ASSESSMENT OF TUBERCULOSIS PREVALENCE TRENDS IN MONTES DE TOLEDO WILD BOAR PRIOR TO VACCINATION

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ABSTRACT

The aims of this work are (1) to obtain a set of indicators suitable for wild boar TB vaccination success assessment under field conditions (2) to use these indicators to describe the initial situation of TB in the vaccination area prior to the start of vaccine deployment (3) to recommend how vaccine success should be measured, based on the available indicators and on the variation of these indicators in space and time (4) to investigate if co-infection with PCV2 is related with the severity of naturally acquired TB in wild boar. Based on a sample of 927 wild boar (Sus scrofa) from Montes de Toledo (Spain), we define 5 TB indicators based on lesions, antibody detection, and culture. Mean lesion prevalence ranged from 29 to 88% by site, mean ELISA prevalence ranged from 27 to 73% by site and of the 77 cultured wild boar, 42 were confirmed as belonging to the MTC by spoligotyping (54%). We suggest that all 5 indicators are valuable, and also propose a classification of wild boar regarding their Tb status into “TB-negative”, “exposed” and “super-excretor”. This study reflects the capital importance of proper disease monitoring prior to starting any disease control. With the presented results, we are able to have a fair knowledge of the background of TB in the future vaccination trial area. Therefore, we are now more aware of the handicaps we face when assessing vaccination success.

Keywords: tuberculosis, wild boar, monitoring, indicators, disease control, co-infection.
INTRODUCTION

Tuberculosis (TB) is a chronic disease caused by *Mycobacterium bovis* and closely related members of the *Mycobacterium tuberculosis* complex (MTC) affecting livestock (causing important economic losses), companion animals, wildlife as well as humans (Gortázar et al. 2012). TB control measures are focused mainly on cattle, through test and cull and movement restriction.

However, TB is also present among goat and pig livestock. In some regions, TB control in livestock is not enough to eradicate the disease and raises concerns regarding the interference of wildlife reservoirs. In Europe, the Eurasian badger (*Meles meles*), cervids of the subfamily Cervinae, and the Eurasian wild boar (*Sus scrofa*) are considered significant MTC maintenance hosts. Outside Europe, known wildlife reservoir situations include the introduced brushtail possum (*Trichosurus vulpecula*) in New Zealand, the cape buffalo (*Syncerus caffer*) in sub-Saharan Africa, and the white-tailed deer (*Odocoileus virginianus*) in North America (Palmer et al. 2012).

In Spain, the wild boar is a native species widely distributed and increasing in numbers (Acevedo et al. 2007) and has been identified as the main wildlife TB reservoir (Naranjo et al. 2008; Boadella et al. 2012). This is the reason why the target species of wildlife TB control in Spain is the wild boar.

Disease control at the human–livestock–wildlife interface should be based on a thorough knowledge of the “natural history” (ecology) of the disease agent and its hosts (Woodford 2009). Disease and population monitoring is a fundamental part of disease ecology. Figure 1 presents a diagram of how new diseases usually lead first to descriptive epidemiology and eventually to risk factor analyses and control actions. Monitoring is needed to identify changes in disease occurrence and to measure the impact of intervention (Boadella et al. 2011a).
Approaches towards disease control in wildlife can be divided into (1) preventive actions that would include barriers, improved hunting carcass hygiene, risk management at waterholes and feeders and so on, (2) wildlife population control measures (culling, selective culling, fertility control), and (3) treatments such as vaccination. Another option is not taking any measures when feasibility and economic reasons prevent us from doing so.

Wildlife vaccination is a strategy generally limited to the most relevant diseases shared with domestic animals or humans. It allowed controlling fox (Vulpes vulpes) rabies in central Europe, demonstrating its value as control tool (Brochier et al. 1991; Pastoret et al. 1996; Cross et al. 2007). More recently, Germany and France have witnessed success in the control of classical swine fever through oral vaccination of wild boar (Rossi et al. 2010). Currently, this control measure is being considered for TB in different geographic regions and in a variety of wildlife hosts as a key element in a long-term strategy to eradicate the disease from cattle (e.g. Ballesteros et al., 2009a; Corner et al. 2009; Tompkins et al., 2009; Chambers et al. 2011; Palmer et al. 2012).

Wildlife vaccination requires a long development process with many steps needed to take into account as summarized in Figure 2.
Briefly, two different vaccines are now available for wild boar, including the traditional Bacille Calmette–Guerin (BCG), an attenuated live strain of *M. bovis*, and a newly developed heat-killed vaccine (MdR, Garrido et al. 2011). Since oral delivery is the most practical means for wildlife vaccination, specific baits and reliable delivery systems that ensure high specificity and acceptance were designed. These baits and delivery cages target 2-4 month old wild boar piglets, the preferred age for vaccination (Ballesteros et al. 2009b, Ballesteros et al., 2011, Gortazar et al. 2012).

Therefore, studies in real settings under conditions of natural MTC transmission are now possible and will hopefully reveal the full potential of protecting wild boar against TB using vaccines. One aspect of paramount importance in vaccination experiments is assessing vaccine efficacy. In the badger in the UK, legal constraints limit the TB tests to those that can be used without killing the subject, including culture of different swabs and samples, gamma interferon testing, and serology (Chambers et al. 2011). In possums in New Zealand, since there are no restrictions on culling, TB tests include both in vivo tests such as lymphocyte proliferation assays and culture of lesion aspirates and postmortem pathology and culture (Tompkins et al. 2009).

Regarding vaccine efficacy assessment in wild boar, in the laboratory trials
carried out between 2008 and 2012, several tools including pathology scores, culture scores, serology, gamma interferon and gene expression were set up to assess wild boar response to vaccination and to experimental challenge with an *M. bovis* field strain (Ballesteros et al. 2009a, Garrido et al. 2011). However, some of these tools are not suitable for field use. For instance, gamma interferon testing requires fresh whole blood samples and gene expression analyses are quite expensive. Hence, diagnostic tools need to be adapted and refined for appropriate field use in large scale experiments.

One characteristic that makes a difference between laboratory experiments and field settings is that in natural (field) situations, co-infections are frequent (Ruiz-Fons et al. 2006, Boadella et al. 2011b). Moreover, it is well known that the intense management of wild boar for increased hunting harvest, through fencing and supplementary feeding, is associated with higher infection risks of directly transmitted pathogens. Porcine circovirus type 2 (PCV2) is considered as the etiological agent of post-weaning multisystemic wasting syndrome (PMWS) an immune-suppressing disease of nursery and growing domestic pigs. Antibodies to PCV2 are frequent among wild boar from the study area, and remained stable during the last decade (Boadella et al. 2012). PMWS might play a role in the population dynamics of intensively managed wild boar populations, through increased piglet mortality (Vicente et al. 2004). PCV2 seroprevalence in Spanish wild boar has been reported to be around 48% (Vicente et al., 2004) meaning that PCV2 circulates at a high rate among the wild boar populations and several PMWS reports in wild boar are published (Ellis et al. 2003; Schulze et al. 2004 Vicente et al. 2004; Sofia et al. 2008; Reiner et al. 2010). One peculiar aspect of PCV2 pathogenesis is lymphoid depletion and facilitation of co-infections.

The aims of this work are:

1. To obtain a set of indicators suitable for wild boar TB vaccination success assessment under field conditions.
2. To use these indicators to describe the initial situation of TB in the vaccination area prior to the start of vaccine deployment.
(3) To recommend how vaccine success should be measured, based on the available indicators and variation of these indicators in space and time.

(4) Additionally, our last objective was investigating if co-infection with PCV2 is related with the severity of naturally acquired TB in wild boar.
MATERIALS AND METHODS

Study area
Montes de Toledo is a mountain chain located in the South of the Central Spanish plateau. The predominating habitat is Mediterranean characterized by evergreen oak Quercus ilex woodlands and scrublands (dominated by Cystus sp, Erica sp, Pistacia sp, Phyllirea sp and Rosmarinus sp) with scattered pastures and small areas of crops (Vicente et al., 2007; de Acevedo et al., 2008), often in savannah like landscape units named dehesas. The climate is typical Mediterranean, with mild to cold winters and hot and summers and limited rainfall (usually less than 600 mm per year) concentrated in spring and autumn and an average temperature of 14.09°C.

Sampling sites included private and public estates (n=27) of Montes de Toledo (39°25' to 39°16'N, 4°05' to 4°23'W), in the region of Castilla La Mancha in South Central Spain. This area has a surface of approximately 36,000 hectares and a height of between 590 to 1010 meters above sea level. Study sites are mainly devoted to recreational hunting for wild boar and red deer (Cervus elaphus) and represent a gradient of situations from intense hunting management (involving fencing, artificial feeding and watering) to a lesser or inexistent management. The study area was divided into East and West because of the existence of geographical barriers.
Figure 3. - Map of the MT area showing the sampling sites and the division of the study area into West and East. Controls areas are composed by various sites and are divided into East controls (red dots) and West controls (blue dots). On the other hand, future treatment sites represent single hunting estates: East treatment site is QM (perimeter delimited in black) West treatment sites are DHC (perimeter delimited in yellow) and LSVLL (perimeter delimited in white).
**Sampling**

Samples were collected from carcasses (n = 987) recovered during the regular hunting seasons (October–February) from 2009/2010 to 2011/2012. After each hunting event a representative sample stratified by age and sex of the hunted animals was randomly selected. Each specimen was subjected to a quick general inspection, collection of biometrical data (total length, thoracic perimeter and hind foot length), tick sampling, and sex and age determination. Age class estimation was done based on tooth eruption patterns: wild boar less than 12 months old were classified as piglets, wild boar between 12 and 24 months were classified as subadults and those over 2 years as adults.

Selected organs were collected and taken to the laboratory for systematic inspection, lesion scoring (see below) and sampling for culture (stored at -20°C until use) and histopathology (fixed by immersion in 10% neutral-buffered formalin). These included the mandibular lymph nodes (LN), tonsils, lungs with tracheobronquial and mediastinal LN, spleen, mesenteric LN as well as other affected organs if detected during inspection. Blood samples were collected from the thoracic cavity. Serum was obtained after centrifugation and stored frozen at –20 °C until use.

**Gross pathology: indicators 1 and 2**

In the laboratory each organ was subjected to careful macroscopic inspection, including serial sectioning. We obtained two indicators: presence/absence of TB-compatible lesions (Indicator 1; Vicente et al. 2006) and TB-compatible lesion score, a new indicator used in the last season (2011-2012; Indicator 2). This new indicator informs on lesion severity and is a simplification of the one proposed by Ballesteros et al. (2009a) for laboratory challenge experiments. The new scoring system was based on lesion size and gives each LN (n=6) and lung lobe (n=7) a standardized value according to the size of the lesion: 0 if no lesion was detected, 1 for lesions smaller than 1cm ("A" lesions) and 2 if lesions detected were >1cm ("B" lesions; Martín-Hernando et al. 2007). By adding up these values we obtained a total score that ranged potentially from 0 to 26.
TB ELISA: indicators 3 and 4

Serology was performed by means of an in-house indirect ELISA technique using bovine purified protein derivative (bPDD) following the protocol described by Boadella et al. (2011b). Sample results were expressed as an ELISA percentage (%) and calculated using the formula: Sample E% = [(sample OD/2 x mean negative control OD) x100]. The cut-off was established at 100% and therefore animals with higher values were considered positive (Indicator 3). This test has 79% sensitivity and 100% specificity in wild boar (Boadella et al. 2011b).

Super excretors (SE) are defined as individuals with more severe (progressed, disseminated) disease that may pose a greater risk of infecting other animals through repeated and/or greater excretion of *M. bovis* (Gallagher et al. 1998; Corner et al. 2006) and therefore contribute disproportionately to infection maintenance (Kramer-Schadt et al. 2009). In addition, SE have a higher mortality rate (Wilkinson et al. 2000) and so probably represent animals with progressive TB that does not resolve. We arbitrarily classified ELISA positive wild boar as “high positive” if the ELISA percentage was >550%. These animals were considered SE (indicator 4). In previous studies we observed a positive relationship between the bPPD ELISA values and total lesion scores in experimentally *M. bovis* challenged wild boar (Garrido et al. 2011). This classification is also similar to the one proposed for badgers (Chambers et al. 2008).
Culture: Indicator 5
Tissue samples of 77 less than two year old wild boar (piglets + subadults) hunted in the 2011-2012 season were submitted for culture.
A mandibular LN and tonsil pool and a thoracic LN pool were tested separately, following the procedures described in Garrido et al. (2011).
Briefly, samples were thoroughly homogenized in sterile distilled water (2 g in 10 ml or equivalently). Five ml of this suspension was decontaminated and processed following the instructions of the manufacturer to inoculate BBL MGIT tubes supplemented with BBL MGIT PANTA and BACTEC MGIT growth supplement (Becton Dickinson). BBLtubes were incubated for 42 days in a BACTEC MGIT 960 System. The remaining 5 ml were decontaminated in hexadecyl-pyridinium chloride at a final concentration of 0.75% (w/v) for 12–18 h. Samples were centrifuged at 2500 x g for 5 min and pellets cultured in Coletsos tubes (bioMérieux) at 37ºC for 4 months. All isolates were spoligotyped in order to confirm the strain (Kamerbeek et al.1997).
Any individual wild boar with a mycobacterial growth confirmed by spoligotyping as belonging to the MTC was defined as culture-positive (Indicator 5).

Histopathology
Tissue samples from 35 culture confirmed M. bovis infected wild boar (aged <2 years, sampling season 2011/12) were routinely processed for histopathology and examined under light microscope by an expert (M.P. Martín-Hernando).

Proposed TB status classification
1) Wild boar without TB compatible lesions, with a negative ELISA and a negative culture result are defined as TB negative.
2) Among the TB positive wild boar:
   - Those that do not match the criteria defining SE (high ELISA values) are called exposed.
   - While those with high ELISA are called SE.

Antibodies against Circovirus: a co-infection indicator
Wild boar sera were tested for antibodies against PCV2 by means of a commercial indirect ELISA (INGEZIM CIRCO IgG, INGENASA, Madrid, Spain).
Following manufacturer instructions, a negative cut-off was calculated as the mean OD of negative controls +0,2 and a positive cut-off was calculated as the mean OD of negative controls +0,25. Samples with OD’s between both cut-offs were considered doubtful.

Statistics
Sterne’s exact method was used to estimate apparent prevalence 95% confidence intervals (CIs). Statistical analyses applied are specified in Results since each analysis required a different test. The p-value was set at 0.05. Data was analyzed using IBM SPSS statistical package version 20 (IBM Corporation, Somar, NY, USA) and STATISTICA (version 7.1; StatSoft, Inc., Tulsa, OK, USA).
RESULTS

TB indicators

Five indicators of TB status were used in this study: (1) gross lesion prevalence; (2) quantitative lesion score; (3) ELISA prevalence; (4) proportion of very high ELISA reactors (SE); and (5) culture prevalence. Prevalence of indicators (1), (3), and (4) varied significantly among age classes but not indicator (5) (Figure 5).

The lesion score (indicator 2) varied with age class ($X^2= 19.9$, $p<0.001$) and correlated positively with ELISA values in all age classes ($r_s=0.527$, $p<0.05$; $r_s= 0.512$, $p<0.05$; $r_s = 0.537$, $p<0.05$ for piglets, subadults and adults, respectively). We tested sex effect over these five indicators and found no significant effect except for indicator (3) ELISA prevalence (Mann-Whitney U test: $Z= 2.3$, $p<0.05$). Thus, sex was not included as a factor in subsequent analyses.

![Figure 5: Prevalences of indicators (1), (3), (4) and (5) for each age class (1= piglets; 2= subadults and 3 =adults). Differences between age classes were tested with the median test or](image_url)
Mann-Whitney U test and statistical values as well as p-values are provided in the lower right corner. Differences were considered statistically significant when \( p<0.05 \) (bold).

Indicators (1), (3) and (4) were available for between-season comparisons. Results on seasonal differences are summarized in Table 1.

All five indicators were available for comparisons among study sites. Numerical data on comparisons among study sites is summarized in Tables 2, 3, 4, 5.

Prevalence differences between East and West controls were analyzed by means of Pearson’s chi-square test or Fisher’s exact test and no significant difference was found \( (p>0.05) \). The mean lesion score was compared between East and West controls with Mann-Whitney’s U test, also with no significant difference \( (p>0.05) \). However, when test sites were included in the east and west parts of the study area, significant differences between the mean lesion score were evidenced \( \text{(East} 3.35\, 95\%\text{IC}=0-6, \text{West} 2.09\, 95\%\text{IC}=0-5, \text{U test}, z=3.5, p<0.001) \).

Of the 77 cultured wild boar, 42 were confirmed as belonging to the MTC by spoligotyping (54.5%). Nine additional wild boar were culture positive but spoligotype negative (results pending). Eleven different spoligotype patterns were identified: SB0119 \( (n=3) \); SB0120 \( (n=1) \); SB0121 \( (n=5) \); SB0134 \( (n=8) \); SB0157 \( (n=5) \); SB0265 \( (n=5) \); SB0339 \( (n=7) \); SB1018 \( (n=1) \); SB1263 \( (n=5) \); SB1316 \( (n=1) \); SB1565 \( (n=1) \). Of these patterns, all belonged to \( M. \text{bovis} \) except for SB0157 which belonged to \( M. \text{caprae} \). Culture prevalence difference between ages is presented on Table 6. Correlation between score and culture results is showed in Figure 6.

Histopathology revealed that 4 \( (11.4\%) \) culture positive wild boar had no microscopic lesions, and that lesions were only microscopic (no gross lesions) in another two confirmed infected wild boar (although for one of them the head LN were missing). Actinomycosis-compatible lesions were observed in 4 wild boar. Histopathology also revealed microscopic thoracic lesions in 7 wild boar where gross inspection only revealed head LN lesions. In total, gross lung lesions or only microscopic lung lesions were recorded in 13 of 25 wild boar with complete samples available \( (52\%) \). Thoracic lesions were less often calcified (and less detectable at visual inspection) than mandibular LN ones.
Figure 6: Relation between lesion score and culture results. The bar graph displays the relationship between the TB-compatible lesion score and culture results in < 2 year old individuals (piglets and subadults). The pie charts evidence the high agreement between culture and score results when score values are under 3 (left) and above 2 (right).

Figure 7.- Small type I tuberculous granuloma in the lung of a gross-lesion negative, culture positive wild boar. These cases are missed when only gross inspection is used. Haematoxilin-Eosin, 4x.
Table 1: Between-season comparison of indicators (1), (3) and (4). Number of samples (n), prevalence (%), and 95% confidence interval (95% CI) stratified by age class (piglets, subadults, adults) and hunting season (2009/2010, 2010/2011, 2011/2012). Results were analyzed by means of Pearson’s chi-square test (2 degrees of freedom in all tests). Differences were considered statistically significant when p<0.05 (bold).

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**Table 2: Lesion prevalence.** Number of carcasses inspected (n), lesion prevalence (%), and 95% confidence interval (95% CI) stratified by age class and site. Associations of age (categorical: piglets, subadults, adults) and site (categorical: West control, DHC, LSVLL, East control and QM) with results were analyzed by means of Pearson’s chi-square test (with continuity correction when needed). Differences were considered statistically significant when p<0.05 (bold). The last row represents means for the whole study area.
| Age Class | Site      | n  | Mean | Median | Range    | Stats | n | Mean | Median | Range    | Stats | n  | Mean | Median | Range    | Stats | n  | Mean | Median | Range    |
|----------|-----------|----|------|--------|---------|-------|----|------|--------|---------|-------|----|------|--------|---------|-------|----|------|--------|---------|-------|----|------|--------|---------|
| Piglets  |           |    |      |        |         |       |    |      |        |         |       |    |      |        |         |       |    |      |        |         |       |    |      |        |         |
|          | West      |    |      |        |         |       |    |      |        |         |       |    |      |        |         |       |    |      |        |         |       |    |      |        |         |
| Control  |           | 17 | 1.18 | 0      | 0-12    | Z= 1.0 | 8  | 3.13 | 0      | 0-19    | Z=0.2 | 34 | 3.24 | 2      | 0-22    | X² = 3.4 | 18 |
| DHC      |           | 7  | 0.57 | 0      | 0-4     | 2 d.f. | 2  | 2.50 | 2.50   | 2-3     | 2 d.f. | 7  | 2.57 | 2      | 0-10    |          |    |
| LSVLL    |           | 1  | 0    | 0      | 0-0     | p> 0.05 | 9  | 0.89 | 0      | 0-4     | p> 0.05 | 12 | 1.08 | 0.5    | 0-3     | p> 0.05 |    |
| Subadults|           |    |      |        |         |       |    |      |        |         |       |    |      |        |         |       |    |      |        |         |       |    |      |        |         |
|          | East      |    |      |        |         |       |    |      |        |         |       |    |      |        |         |       |    |      |        |         |       |    |      |        |         |
| Control  |           | 21 | 2.14 | 2      | 0-8     | Z= 1.0 | 32 | 1.38 | 0.5    | 0-5     | Z=1.4 | 44 | 4.39 | 4      | 0-17    | 1 d.f. | 17 |
| QM       |           | 8  | 2.88 | 2.5    | 0-6     | p> 0.05 | 7  | 2.86 | 4      | 0-6     | p> 0.05 | 17 | 6.29 | 4      | 0-20    | p> 0.05 |    |
| Adults   |           |    |      |        |         |       |    |      |        |         |       |    |      |        |         |       |    |      |        |         |       |    |      |        |         |
| West     |           |    |      |        |         |       |    |      |        |         |       |    |      |        |         |       |    |      |        |         |       |    |      |        |         |
| Control  |           | 21 | 2.14 | 2      | 0-8     | Z= 1.0 | 32 | 1.38 | 0.5    | 0-5     | Z=1.4 | 44 | 4.39 | 4      | 0-17    | 1 d.f. | 17 |
| DHC      |           | 7  | 0.57 | 0      | 0-4     | 2 d.f. | 2  | 2.50 | 2.50   | 2-3     | 2 d.f. | 7  | 2.57 | 2      | 0-10    |          |    |
| LSVLL    |           | 1  | 0    | 0      | 0-0     | p> 0.05 | 9  | 0.89 | 0      | 0-4     | p> 0.05 | 12 | 1.08 | 0.5    | 0-3     | p> 0.05 |    |
| East     |           |    |      |        |         |       |    |      |        |         |       |    |      |        |         |       |    |      |        |         |       |    |      |        |         |
| Control  |           | 21 | 2.14 | 2      | 0-8     | Z= 1.0 | 32 | 1.38 | 0.5    | 0-5     | Z=1.4 | 44 | 4.39 | 4      | 0-17    | 1 d.f. | 17 |
| QM       |           | 8  | 2.88 | 2.5    | 0-6     | p> 0.05 | 7  | 2.86 | 4      | 0-6     | p> 0.05 | 17 | 6.29 | 4      | 0-20    | p> 0.05 |    |
| Total    |           | 54 | 1.70 | 1      | 0-12    |        | 58 | 1.76 | 0.5    | 0-19    |        | 114| 3.87 | 3      | 0-22    |        |    |

**Table 3: TB-compatible lesion score.** Number of carcasses inspected (n), mean, median and range of values stratified by age class and site. Associations of age (categorical: piglets, subadults, adults) and site (categorical: West control, DHC, LSVLL, East control and QM) with results were analyzed by means of Mann-Whitney’s U test and median test. Differences were considered statistically significant when p<0.05 (bold). The last row represents means for the whole study area.

*Due to small sample size of LSVLL, Mann-Whitney U test was just performed for West control and DHC.

** Due to small sample size of DHC, Mann-Whitney U test was just performed for West control and LSVLL.
Table 4: Prevalence estimated by ELISA. Number of sera tested (n), ELISA prevalence (%) and 95% confidence interval (95% CI) stratified by age class and site. Associations of age (categorical: piglets, subadults, adults) and site (categorical: West control, DHC, LSVLL, East control and QM) with results were analyzed by means of Pearson’s chi-square test (with continuity correction when needed). Differences were considered statistically significant when p<0.05 (bold). The last row represents means for the whole study area.
### Table 5: Proportion of superexcretors.

Number of sera tested (n), superexcretor percentage (%) and 95% confidence interval (95% CI) stratified by age class and site. Associations of age (categorical: piglets, subadults, adults) and site (categorical: West control, DHC, LSVLL, East control and QM) with results were analyzed by means of Pearson’s chi-square test (with continuity correction when needed). Differences were considered statistically significant when p<0.05 (bold). The last row represents means for the whole study area.

<table>
<thead>
<tr>
<th></th>
<th>Piglets</th>
<th>Subadults</th>
<th>Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Prevalence (%)</td>
<td>95% CI</td>
</tr>
<tr>
<td>West</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>124</td>
<td>9.7</td>
<td>4-15</td>
</tr>
<tr>
<td>DHC</td>
<td>17</td>
<td>0</td>
<td>0-20</td>
</tr>
<tr>
<td>LSVLL</td>
<td>5</td>
<td>0</td>
<td>0-50</td>
</tr>
<tr>
<td>East</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>104</td>
<td>12.5</td>
<td>6-19</td>
</tr>
<tr>
<td>QM</td>
<td>47</td>
<td>8.5</td>
<td>0-16</td>
</tr>
<tr>
<td>Total</td>
<td>297</td>
<td>9.8</td>
<td>6-13</td>
</tr>
</tbody>
</table>
### Table 6: Culture prevalence.

Number of animals with mandibular lymph nodes plus tonsil and/or thoracic lymph nodes cultured (n), culture prevalence (%) and 95% confidence interval (95% CI) stratified by age class and site. Associations of age (categorical: yearlings, subadults, adults) and site (categorical: West control, DHC, LSVLL, East control and QM) with results were analyzed by means of Pearson’s chi-square test or Fisher’s exact test. Differences were considered statistically significant when p<0.05 (bold). The last row represents means for the whole study area.

<table>
<thead>
<tr>
<th></th>
<th>Yearlings</th>
<th></th>
<th>Subadults</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Prevalence (%)</td>
<td>95% CI</td>
<td>Stats</td>
</tr>
<tr>
<td><strong>West</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>75</td>
<td>33-100</td>
<td>$X^2=5.4$</td>
</tr>
<tr>
<td>DHC</td>
<td>7</td>
<td>85.7</td>
<td>60-100</td>
<td>2 d.f.</td>
</tr>
<tr>
<td>LSVLL</td>
<td>2</td>
<td>0</td>
<td>0-78</td>
<td>p=0.06</td>
</tr>
<tr>
<td><strong>East</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>15</td>
<td>80</td>
<td>60-100</td>
<td>Fisher’s test</td>
</tr>
<tr>
<td>QM</td>
<td>4</td>
<td>75</td>
<td>25-99</td>
<td>p=0.6478</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>32</td>
<td>75</td>
<td>60-90</td>
<td></td>
</tr>
</tbody>
</table>
Co-infection
Assessment of co-infection with PCV2 yielded the following results: Serum antibodies against PCV2 were found in 89.8% of the tested wild boar, including 85.7% of the piglets, 83.6% of the subadults and 94.6% of the adults. When analyzing the last season in order to test if PCV2 relates somehow with severity of naturally acquired TB (by using lesion score) we found that in piglets (n=49), PCV2 ELISA optical densities (ODs) correlated with the TB lesion score ($r_s=0.32$ $p<0.05$). This correlation was even stronger when only TB positive piglets (n= 25) were considered ($r_s=0.5$ $p=0.01$). In contrast, no such correlations between PCV2 ODs and TB lesion score were evidenced for subadult (n=55) and adult wild boar (n=112), respectively.
DISCUSSION

Nowadays, the implication of wildlife in transmission and maintenance of diseases that concurrently affect humans and livestock is widely recognized and an effort is being made to implement integrated control strategies (Boadella et al. 2012). The aim of these measures is to avoid negative consequences on public health, economy (losses because of trade barriers, decreased production, testing and culling, sacrifice in emergency situations…) and wildlife health and conservation. Even though this work is focused on vaccination, we would like to remark the capital importance of an integrated approach since there is no silver bullet for TB control and it is unlikely that a single control measure is capable of controlling infections at large spatial scales and being universally applicable (White et al. 2008).

Based on the results obtained in this study we gain valuable background information of our treatment and control sites that help us deciding the proper way to assess vaccine efficacy in a near future. This information provides (1) a clear overview of current TB status in wild boar in Montes de Toledo, (2) insights into the most suitable tools for estimating the effect of vaccination on TB prevalence and the proportion of individuals with severe TB and (3) evaluation of the natural inter-site and inter-season variation of five different TB indicators. In addition, we obtain new knowledge on the interaction between TB and PCV2 in wild boar.

Regarding the current TB status in wild boar in Montes de Toledo, our results show high prevalences, even in early life stages. This result concurs with previous studies (Vicente et al. 2006) made in Ciudad Real province and its boundaries (study area included MT but also Sierra Morena mountain chain) that reported macroscopic TB-like lesions (indicator 1) detection in up to 100% of the sampled wild boar (mean site prevalence 42%) and up to 50% in red deer (mean site prevalence 14%). Regarding cattle TB, prevalences of skin-test
reactors were 11.28% in the Horcajo veterinary unit and 4% in the Malagón veterinary unit in 2011 (Source: Regional Government Animal Health Authority). This evidences that our study area is a hotspot for TB and prevalences are among the highest reported for wildlife along with those reported in Doñana National Park (NP) - 52.4% - by culture from tissue samples, indicator (5) (Gortázar et al. 2008). It is remarkable that both areas (MT and Doñana NP) display high prevalences even though one is intensively managed due to its important commercial hunting industry whereas the other is a natural, almost unmanaged environment.

The accuracy of our data relies on the indicators of TB status used and the sample size. Fair sample sizes allow us to be much more confident in our results. Control areas are composed by various sites whereas treatment sites represent single hunting estates and therefore sample sizes achieved are lower. The observed variation also depends on the number of seasons we have been sampling in each particular hunting estate. We use 5 different indicators because of their different biological meanings.

As defined in the methods section, wild boar with an extremely high ELISA value are defined as SE. However, it is most likely that wild boar with considerable lung lesions do also significantly contribute to excretion. Thus, based on our results indicating that culture was consistently positive above a certain lesion score (Figure 6), we decided to correlate the lesion score with the ELISA value. We suggest that individuals with either a high TB lesion score (>11), or a high ELISA test result (>550%) are likely SE. Hence, we propose to widen the definition of SE by considering both indicators in parallel (i.e. one positive means the individual is defined as SE).
Figure 8. - Individuals considered super-spreaders (green area) by current definition (high ELISA results and/or high score values).

Figure 9. - Super-spreaders, other criteria for its definition could divide them into three categories:
1. Individuals with high ELISA results (yellow area).
2. Individuals with high score values (orange area).
3. Individuals with both high ELISA and score values (red area).

Table 7 shows how the 133 less than 2 year old wild boar sampled in 2011/12 would be divided among the three TB status categories.

<table>
<thead>
<tr>
<th>TB test results</th>
<th>n</th>
<th>%</th>
<th>Proposed name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesion negative AND ELISA negative AND culture negative</td>
<td>53</td>
<td>39.8</td>
<td>TB negative</td>
</tr>
<tr>
<td>Lesion positive OR ELISA positive OR Culture positive NOT fitting SE criteria</td>
<td>76</td>
<td>57.1</td>
<td>Exposed</td>
</tr>
<tr>
<td>Lesion positive OR ELISA positive OR Culture WITH Score &gt;11 AND/OR ELISA values &gt;550</td>
<td>4</td>
<td>3</td>
<td>Super-excretor</td>
</tr>
</tbody>
</table>

Table 7: Individuals belonging to each category in the epidemiological classification. All individuals under 2 years of the last season included (n=133), even if some of the tests was not available for some subjects.

Table 8 shows that 57% of wild boar under 2 years become exposed and just 3% become SE, the most epidemiologically relevant category. No animals meet
both SE criteria at the same time, indicating that those 4 shown on Figures 8 and 9 are adults. This classification has similarities to the one used in badgers in the UK (Chambers et al. 2008). The advantage of working with wild boar is the availability of carcasses during the hunting season that enables us to gain two relevant indicators (1 and 2).

Out of all the indicators, the lesion score is a newly proposed and very accurate tool to define lesion severity and lesion distribution and to recognize animals with generalized lesions and likely thoracic involvement (individuals that, therefore, may have an epidemiological implication as spreaders, Gavier-Widen et al. 2001). Therefore, the lesion score may be a better reflection of subtle changes expected due to vaccine protection in comparison with lesion presence/absence criteria and others. This score is actually a simplification of the one used in experimental vaccination and challenge studies (Ballesteros et al. 2009a; Garrido et al. 2011). However, we adapted it to field conditions where the number of wild boar is higher and we face time and logistic constraints for the number of organs to sample per individual. Our field lesion score is simplified regarding the number of sites inspected in the laboratory (6 LNs and 7 lung lobes) though a complete macroscopic exam of other organs is performed in the field.

As evidenced in Figure 6, the score has a good agreement with culture results: animals with score higher than 2 have 95% of probability of being culture positive. Culture is the gold standard technique for diagnosing TB but is costly from an economic and timing perspective, especially when working at a population level. So, if facing economic or time constraints, we could eventually skip culturing for animals with score higher than 3. It should also be noted that not all individuals with positive culture result exhibit lesions detectable by standard macroscopic inspection.

Histopathology revealed microscopic lesions in 2 wild boar with no gross lesions. This would add 6% of prevalence to the lesion indicator. However, histopathology also revealed 4 cases of presumptive actinomycosis. If these
lesions had occurred in wild boar of unknown TB-status and without histopathology, these could have been confounded with TB-like lesions during gross inspection. In a balance, additionally detected microscopic lesions would compensate for false-positive actinomycosis cases, yielding a similar final prevalence when comparing gross lesion inspection and microscopic inspection. Of course, histopathology is an important added value, but the time required is almost unaffordable in large scale studies, as opposed to controlled small scale experiments (e.g. Garrido et al. 2011 vs. Vicente et al. 2006).

Variation through sites gives us an idea on how different initial situations as evidenced on Results. From these data we can infer that wild boar from control sites are not a good reference to compare with treatment sites, since the current TB prevalence is quite different. However, control sites will allow recording if the general TB prevalence trend is increasing or decreasing during the treatment period.

Regarding TB indicator variation through time, we were surprised by the finding that serology indicators changed between seasons, even if only three seasons were included in this study. ELISA prevalence was significantly different just in piglets, perhaps due to different infection pressure. But interestingly, the proportion of high ELISA values (super-spreaders) was highest in piglets in season 1, in sub-adults in season 2, and in adults in season 3. This change could perhaps represent an epizootic wave of higher TB exposure, starting with an extremely high infection pressure among piglets in season one. Since inter-season changes in TB indicators were evident (at least the ones based in serology), once again, historic data for a treatment population are no good reference for the assessment of vaccination success.

Recommendations:
1) Use of as many indicators as possible. Paramount importance of quality and of biological meaning of the different indicators.
2) Potential usefulness of score.
3) Variability in space prevents us from making direct comparisons among sites.
4) Variability in time prevents us from making direct comparisons before/after treatment.

Concerning co-infection, we suggest that early contact of wild boar piglets with PCV2 could contribute to increase wild boar piglet susceptibility to generalized TB. This hypothesis would need experimental confirmation, but is supported by the fact that no such link between PCV2 and TB was evidenced in older age groups. If confirmed, this finding would have implications regarding the likelihood of co-infected wild boar piglets to become significant TB excretors.


CONCLUSIONS

1. - This study reflects the capital importance of proper disease monitoring prior to starting any disease control. With the presented results we are able to have a fair knowledge of the background of TB in the future vaccination trial area. Therefore, we are now more aware of the handicaps we face when assessing vaccination success.

2. - We define 5 TB indicators based on lesions (1 and 2), antibody detection (3), culture (5), and a combination of antibody detection and lesions (4). We suggest that all 5 indicators are valuable, and also propose a classification of wild boar regarding their Tb status into “TB-negative”, “exposed” and “super-excretor”.

3.- Among the three possible ways of measuring vaccine efficacy (changes between treatment and control sites; changes in time in treatment sites; differences between wild boar consuming or not consuming vaccine baits), the third is expected to be the most accurate one. It is important to sample as many wild boar as possible in the treatment sites, while keeping enough sampling effort in the control sites to allow monitoring the general TB trends in the study area.
ACKNOWLEDGEMENTS

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