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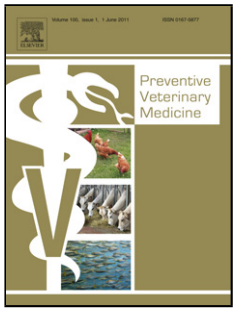

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Impact of piglet oral vaccination against tuberculosis in endemic free-ranging wild boar populations

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Abstract

The Eurasian wild boar (*Sus scrofa*) is the main wild reservoir of the *Mycobacterium tuberculosis* complex in Mediterranean woodlands and a key risk factor for cattle tuberculosis (TB) breakdowns. Wild boar vaccination therefore has the potential to be a valuable tool for TB control. We tested two orally delivered vaccines, heat-inactivated *Mycobacterium bovis* (IV) and BCG, in four sites (two per vaccine type: one Managed and one Natural or unmanaged) during four years. TB was also monitored in 15 unvaccinated sites (spatial control), as well as in all sites from one year prior to intervention (temporal control). The rationale is that by vaccinating 2-6 month old wild boar piglets we can reduce disease at the population level during the study period. This is achievable due to the fast turnover of wild boar populations. Vaccine baits were deployed using selective piglet feeders and this method proved highly successful with uptake rates of 50 to 74% in Natural sites and 89 to 92% in Managed sites. This is relevant for the potential delivery of vaccines to control other diseases, too. Local wild boar TB prevalence at the beginning of the study was already high ranging from 50 to 100%. TB prevalence increased in unvaccinated sites (6%), while a significant decline occurred in the Managed IV site (34%). Changes recorded in the remaining sites were not significant. The short-term impact of vaccination observed in the field was complemented by mathematical modelling, representative of the field system, which examined the long-term impact and showed that vaccination of piglets reduced prevalence and increased abundance at the population level. We conclude that IV could become part of integrated TB control schemes, although its application must be tailored for each specific site.

Keywords: BCG, epidemiological modelling, field vaccination, heat-inactivated *Mycobacterium bovis*, tuberculosis control, wild boar.

INTRODUCTION

Vaccination is an effective tool to prevent, control and eradicate infectious diseases (Rappuoli et al., 2002). However, technical and logistical difficulties, coupled with the high cost of vaccinating free-ranging wildlife have limited its application to diseases that have a significant impact on public health, economy or conservation (Cross et al., 2007). The turning point in wildlife vaccination was the successful use of an oral vaccine to control fox (*Vulpes vulpes*) rabies in Europe (Freuling et al., 2013). This success prompted research into field vaccination strategies to control other relevant diseases in wildlife, including animal tuberculosis (TB). TB is a chronic infection caused by *Mycobacterium bovis* (*M. bovis*), Myc*obacterium caprae* (*M. caprae*) and other members of the *Mycobacterium tuberculosis* complex (MTC). It is a zoonosis, although the number of human cases is now low in industrialised countries (Langer and LoBue, 2014) and therefore the impact of animal TB is mainly socio-economical, derived from eradication campaign costs, associated movement restrictions, and indirect losses to both the livestock (Zinsstag et al., 2006) and regional hunting industries (Barasona et al., 2016). Additionally, animal TB causes concern for the conservation of endangered species, e.g. the Iberian lynx (*Lynx pardinus*; Gortázar and Boadella, 2014).

The majority of TB-control efforts focus on cattle (Reviriego Gordejo and Vermeersch, 2006). However, TB is a well recognized example of multi-host infection and is unlikely to be eradicated without targeting all relevant hosts (Gortázar et al., 2015). The MTC host network in Mediterranean woodland habitats of the Iberian Peninsula is complex and includes several relevant domestic and wild host species (Gortázar and Boadella, 2014). The native Eurasian wild boar (*Sus scrofa*) is considered the main wild reservoir for MTC in this region (Naranjo et al., 2008). This suid is also regarded as a

key risk for cattle TB breakdowns (Hardstaff et al., 2014), mostly through indirect contact (Cowie et al., 2016; Kukielka et al., 2013). Wild boar are consequently an additional target species for TB control. Evidence suggests that wild boar management interventions, such as biosafety measures that reduce wildlife–cattle contact rates (Barasona et al., 2013) or culling of wild boar (Boadella et al., 2012), may reduce TB prevalence in sympatric ruminants such as cattle and red deer (*Cervus elaphus*). In this context, wild boar vaccination might represent a valuable additional tool for TB control in Mediterranean Iberia.

Proof of principle of TB disease reduction by vaccination with the live attenuated *M. bovis* Bacillus Calmette-Guérin (BCG) has been demonstrated for several wild reservoirs in controlled experiments (Buddle et al., 2006; Lesellier et al., 2006; Nol et al., 2008). Further field experiments have been conducted in brush-tailed possums (*Trichosurus vulpecula*; Corner et al., 2002; Tompkins et al., 2009; Nugent et al., 2016) and Eurasian badgers (*Meles meles*; Chambers et al., 2011; Gormley et al., 2017) with promising results regarding protection (see summary in Supplementary Material SM1).

Two orally administered vaccine candidates have been tested in laboratory trials for their effectiveness at controlling TB in wild boar: BCG (Ballesteros et al., 2009a; Garrido et al., 2011; Gortázar et al., 2014) and heat-inactivated *M. bovis* (IV; Garrido et al., 2011; Beltrán-Beck et al., 2014a). Both vaccines decrease disease severity, reducing lesion and culture scores, when compared to unvaccinated controls. Additionally, an injectable version of the IV vaccine successfully reduced TB lesion prevalence on a wild boar farm (66% reduction; Díez-Delgado et al., 2017). Vaccine safety and field

species-specific delivery have been assessed in additional trials (Beltrán-Beck et al., 2014b).

Both BCG and IV vaccines are prophylactic and non-sterilising. Thus, as in other TB vaccines, their protective effect is expected to reduce the severity of the disease and subsequent transmission, rather than curing or completely preventing infection. The vaccines are formulated for oral delivery, as oral administration via baits is the most practical means for wildlife vaccination on large scales (Cross et al., 2007). This, coupled with complementary tools such as species-specific baits (Ballesteros et al., 2009b) marked with chemical compounds (Ballesteros et al., 2011) and selective baiting stations (Ballesteros et al., 2009c), enables a targeted vaccine delivery and the assessment of bait uptake.

Extensive field trials are needed to assess vaccine performance in free-ranging populations using oral delivery. This study reports the results from a large-scale (ca. 460 km^2) four-year wild boar oral vaccination experiment that began in 2012 and was implemented in a high prevalence area of Montes de Toledo, Spain. The field trial targeted vaccination of 2-6 month wild boar piglets with the rationale that disease prevalence could be reduced within the four-year trial period. Piglets were chosen as the target age class (Ballesteros et al., 2009c) as they are more likely to be uninfected and thus suitable for vaccination (age is a risk factor for this chronic disease, O´Brien et al., 2002; Vicente et al., 2013). Moreover, given the fast population turnover of wild boar in the study area (where wild boar are extensively hunted), most subadult and adult wild boar will have been vaccinated by the end of the fourth year. This will enable the population effect of the vaccine to be assessed.

To underpin the field studies and to assess the impact of piglet vaccination on the epidemiological dynamics we also developed a mathematical model of wild boar TB interactions. Mathematical models are crucial tools for understanding how disease management strategies modify host and pathogen dynamics and have a long history of contributing to the understanding of the effectiveness of vaccination programmes (Keeling and Rohani, 2008; Scherer and McLean, 2002). Moreover, while the field study considered the short-term impact (after four years) of vaccination, the model can assess the long-term impact on prevalence and population abundance and test the consequences of vaccination success and of vaccine cessation on the resultant epidemiological dynamics.

This study therefore combines field trials of a four-year wild boar vaccination experiment with a mathematical modelling study of the field system. Our aims were to: first assess bait uptake rates under field conditions; second assess vaccine impact measured as changes in TB prevalence in the wild boar population based on pathology; and third mathematically model field vaccination in order to gather additional long-term insights into the influence of different levels of vaccination on disease prevalence and population density. Our hypothesis was that wild boar piglets would be efficiently targeted and that both IV and BCG would lead to measurable reductions in TB prevalence.

MATERIALS AND METHODS

The experiment was conducted under a research license (828493/2011) issued by D.G. Agricultura y Ganadería, Junta de Castilla- La Mancha. Post-mortem inspection and

sampling were performed on hunter-harvested wild boar. No animals were culled for the experiment.

Study area

The study was conducted in Montes de Toledo, a mountain chain located in Central Spain (39º 25' to 39° 16'N, 4° 05' to 4° 23'W). This region has a Mediterranean wood and scrubland habitat dominated by evergreen oaks (*Quercus* sp.). The climate is typically Mediterranean, with mild to cold winters, hot summers and rainfall mostly limited to spring and autumn.

The study area is composed of an array of privately owned hunting estates, communal lands and natural areas representing a gradient of wildlife management levels. Natural or unmanaged populations are those in which free-living individuals live on open lands where no supplementary feeding takes place, while managed populations generally maintain high densities through supplementary feeding and fencing. Both of them, natural and managed populations are hunted (with differences in economical profitability). Land use changes have favoured the upsurge of a commercial hunting industry that is economically relevant for the area, in which the main big game species are red deer and wild boar (Vicente et al., 2013).

In this TB endemic area, wild boar TB-compatible lesion (TBCL) prevalence ranges from 52% to 70% and has increased over time (Vicente et al., 2013). Lower (12%) and relatively stable TBCL prevalence has been described for red deer (Vicente et al., 2013).

A total of 19 sites were selected for TB monitoring, of which two privately owned hunting estates and a natural park were devoted to vaccination (96 km^2) and the remaining sites, representative of the whole management spectrum, were pooled and used as control (n=15, ca. 360 km^2; further site characterization is provided in Table 1 Supplementary Material SM2).

BCG was deployed on one of the privately owned estates (Managed BCG) and IV on the other (Managed IV). The natural park was divided into two areas (accounting for two sites) delimited by a topographical barrier. BCG was deployed in the north area (Natural BCG) while IV was deployed in the south area (Natural IV; Figure 1). Thus, Natural BCG and Natural IV sites w~~h~~ere separated by the main road that separates the north and south mountain chains and an exposed flat area of open grassland.

Rationale

Given the high wild boar TB prevalence in the study area (63%) and the well-documented increasing trend (Vicente et al. 2013), measures to control TB in this species are needed. We propose that vaccination represents a chance to reduce TB in wild boar and can be readily implemented and assessed in the context of the existing monitoring scheme.

Piglets as target of vaccination

TB is a chronic infection that progresses slowly until it eventually kills the animal (Barasona et al. 2016). TB vaccines are preventive, thus in order to protect, the animal must be uninfected. Since increasing age is a well-known TB risk factor (O´Brien et al., 2002; Vicente et al., 2013), 2-6 month old piglets are the vaccination target as they are

less likely to be infected. Moreover, vaccinating in early life could prevent the generalization of the disease. Generalized individuals are those with disseminated lesions that excrete large concentrations of mycobacteria (Santos et al., 2015; Barasona et al., 2017). These are known as super-shedders and are believed to be the major drivers of infection maintenance within populations, and thus are key targets for disease control (Kramer-Schadt et al. 2009).

Assessing disease at population level

In the study area wild boar are regularly hunted, i.e. hunting is an inherent feature of the study sites. This provides a suitable framework for data and sample collection that is, indeed, used for the national wildlife monitoring programmes (MAPAMA, 2017). Thus, an effective control strategy should integrate in this set-up monitoring framework that also enables the assessment of the impact of the intervention. Monitoring based on sampling hunter-harvested wild boar provides a solid means for wildlife TB assessment, although it has some limitations. One of them is that hunters do not target piglets as they lack trophy value, so this age class is under represented. Also, assessing protection is difficult when piglets are vaccinated in summer and sampled 2-6 months later. Moreover, the effects of vaccination need to take place at a population level, i.e. cause a decrease in prevalence in the overall population. Since hunting leads to a fast population turnover, most of the population will belong to a vaccinated cohort by the end of the experiment (four year vaccination). In summary, vaccine assessment is performed over the whole population and results are expected by the fourth year.

Vaccination program

Vaccines

The live attenuated BCG vaccine was derived from Danish *M. bovis* (CCUG strain 27863) and was prepared as described elsewhere (Ballesteros et al., 2009a; Garrido et al., 2011; Gortázar et al., 2014). Vaccine doses consist of 0.15 ml of a suspension containing 10^6 c.f.u. (the dose tested in Ballesteros et al., 2009a; Garrido et al., 2011; Gortazar et al., 2014). Vaccine doses were placed in sterile airtight polypropylene 0.2 ml vials (VWR®, Radnor, Pennsylvania, USA). BCG was freshly prepared for each vaccination cycle and stored at 4ºC until deployment (24 to 72 hours).

The IV vaccine was derived from a heat-inactivated field isolate obtained from naturally infected wild boar (Neiker1403, spoligotype SB0339) and was prepared as described in Garrido (2011). Each IV vial contained the equivalent of 10^7 c.f.u. in 0.2 ml of PBS.

Vaccine delivery

(i) Baits

BCG and IV vaccine vials were deployed in specific baits for wild boar piglets (Ballesteros et al., 2009b). The baits have a hemispherical shape (3.4 x 1.6 cm) and are made with piglet feed, wheat flour, paraffin, sucrose, and cinnamon-truffle powder attractant (Ballesteros et al., 2009b). These baits have proved stable, safe and effective as regards reaching the target species and age class in the field (Ballesteros et al., 2011). A chemical marker, iophenoxic acid (IPA; PR EuroCHEM Ltd., Cork, Ireland), was added to the baits (as described in Ballesteros et al., 2011) to determine the proportion of wild boar piglets consuming baits (bait uptake). Two IPA derivatives, each associated with a vaccine type, were employed. Propyl-IPA was associated with BCG baits and ethyl-IPA with IV baits.

(ii) Selective piglet feeders spatial distribution

Baits were placed in selective piglet feeders (Ballesteros et al., 2009c). Experimental areas were divided into a 2 km^2 grid by means of GIS analysis (QGIS version 1.8.0 Lisboa). Two piglet feeders were distributed in each grid and were separated by approximately 100 meters to avoid monopolisation by any dominant family group. They were placed in the vicinity of a permanent waterhole (to ensure wild boar passed by) in a spot where they received afternoon shade (to avoid extreme heat). Managed sites (BCG and IV) had 10 pairs of piglet feeders each and Natural sites 14 pairs each (total piglet feeders =96). A detailed map of piglet-feeder distribution is provided in Figure 1 Supplementary Material SM2.

Vaccination schedule

Vaccination took place in summer to target the main peak of 2-6 month old wild boar after weaning and thus, able to consume baits and immunologically mature. Moreover, in summer natural food resources are at their lowest in Mediterranean habitats, which potentially enhances bait consumption (Ballesteros et al., 2009a). To increase the use of feeders by wild boar and to limit bait uptake by non-target species maize was pre-baited 2-5 times a week for 8 weeks prior to vaccine deployment (Kaden et al., 2000; Ballesteros et al., 2011). Also, sham baits (without vaccine or markers) were placed to habituate wild boar piglets to baits.

The vaccination campaign included three cycles that consisted of three nights each. Two consecutive cycles took place in early summer (end of June-July) and one in late summer (end of August-September). Twenty baits per piglet feeder were deployed each day at dusk, leading to a total of 17280 vaccine baits per year (180 baits/km^2 and year)

during four consecutive years. Non-consumed baits were retrieved the next morning and fresh vaccine baits were newly placed each day (the vaccine spent a maximum of 12 hours in the environment).

Vaccine impact assessment

Hunter-harvested wild boar (n=1158) were sampled during the normal hunting season (October to February) from 2011-12 to 2015-16. Samples obtained prior to vaccination (hunting season 2011-12, "control year" hereafter) served as pre-intervention background, providing baseline data on infection and disease. A representative sample stratified by the age and sex of the hunted animals was randomly selected at each hunting event. Each specimen was subjected to sex and age determination, blood collection from the cavernous sinus (Arenas-Montes et al., 2013) and a general inspection of the whole carcass. Age was assessed on the basis of tooth eruption patterns (Saenz de Buruaga et al., 1991) and coat, establishing four categories: wild boar under 6 months were classified as very young piglets (n=24) which are the vaccination target, those from 6 to12 months were classified as piglets (n=227) and were sampled to assess bait uptake and vaccine impact, those between 12 and 24 months as yearlings (n=309), and those over 2 years as adults (n=598).

Organ samples taken in the field include the mandibular lymph nodes (LNs), tonsils, lung with tracheobronchial LNs and mediastinal LN, spleen, and mesenteric LNs (Martín-Hernando et al., 2007). TBCL presence and lesion scoring were recorded by carrying out detailed inspections in the laboratory.

Prevalence was used to estimate vaccine impact (which is the common approach in

wildlife TB studies e.g. Nugent et al., 2016; Díez-Delgado et al., 2017), as incidence is difficult to estimate in free-ranging wildlife (Delahay et al., 2013). Lesion presence is a recognised monitoring system to assess wildlife TB, since it is more practical and cost effective compared to culture, especially when working at a population level (Rodwell et al., 2001; Vicente et al., 2013). Lesion scoring is useful to determine the degree of vaccine-induced protection in laboratory trials, thus a simplified lesion scoring method was developed to report on lesion severity in field trials (Díez-Delgado et al., 2014a). Also, recording affected organs and cavities provides valuable information to determine disease severity (generalization) and infer infectiousness (Barasona et al., 2017). Briefly, the lesion score is based on lesion size (0 if no lesion is present, 1 for lesions <1 cm and 2 for larger lesions) and inspection of the routine target organs (considering each lung lobe separately and excluding the tonsils). An individual's total lesion score ranges from 0 to 26. Individuals with lesion scores >0 are defined as TBCL positive.

Processed tissues were stored at -20ºC. In order to confirm *M. bovis* or *M. caprae* presence, mandibular LN and tonsil pool plus a thoracic LN pool were cultured following the procedures described in Garrido (2011) and all isolates were spoligotyped (Kamerbeek et al., 1997).

Bait uptake assessment

Free-ranging wildlife does not allow for individual identification or individual assignation to a vaccine status unless marked and captured several times. Therefore, bait uptake was used as a proxy for vaccine coverage (proportion of individuals that have received a vaccine; Ballesteros et al., 2010; Beasley et al., 2015).

Bait uptake is determined by the presence of a chemical marker in serum (IPA derivatives). The IPA derivatives analysis was carried out following the extraction method and LC/ESI-MS analysis described in Ballesteros (2010). Markers are detectable in serum for at least 18 months after bait ingestion (Ballesteros et al., 2010). Marker presence is, therefore, used to estimate bait uptake by individual wild boar piglets in the vaccination campaign prior to the hunting season. Discriminating whether older (>12months) wild boar consumed bait as piglets or as older individuals is not possible when the marker is used over several consecutive years. Results of marker presence in older wild boar can therefore not be used to relate individual vaccine status to individual outcome.

Statistics

Descriptive analysis, predictors, and logistic regression

Changes in temporal trends within the same site were analysed using a Chi square test or Fisher exact test (two tailed) when required.

In order to assess vaccine impact (defined as the combined probability of bait uptake and protection) for each site as compared to control sites, a logistic regression model was fitted using lesion presence as a dependent variable. Predictors tested in the model were known drivers of TB (Vicente et al., 2013): age (<12 months, 12 to 24 months and >24 months old), rainfall (m), relative wild boar abundance, years (1 to 4); and initial TB prevalence (proportion), to account for the situation prior to intervention.

Data on study area rainfall were obtained from the National Agency of Meteorology, Station 4184. The cumulative annual rainfall was calculated from September to August

to match sampling years rather than natural years. Wild boar populations were monitored by obtaining annual relative wild boar abundance estimates based on a dropping frequency index (FBII; Acevedo et al., 2007) for the vaccinated sites (n=4) and the majority of control sites (n=11).

All analyses and data visualisation were undertaken with the R statistical package (R Development Core Team, 2015) and the ggplot2 package (Wickham, 2009). Significance was fixed at $p < 0.05$. The 95% confidence intervals (CI) were calculated using bootstrapping.

Modelling

A mathematical model representing the key processes in the field system was developed to answer questions that could not be tested in the experimental trial and to gain insight into the mechanisms that govern the dynamics of vaccinating against TB in wild boar. The model reflects a single geographical estate containing a homogeneously mixed population with parameters that are representative of the field-trial sites. The model is deterministic and compartmental and uses a system of ordinary differential equations to represent the dynamics of susceptible, infected (which have TBCL but are not infectious) and generalized (which have lesions in more than one anatomical region and are considered to be infected, infectious and suffer high disease-induced mortality) individuals for piglet, yearling and adult age-classes. Piglets that are successfully vaccinated (those that receive the vaccine and are receptive to immunisation) have a reduced chance of infection and if infected a reduced rate of progression to the generalized class.

Two different scenarios representing our vaccination sites were modelled: (a) a site with medium initial prevalence where piglets have a low chance of infection prior to vaccination and (b) a site with higher initial prevalence and greater rates of transmission combined with a greater proportion of piglets infected prior to vaccine delivery (through pseudo-vertical transmission from parent to offspring; piglets not receptive to immunization). Three situations were addressed: (i) the influence of different levels of vaccination success (which combines the effects of both coverage and efficacy by representing the proportion of effective immunisations) on disease prevalence, (ii) the influence of continued vaccination (25 years) and eventual cessation on population density, and on (iii) disease prevalence. The model framework, parameterisation and interpretation are further explained in Supplementary Material SM3.

RESULTS

Bait uptake

The proportion of wild boar with chemical marker presence in serum by site and age class is displayed in Figure 2. Piglets from Natural sites had lower uptake rates (50 to 74%) than those from Managed sites (89 to 92%). The chemical marker was detected as well in older (>12 months) wild boar (42-59%).

The topographical barrier separating different vaccine types on the Natural sites was not fully effective: consumption of both vaccine types (presence of both markers) was detected in 22-39% of vaccinated wild boar from the Natural sites.

Vaccine impact

Figure 3 presents the observed temporal trend of TBCL prevalence in the overall population and in the piglet age class. The agreement between TBCL and culture had a kappa value of 0.56 (raw data on TBCL, lesion score and culture are listed in Supplementary Material SM4).

TBCL prevalence increased steadily but not significantly when compared with the control year in the Control sites (6% increase, X^2 = 0.922, 1 d.f., p>0.05) during the study period. No individual control site had a consistently declining trend in TBCL prevalence (Table 1 Supplementary Material SM2). With regard to the vaccinated sites, a significant decline occurred on the Managed IV site (34% reduction since control year; X^2 = 7.665, 1 d.f., p<0.01). Vaccination on this site appeared to prevent infection and reduce disease severity (see culture and score data in Supplementary Material SM4). No significant changes were recorded on the remaining sites (p>0.05). The inter-annual variability in TBCL prevalence was marked on the Natural sites (Figure 3). No significant trend was recorded for any site in the piglet age class (p>0.05).

No significant differences in lesion scores were detected among vaccinated and control groups, probably due to heterogeneities in lesion evolution and challenge and to the simple scoring system used on field.

Table 1 displays the results of the logistic regression model. Vaccination had a significant effect when IV was used on the Managed site (p<0.001). However, its effect was negligible for the sites on which BCG was deployed (p>0.05) and for the Natural IV site (p>0.05). Other significant variables explaining TBCL presence in our model were increasing age, low rainfall and initial prevalence.

Modelling

Two scenarios representing our vaccination sites were investigated: (a) similar to Managed sites (medium initial prevalence where piglets have a low chance of infection prior to vaccination) and (b) similar to Natural sites (high initial prevalence and greater rates of transmission combined with a greater proportion of piglets infected prior to vaccine delivery).

Effects of vaccination success on disease prevalence

Figure 4 a(i) & b(i) (see also Supplementary Material SM3) shows that as the proportion of successfully vaccinated piglets (those effectively immunised) increases, TBCL prevalence decreases (38% and 30% decrease, respectively, when vaccination success is 100%). This decrease in total prevalence is driven by a reduction in the density of generalized individuals and is greatest when piglets have a lower risk of infection prior to vaccination (Figure 4a(i)).

Effects of continued vaccination (25 years) and eventual cessation on population density and disease prevalence

Figure 4 a(ii) & b(ii) shows the epidemiological dynamics for a 25-year vaccination programme, with a vaccine success-rate of 75%. By the end of the vaccination period the proportion of the population belonging to the piglet age class is 26% in Figure 4a and 34% in Figure 4b and therefore vaccination of piglets against TB is an effective method of TB control (see also Supplementary Material SM3). It indicates that there is an initial reduction in the level of infected and generalized individuals, which lowers disease transmission and consequently leads to a decrease in prevalence. The impact of

vaccination is largest when there is a reduced chance of piglet infection prior to vaccination and a lower initial prevalence. In the set-up that is most similar to Managed site IV the model predicts a 35% decrease in TB prevalence after 4 years (Figure 4a(iii)). This is comparable with the 34% decrease reported in the field study. A consequence of the vaccine induced reduction in prevalence is a reduction in population mortality due to a decrease in disease-induced death. This drives an increase in total population density which in the long-term allows the density of infected and generalized individuals to return to their pre-vaccination levels. Therefore, the long-term reduction in disease prevalence shown in Figure 4 a(iii) & b(iii) is a consequence of an increase in total population density rather than a decrease in the density of infected and generalized individuals. These model results highlight how observations from the early years of a vaccination programme may not provide a clear picture of the effectiveness of a long-term vaccination strategy, since the benefits of vaccination on reducing the level of infection in the early years are countered by the subsequent increase in total population density.

The model results also indicate that when the vaccination programme is stopped there is an initial increase in disease prevalence and density of infected and generalized wild boar before levels return to those prior to vaccination. This is a consequence of the elevated population density resulting from vaccination and of the temporary nature of vaccine-derived immunity (see also Supplementary Material SM3).

DISCUSSION

Contrary to our expectations we found no consistent reduction in prevalence after vaccination, the exception being IV vaccination on the Managed site. Here, under conditions of 90% bait uptake and 50% initial disease prevalence, IV appeared to prevent infection and reduce disease severity, lowering TBCL prevalence in the population by 34% after four years in a context of increasing prevalence in control sites. Model results confirmed that successful vaccination of piglets could lead to the observed reduction in prevalence but also predicted an increase in the overall host population density due to vaccine-derived reductions in disease-induced mortality in the long-term.

Achieving an adequate level of bait uptake is as important to the success of the strategy as vaccine efficacy (Massei et al., 2010). However, this goal is difficult to achieve and assess in free-ranging populations. Bait uptake by piglets is commonly a limiting factor in oral vaccination via baits (Kaden et al., 2000), but this trial was able to reach more than 70% (which is a reported threshold to achieve effective intervention; Anderson et al., 2013) of this age class on three of the four sites. This is relevant as regards the potential of vaccination for controlling other diseases, e.g. classical swine fever, in the case of its eventual emergence in Mediterranean regions. In this study, higher uptake was achieved in populations used to being fed and to human presence, i.e. managed hunting estates. Therefore species management, a reported risk factor for TB (Vicente et al., 2013), can be helpful in vaccine delivery whilst naïve populations might take longer to get used to new food sources (Delahay et al., 2003). While the bait uptake in piglets was high the presence of the marker was also recorded in a proportion of older individuals (42-59%). This could be due to marker persistence for greater than 18 months or indicate that, despite using piglet feeders, some older wild boar gained access

to baits. In previous studies that tested the viability of oral bait delivery (Ballesteros et al., 2011), older individuals gained access to baits in a proportion ranging from 8 to 43%. While the effect of vaccination on adults is unknown we speculate that it could act as a protective vaccination or revaccination, prolonging the individuals' immunity (as long as they are uninfected). It will nevertheless decrease bait availability for piglets.

There has been a dramatic increase in the prevalence of TBCL in wild boar over the last 20 years. For example, populations in Mediterranean Spain have shown a 26% increase in prevalence between 2000 and 2012 (Vicente et al., 2013). A similar increasing trend was observed in the control sites in this study, with a 10% increase in prevalence during the five-year study period. Our vaccination results should be interpreted in this context. Such findings make it increasingly important to assess new methods that can be used to reduce TB in wild boar. In this study we assess the potential of piglet vaccination as a tool for managing TB in wild boar.

Piglet vaccination using IV was successful in achieving a significant 34% reduction in TBCL prevalence at the population scale in the Managed site (90% piglet bait uptake and moderate, 50%, initial TBCL prevalence). The model results confirmed this (Figure 4) showing that piglet vaccination could lead to the rapid reduction of prevalence. The model also highlighted that a similar (albeit reduced) decrease in prevalence would occur even if there was a high chance of piglets becoming infected prior to vaccination. This demonstrates that the vaccination of piglets using IV can be a valid and effective TB control tool in wild boar.

In contrast, the impact of IV in the Natural site was negligible. This suggests that the effect of vaccination may be context dependent. Vaccine performance can be affected by initial prevalence (which affects exposure to infection), pre-existing infection (since the vaccine is not curative), population dynamics, and may vary over time and space (Halloran et al., 1997; Kaden et al., 2000; Gormley and Corner, 2011). Potential mechanisms for the different IV vaccine impact in the two sites in our study are: heterogeneous exposure to MTC, different levels of vaccination success at the two sites and inter-population mixing at the natural site.

First, exposure heterogeneity (in terms of infective dose and number of reinfections) could explain the different results obtained for IV as vaccines are believed to offer better protection against a light challenge of infection (Clemens et al., 2011). In our study, the Managed IV site was characterised by a moderate initial prevalence (50%), no generalization (lesions restricted to mandibular LNs) and low infection pressure for piglets, whereas the Natural IV site was characterised by a high initial prevalence (77%), a moderate proportion of generalized individuals (36%) and a high proportion of diseased 12-month-old wild boar (86%). Although the challenge dose is unknown in field trials, in the latter setting the potential exposure might have been sufficiently intense to resemble the challenge in laboratory trials, in which all individuals develop the disease despite receiving the vaccine. While the mathematical model does not explicitly include the intensity of exposure to infection the model results suggest that both increased transmission and the proportion of infected piglets prior to vaccination reduce the impact attainable through vaccination (Figure 4).

Secondly, bait uptake achieved on both IV sites was not significantly different (92 and 74%), but nevertheless the proportion of successfully vaccinated individuals (those that

received vaccine and were receptive to immunization) might have been. The likely higher level of infected piglets at the time of vaccination and the consumption of both vaccines (with possible non-protective outcomes; Díez-Delgado et al., 2014b) may have decreased the proportion of effectively immunised individuals and act as a confounder in the interpretation of vaccine impact based on vaccine type in Natural sites.
Thirdly, permeable fences in the Natural IV site allowed inter-population mixing (immigration/ emigration). These movements complicate the assessment of vaccine efficacy (dilution effect) and may act as a source of infection. Therefore we predict that enclosed and well-delimited (wild boar-proof fenced) populations will benefit most from vaccination.

We found no evidence of reduction of prevalence in any of the sites where BCG was deployed, as prevalence remained stable. BCG is known to confer variable protection in humans and cattle (Fine, 1995; Buddle et al., 2013) and field trials in which BCG failed to provide any protection have been reported (in humans e.g. Colditz et al., 1994 and wildlife e.g. de Klerk et al., 2010). Field trials deploying BCG in other wildlife hosts have demonstrated protective effects on vaccinated individuals (Supplementary Material SM1). While this study confirmed BCG viability (Beltrán-Beck et al., 2014b), we cannot rule out interference owing to non-tuberculous mycobacteria priming, genetic differences, nutritional status or co-infections (Fine, 1995; Buddle et al., 2013).

A limitation of this study is that despite having four treatment sites we lack replication as they are divided by type of management and vaccine. Differences among sites beyond the tested confounders might have influenced the outcome.

The results from the mathematical modelling study indicate that the long-term use of piglet vaccination could reduce TB prevalence and thereby control TB in wild boar but would not be sufficient to achieve eradication. The model provides important insight on how the epidemiological dynamics respond to vaccination. Disease prevalence reaches its minimum value around 5 years after the start of the vaccination campaign (roughly the time frame of this field experiment). Thereafter, the reduction in disease prevalence, and associated reduction in disease-induced mortality at the population level, leads to an increase in population abundance. This finding could be tested in future wildlife vaccination programmes against virulent pathogens. This increase in population abundance also implies that increased hunting or population control may be required in order to balance the consequences of vaccination on population dynamics.

Furthermore, a consequence of the elevated population abundance is that disease prevalence can temporarily increase, beyond the pre-vaccinated level, if vaccination is stopped. The implication is therefore that disease management through vaccination requires a long-term commitment to maintain the reduction in disease prevalence. The oral bait method applied in this study provides an effective method for such long-term vaccine deployment.

CONCLUSIONS

Our efforts to deploy bait in free-ranging wild boar populations provided practical insights into the logistics of oral vaccination in Mediterranean ecosystems. Oral IV can contribute to TB control in its main Iberian reservoir, the wild boar. However, this study showed that IV performance could be context dependent. The study also showed that vaccination can have complex consequences on the population and epidemiological

dynamics and this suggests that long-term disease control strategies need to be integrated with other wildlife management tools.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Figure captions

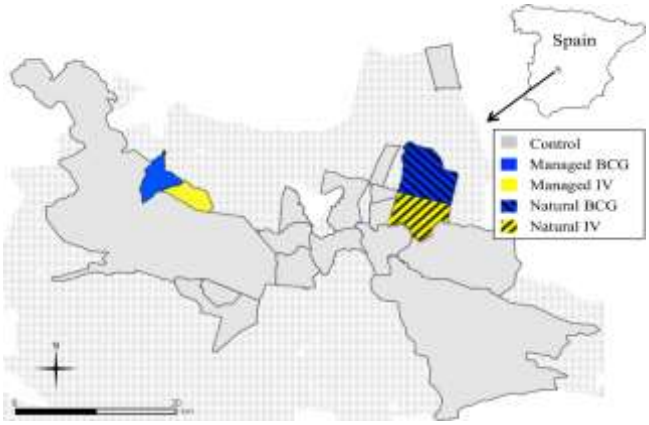

Figure 1. Study area, Montes de Toledo, central Spain.

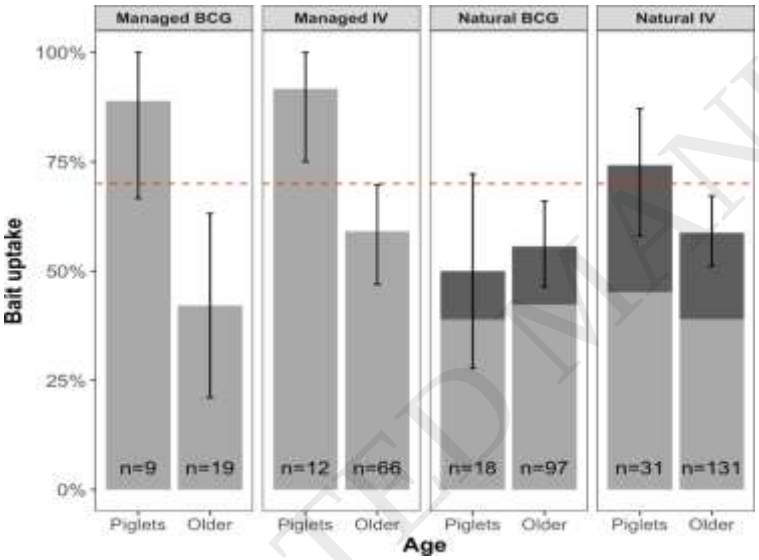

Figure 2. Bait uptake. Proportion of wild boar individuals positive to chemical marker detection by site and age class (piglets = wild boar <12 months; older = wild boar >12 months). Bars are the percentage of individuals positive to detection of chemical marker, light grey bar represents single chemical marker detection and dark grey the presence of both markers. Error bars are bootstrap 95% confidence intervals (CI). Horizontal dashed line stands for the minimum theoretical 70% uptake threshold required to achieve an effective intervention (Anderson et al., 2013).

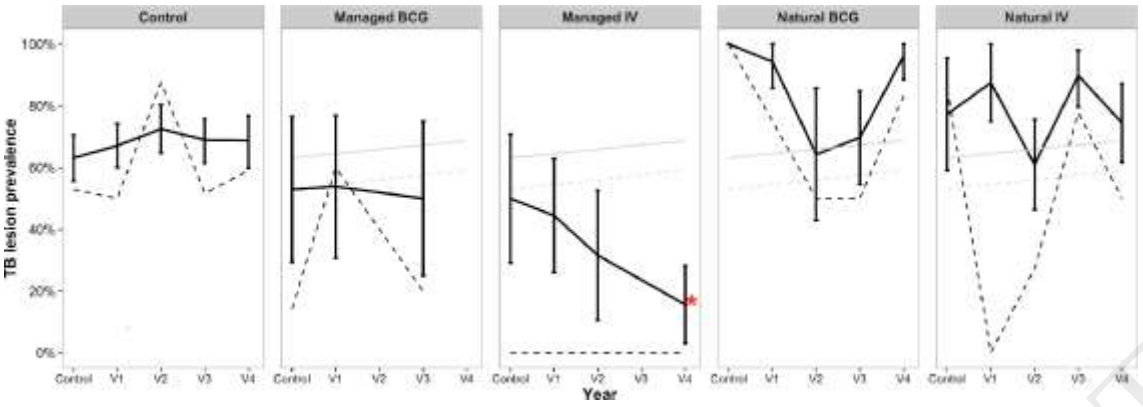

Figure 3. Temporal trend of tuberculosis (TB) lesion prevalence of piglets and total population by site. The dashed line represents piglet age class and the solid line the total population. Background information: the average trend for total population (solid line) and piglets (dashed line) found on the control site appears in light grey in the vaccine site figures. Error bars are bootstrap 95% confidence intervals (CI). Asterisk indicates a significant at $p < 0.01$ decline in prevalence as compared to pre-vaccination levels.

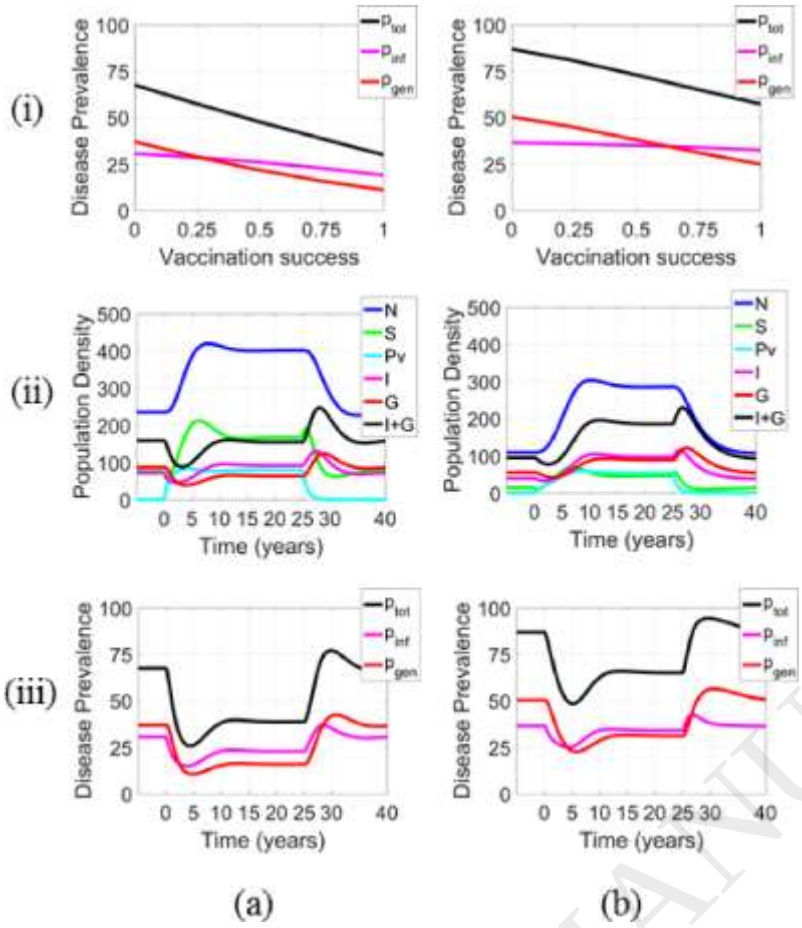

Figure 4. Modelling results for wild boar vaccination against tuberculosis. Column (a) represents a site with medium disease prevalence on which piglets have a low chance of infection prior to vaccination (default disease transmission rate and no pseudo-vertical transmission) and so is similar to a Managed site. Column (b) represents a site with higher initial prevalence and greater rates of transmission combined with a greater proportion of piglets infected prior to vaccine delivery (double transmission rate and 100% pseudo-vertical transmission) and so is similar to a Natural site. Row (i) shows disease prevalence against proportional vaccination success, *vp*, with results determined at the stable endemic steady state when the specified level of vaccination is included; (ii) shows changes in population density against time (years) for a vaccination level of 75% (*vp* = 0.75) during 25 years of continued vaccination and eventual cessation; and (iii) shows changes in disease prevalence against time (years) for a

vaccination level of 75% (*vp* = 0.75) during 25 years of continued vaccination and eventual cessation. N (blue) represents the total population density, I (magenta) represents the total density of infected but not generalized, G (red) the total density of generalized, S (green) the total density of susceptibles and Pv (cyan) the total density of vaccinated piglets. *ptot* (black) is the proportion of the total population infected with TB (*ptot* = (I+G)/N), *pinf* (magenta) is the prevalence of infected but not generalized (*pinf* = I/N); and *pgen* (red) is the prevalence of generalized infection (*pgen* = G/N).

Table 1. Results of the logistic regression model of tuberculosis compatible lesion presence. Estimates (B), estimate associated standard error (SE) and p-value are shown. Reference values for age class and site variables are "<12 month old" and "control" respectively.

Predictor		B	(SE)	
(Intercept)		-0.542	(0.543)	
Age	Yearlings	1.044	(0.233)	***
	Adults	1.424	(0.216)	***
Rainfall		-1.394	(0.537)	**
FBII		-0.396	(0.361)	
Site	Managed BCG[a]	-0.500	(0.419)	
	Managed IV	-1.490	(0.296)	***
	Natural BCG	-0.058	(0.384)	
	Natural IV	0.262	(0.259)	
Initial prevalence		2.043	(0.541)	***
Year		-0.067	(0.079)	

[a] Only results of three vaccination years available
*** p<0.001 ** p<0.01 * p<0.05