g. the restriction of the use of supplementary feeding or an increase in hunting pressure), which may represent an excellent starting point for controlling porcine brucellosis. The pathogenicity of *B. suis* in wild boar has yet to be clarified (Godfroid, 2002), although the most recent research suggests that *B. suis* bv2 has little influence on reproductive parameters (ovulation rate, litter size or partial resorption index) in female wild boars (Ruiz-Fons et al., 2006). To date, no work has explored the effect of *B. suis* bv2 on male wild boars.

The main aim of this work was to evaluate whether or not the interaction between (i) host- (age, sex and population density), (ii) environmental- (geographical location) and (iii) human management-related risk factors (presence/absence of outdoor domestic pigs) influences the probability of *B. suis* bv2 infection in wild boars. Thus, we studied the influence of a previous contact with *Brucella* spp. on reproductive parameters in both female (breeding status and fertility) and male wild boars (weight of testes) and assessed the variability of the *B. suis* isolates that we obtained. Furthermore, this work represents the first fine-scale survey of porcine brucellosis in wild boar in Extremadura, a vast region in south-western Spain where rural economy is largely based on extensive pig farming.

Material and Methods

Study area

This study was carried out in Extremadura, a Mediterranean region in south-western Spain with mountains peaking at 685 m a.s.l. Average annual precipitation reaches 623 mm and is concentrated in the period November–April. The mean annual temperature averages 17.7°C, January being the coldest and July the warmest months of the year. The vegetation is characterized by abundant *Quercus ilex* and *Q. suber* forests with understoreys dominated by *Quercus coccifera*, *Cistus ladanifer* and *Erica arborea*.

Animals from eleven unfenced hunting estates with no artificial management practices in three different areas, *Sierra de San Pedro* (39°27′0″N–5°19′0″O), *Monfragüe* (39°50′27″N–6°01′48″O) and *Las Hurdes* (40°30′N–6°30′O, see Fig. 1), were sampled during the study period (2004–2005).

Wild boar density in the study area ranged from 2 to 10 individuals per square kilometre.

This population parameter was estimated in summer by counting the maximum number of boars per counting session (4 h previous to nightfall) observed in pre-determined open areas. In these places, boars are baited every week with

Fig. 1. Map showing the location of areas where wild boar was sampled for exploring the role of host, environmental and human management risk factors on the probability of *B. suis* bv 2 infection in the region of Extremadura, south-western Spain. (SP, Sierra de San Pedro; MF, Monfragüe; LH, Las Hurdes). Solid dots represent estates with domestic pig farms; circles represent estate without pig farms. Small map in the upper right corner represents the localization of the studied area within Spain.

corn and counted using binoculars from fixed points less than 300 metres away. These counts took place from July to September, a period in which food is scarce in these Mediterranean ecosystems. All counting points in each hunting estate were visited simultaneously to avoid double counts of boars. Therefore, both the number of fixed points and the number of counting sessions can be considered to be constant for each hunting estate.

Because domestic pigs are a reservoirs of *B. suis* in Spain (Muñoz et al., 2010), the presence of domestic pigs was recorded for each hunting estate. It is important to note that in these hunting estates, no physical barrier separates domestic pigs from wild boar, which share pastures, food and water points, thereby making the contact among these animals very likely. Outdoor pig farms were present in six of the eleven studied estates (Fig. 1).

Wild boar data

All the 204 wild boars studied were hunt harvested during the 2004–2005 hunting season between October 12 and February 28. Once an animal was shot, its sex was determined by direct observation of genitalia, and its age was estimated based on its tooth replacement and eruption patterns, and as well by dental attrition (Boitani and Mattei, 1992).

After sex and age determination, animals were dissected to extract their reproductive tracts, which were stored in plastic bags and maintained at 4°C in cool boxes and then examined within 20 h. Immediately afterwards, blood samples were collected from the heart or the thoracic cavity of the wild boar and kept at 4°C. Subsequently, blood samples were centrifuged a 3000 rpm for 10 minutes at the Animal Health Department of the University of Extremadura (Spain). Only sera of acceptable quality (sera perfectly separated from the coagulum and without any kind of residual) (n = 204) were selected for serology and stored at −20 °C until further analysis.

In the laboratory, 188 reproductive organs were studied. After removal of the Vas deferens (n = 85), the testes were blotted dry with a paper towel and weighed to the nearest 0.01 grams. Any partially damaged organs were excluded from our study. To assign breeding status, the reproductive tracts of females (n = 103) were also analysed, with particular attention given to the ovaries and the presence of embryos or foetuses. Female breeding status (breeding or non-breeding) was assessed by detecting the presence of embryos, foetuses or extended teats. When none of these features were present, females were considered to be non-breeding. In pregnant females, the number of foetuses was counted to estimate potential fertility (Groot Bruinderink et al., 1994). The presence of gross lesions was evaluated in both female and male reproductive organs.

Serological and bacteriological study

Brucella spp. antibodies were detected using as per the manufacturer's instructions, a commercial indirect enzyme-linked immunosorbent assay kit (ELISA), Ingezim Brucella Porcina (Ingenasa, Madrid, Spain), with 98.6% sensitivity and 100% specificity in domestic pigs. True prevalence at 95% CI of *B. suis* was estimated taking into account apparent prevalence (number of animals tested positive/number of animals tested) and both the sensitivity and specificity of the ELISA test (Rogan and Gladen, 1978).

Of the 204 wild boars studied, 188 were included in the bacteriological study (85 testicular and 103 vaginal swabs taken before performing the necropsies). Inoculations were carried out in a *Brucella* medium (OIE, 2009) enriched with 10% horse serum, incubated at 37°C for at least 10 days in an atmosphere containing 5% CO_2. Putative Brucella-positive cultures were further analysed using standard bacteriological procedures. DNA was extracted using a QIAamp DNA minikit (QIAGEN, Hamburg, Germany); isolates were identified as *Brucella* species using a previously described polymerase chain reaction technique (Romero et al., 1995). To assess the precise bv of the *B. suis* strains isolated, the INgene Bruce-ladder suis kit (Ingenasa, Madrid, Spain) was used as per the manufacturer's instructions. Finally, to assess the variability of wild boar *B. suis* isolates, a multiple-locus variant-repeat assay (MLVA) based on a subset of 16 tandem repeat loci (MLVA-16) was performed following previously described procedures (Le Flèche et al., 2006; Al Dahouk et al., 2007). More information concerning this analysis can be found in the Data S1 (supporting information).

Statistical Procedures

A set of specific statistical models were evaluated to explore the effect of a previous contact with *B. suis* (as explanatory variable) on breeding status (categorical variable with two modalities: 1 = the sow was breeding and 0 = the sow was not breeding) and fertility (an ordinal variable ranging from one to six foetuses) of sows or testes weight of boars (a continuous variable). The effect of age (in months) was included as covariate in all statistical models due to its clear effect on the reproductive performance of female (Fonseca et al., 2011) and male (Rathje et al., 1995) wild boars. Boar density was also included as a covariate because competition for food causes density dependence in both birth and death rates of wild boar populations (Melis et al., 2006). Additionally, the season of harvesting and its interaction with *B. suis* infection were taken into account in the case of males (Mauget and Boissin, 1987).

Each response variable required different statistical modelling with specific error structures. For example, generalized linear models (GLM) were used for both breeding status (binomial errors and logit link function) and the potential fertility of females (Poisson errors and log link function), whereas additive models (Gaussian errors) were used for adjusting the testes weight in males.

In all cases, we followed an information–theoretic approach based on the Akaike information criterion corrected for small sample sizes (AICc) (Burnham and Anderson, 2003). We also estimated the Akaike weight ($w_{i,}$), that is, the relative likelihood of the model given the data available. This statistical procedure was performed using the package 'mgcv', version 1.7-2 (Wood, 2011, for additive models) of the statistical software R.

We used a tree modelling approach to identify major risk factors linked to the presence of anti-Brucella antibodies. Classification and regression trees are flexible and robust analytical statistical tools that are ideally suited for the analysis of complex ecological (De 'Ath and Fabricius, 2000) and epidemiological data (Friedman and Meulman, 2003). In our case, *Brucella* seroconversion (i.e. a discrete nominal variable with two categories, 1 = when boar was *Brucella* seropositive and 0 = otherwise) was initially explained by the following risk factors: host factor as sex (male or female), age (in months) and wild boar density in summer (the winter wild boar density was also estimated but was excluded from our statistical analysis because it showed some degree of correlation with summer densities [$\beta = 0.5$, SE = 0.13, $P < 0.01$, $R^2 = 8\%$]); the environmental factor as geographical area (Monfragüe, Las Hurdes or Sierra de San Pedro) and human factor as the presence/absence of outdoor domestic pigs in each hunting state. Finally, the explained deviance for the final tree and predictions for specific values of the response variables were also generated. This statistical procedure was performed using the package 'tree' version 1.0–29 (Ripley, 2012, for fitting tree regression models) of the statistical software R version 2.15. 1 (R Development Core Team, 2012).

Results

Serology and bacteriology

The ELISA test was applied to 204 wild boars, of which 121 were found to be seropositive to *Brucella* (59.3%, 95% CI, 52.2–66.1). In the 188 culture attempts, a total of seven isolates were obtained (3.7%, 95% CI, 1.6–7.8). The seven culture positive animals (three females between 0.8 and 4 years old and four males between 1.5 and 5.5 years old) were also positive in the ELISA test.

All the isolates obtained were identified as *B. suis* bv 2 using the INgene Bruce-ladder suis kit. In addition, the MLVA-16 assay revealed that six of the seven isolates from Sierra de San Pedro were closely related (i.e. more than 90.9% similarity). Further information concerning this analysis can be found in the Data S1 (supporting information).

Reproductive parameters

In terms of the breeding status of sows, 68 of the 103 (66%) females studied were breeding females. The mean number of foetuses in pregnant females was 3.5. Foetuses were counted in 35 of 68 pregnant females, with a minimum of two and a maximum of five.

The results of our model selection are shown in Table 1. According to our model selection procedure, the effect of age was sufficient ($w_{\text{Age}} = 0.62$, deviance explained = 25.7%) to explain the observed variability on the breeding

Table 1. Model selection for exploring the influence of *Brucella suis* biovar 2 (Bsbv2) on selected reproductive parameters of 103 female and 85 male wild boars hunt harvested in several hunting states in Extremadura, south-western Spain. For breeding status and potential fertility ($n = 36$), we used generalized linear models with specific error structures in each case, whereas for the effect on testis weight, additive models were used

Biological models	K	AICc	Δi	*wi*
Female wild boar				
Breeding status				
Age	**3**	**102.31**	**0**	**0.62**
Age + Bsbv2	**1**	**104.14**	**1.83**	**0.25**
Age + Bsbv2 + Wild boar density in summer	3	105.47	3.16	0.13
Potential fertility				
Mo	**1**	**124.30**	**0**	**0.51**
Wild boar density in summer	**2**	**126.17**	**1.87**	**0.19**
Age	2	126.38	2.08	0.19
Wild boar density in summer + Bsbv2	3	128.56	4.26	0.06
Age + Bsbv2	3	128.76	4.46	0.05
Age + Bsbv2 + Wild boar density in summer	4	131.01	6.71	0.01
Male wild boar				
Testis weight				
Age + Season	**6**	**854.79**	**0**	**0.48**
Age + Bsbv2	6	856.85	2.06	0.17
Age + Season + Bsbv2	7	857.57	2.78	0.12
Age	5	857.74	2.95	0.11
Age + Season * Bsbv2	8	857.81	3.02	0.10

Age was in years, Season = autumn (October-December) and winter (January–February). K = number of parameters, including intercept; AICc = Akaike information criterion corrected for small sample sizes; ΔAICc = difference of AICc with respect to the best model; *wi* = Akaike weight; Mo = null model only with the constant term. For breeding status and testis weight only models with ΔAICc less than 10 were shown. In bold, the best models.

status of sows. In fact, the probability of being pregnant increased with age ($\beta_{Age} = 1.18$, SE = 0.27), pregnant females being on average 1.5 years older (mean age of pregnant females = 2.8, min = 6 months max = 5.5 years) than their non-pregnant counterparts (mean age of pregnant females = 1.1 years, min. = 8 months, max. = 4 years). The competing model $w_{Age + Bsbv2} = 0.25$, deviance explained = 34.5% suggests that *B. suis* bv 2 contact diminishes the probability of becoming pregnant ($\beta_{Bsbv2} = -0.25$, SE = 0.55).

Our model selection suggested that none of the explanatory variables considered was able to explain the observed variability in apparent fertility ($w_{Mo} = 0.25$). However, wild boar density in summer did have a slight negative effect ($\beta_{\text{Wild boar density in summer}} = -0.08$, SE = 0.13), as suggested by the second competitive model. In fact, only 4% of the observed variability in the number of foetuses can be explained by density dependence during the dry season. Additionally, the three *B. suis* bv 2 culture positive females (i.e. actively infected animals) were in anoestrus without macroscopic lesions in their reproductive organs.

Testes weight clearly depended on age and season ($w_{Age + Season} = 0.48$), these two factors explaining 57.3% of variability in weight. Regardless of age, in autumn (October to December) boar testes weighed about 26 g more than in winter. However, despite the fact that the second candidate model included the effect of *B. suis* bv 2, this effect had little support ($\Delta i > 2$) for explaining the observed patterns. Therefore, these results can be regarded as spurious because *Brucella* prevalence in males was greater in autumn (68.9%, 59.85–78.02 at 95% CI) – when testes are heavier – than in winter (0.42%, 0.28–0.46 at 95% CI). Nevertheless, despite this apparent lack of effect, three-four *B. suis* bv 2 positive testes showed marked bilateral orchitis with the presence of adherences and purulent exudates in the scrotum (Fig. 2).

Fig. 2. Orchitis with presence of adherences and purulent exudates. Scrotum has been cut open allowing to see exudates and adherences.

Risk factors for B. suis infection

The most parsimonious tree model was fitted using only three variables (wild boar density in summer, age and sex) and explains 12.1% of the probability of *Brucella* seroconversion (Fig. 3). Specifically and regardless of the age or sex of wild boar, the highest probabilities of seroconversion (0.71, Fig. 3) were found in hunting estates with wild boar densities in summer over 2.5 individuals per 100 ha. Conversely, when densities were below 2.5 boars/100 ha, the probability of *Brucella* contact depended mainly on the age and gender of pigs: males and females over 22 months old had the greatest probability of *B. suis* bv 2 contact ($P = 0.63$), followed by females under 22 months ($P = 0.57$), and finally by males under 22 months ($P = 0.26$), in which *Brucella* contact was occasional. Neither the presence of outdoor domestic pigs nor the geographical location influenced seroconversion against *Brucella* spp.

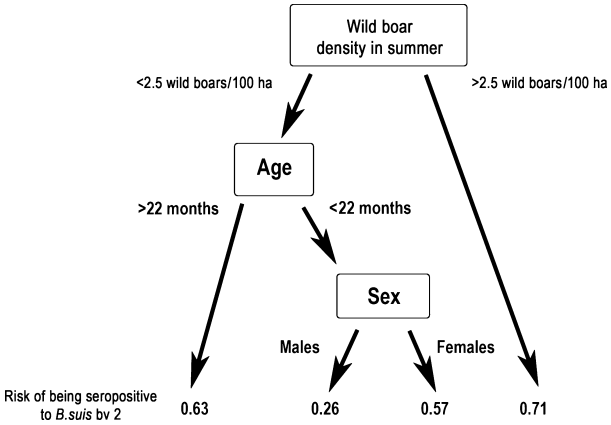

Fig. 3. Tree-based modelling representing the most important risk factors for explaining the probability of seroconversion against *B. suis* bv 2 in wild boar hunt harvested in hunting states in Extremadura, southwestern Spain.

Discussion

The prevalence of anti-Brucella antibodies observed in our study area is in line with that obtained in the most recent national survey (Muñoz et al., 2010) and reveals Extremadura to be one of the Spanish regions with the highest *Brucella* seroprevalence. However, despite the large number

of *Brucella* seropositive animals, only seven isolates were obtained, all identified as *B. suis* bv 2. It is important to note that the presence of specific antibodies against *Brucella* does not necessarily imply active infection (Godfroid, 2002). In domestic pigs experimentally infected with *B. suis*, pathogen isolation became impossible a mere 4 months after inoculation (Deyoe, 1972), an observation that may explain the small number of active genital infections relative to the amount of seropositive animals at the time of sampling. Moreover, we should take into account the fact that the existence of false-positive serological reactions induced by gram-negative bacteria sharing common epitopes with *Brucella* might cause an overestimation of the apparent seroprevalence against *Brucella* (Muñoz et al., 2010).

Wild boar density in summer was the most important factor for explaining *B. suis* bv 2 prevalence in our study area, probably because it is correlated with a higher contact rate of animal at specific points during the dry period when food and water is scarce (Fernández-Llario, 1996). In agreement with this finding, a recent study has reported a parallel increase of *Brucella* spp. prevalence and of the wild boar population size in Switzerland (Wu et al., 2011). However, surprisingly, other studies conducted in Spain (Muñoz et al., 2010) have failed to detect any kind of density dependence, possibly because Brucella-free intensive game exploitations with high wild boar densities were included. In our case, we only analysed data from unfenced estates with densities of 2–10 wild boars per square kilometre, while in other works a number of different types of populations (open, fenced and farmed) have been studied. In these kinds of populations other risk factors may be more relevant than wild boar density.

In addition, *B. suis* bv 2 infections seem to depend on age and sex, wild boar males under 22 months being the group with the lowest prevalence. Considering that sexual activity in male wild boars under 2 years old is limited (Fenández-Llario, 2005), our results suggest that transmission of *B. suis* mostly occurs through mating. It is in agreement with the results found in previous studies where the presence of anti-Brucella antibodies depended on sex and age of wild boar, being animals sexually active those with higher prevalence (Bergagna et al., 2009).

In contrast to recent studies from the same region (Muñoz et al., 2010), the existence of outdoor pig farms did not appear to have any type of influence on the observed risk of *B. suis* infection. However, we should be aware that our way of characterizing the effect of domestic pigs (i.e. presence or absence of outdoor exploitations) is a poor subrogate for determining the risk of interactions with domestic pigs. More accurate estimates of outdoor pig densities sharing habitats with wild boar and the knowledge about their serology status against Brucella would allow us to improve our understanding of the role of livestock in the maintenance and spread of *B. suis* bv 2.

According to our results, a previous contact with *B. suis* bv2 does not appear to have any influence on wild boar reproductive parameters, which were mainly shaped by the age of individuals. Only the breeding status of females seemed to be slightly affected by a previous contact with Brucella (which diminishes the probability of becoming pregnant), but further research considering additional measurements of fecundity (e.g. intrauterine mortality) is still needed to address this question. Indeed, we used seropositivity and not active genital infection with *B. suis* to evaluate the influence of *Brucella* on the studied reproductive parameters, and it has been reported that some time after *B. suis* infections in domestic pigs, reproductive parameters can revert to normal again (MacMillan et al., 2006).

Interestingly, three females with active infections turned out to be non-breeding females, whereas three of four infected males had manifest orchitis. However, due to the low number of infected animals found in this study, we cannot conclusively determine what effects active genital infection with *B. suis* had on these reproductive parameters, and further research with larger numbers of infected animals are needed to analyse this effect. Even so, the lack of influence on apparent fertility and the few cases of genital infections detected suggest that *B. suis* does not shape reproductive success in wild boar at population level, as has been suggested by other studies of wild boar populations with great seroprevalence against *Brucella* that have increased constantly (Wu et al., 2011).

The isolation of the oetiological agent of porcine brucellosis in our sample confirms that *B. suis* is present and circulates in the wild boar population in Extremadura. Adult, sexually active boars are potential spreaders of porcine brucellosis in outdoor pig farms that share the same habitat, above all in hunting estates with overpopulations of wild boar. Although our statistical modelling explained a moderately low proportion of the observed *B. suis* seroprevalence, our results suggest that wild boar density in summer may be a risk factor. Thus, management measures aiming at reducing wild boar densities may contribute to the control of porcine brucellosis. This measure will be extremely important in hunting estates with summer wild boar densities over 2.5 wild boar/100 ha and, particularly, in those estates that also have outdoor pig farms.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Variability of *B. suis* bv 2 isolates assessed by MLVA