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Original Contribution

Vaccination Against Porcine Circovirus-2 Reduces Severity of Tuberculosis in Wild Boar

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Abstract: Tuberculosis (TB) in wild boar (*Sus scrofa*) may be affected by coinfections with other pathogens, such as porcine circovirus type 2 (PCV2). Therefore, sanitary measures focused on controlling PCV2 could be useful in reducing the impact of TB in this wild suid. The aim of this study was to explore whether vaccination against PCV2 targeting young animals affects TB prevalence and TB severity in wild boar. The study was conducted on a game estate in mid-western Spain. Seventy animals of ages ranging from 4 to 8 months were captured, individually identified, vaccinated against PCV2 and released, forming a vaccinated group. Not-captured animals cohabiting with the vaccinated wild boar constituted the control group. Animals from both groups were hunted between 2013 and 2016 and a TB diagnosis based on pathological assessment and microbiological culture was made in all of them. The effect of PCV2 vaccination on TB prevalence and severity was explored using generalized lineal models. Whereas TB prevalence was similar in vaccinated and control groups (54.55 vs. 57.78%), vaccinated animals showed less probabilities to develop generalized TB lesions. Furthermore, mean TB severity score was significantly lower in vaccinated animals (1.55 vs. 2.42) suggesting a positive effect of PCV2 vaccination.

Keywords: PCV2, Tuberculosis, Vaccination, Wild boar

Introduction and Purpose

Bovine tuberculosis (TB) is a chronic bacterial disease mainly caused by *Mycobacterium bovis* that leads to significant economic losses worldwide (Hermoso de Mendoza et al. 2006; Harris et al. 2014). Furthermore, *M. bovis* poses a serious threat to public health as a zoonotic pathogen (Müller et al. 2013) and may have an impact on the preservation of endangered species, such as the Iberian lynx (*Lynx pardina*) (Gortázar et al. 2008).

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In recent decades, bovine TB has been eradicated from many European nations (e.g. France, Germany) (EFSA 2014). However, in other countries, such as Spain, bovine TB prevalence remains stable, despite great efforts made through official eradication campaigns (RASVE 2015). A high prevalence of bovine TB found in mid-western areas of Spain has been related to the presence of wild reservoirs of *M. bovis*, mainly wild boar (*Sus scrofa*) (Gortázar et al. 2005). Thus, measures focused on controlling TB in this wild reservoir will be necessary to eradicate bovine TB in cattle from these areas (García-Bocanegra et al. 2012).

TB prevalence and severity in wild boar have been related to the existence of other concomitant pathogens that frequently infect these animals. Thus, TB prevalence has been positively associated with a high prevalence of porcine circovirus type 2 (PCV2) (Risco et al. 2013a). Furthermore, TB-affected wild boar that are also coinfected with pathogens, such as PCV2 or *Metastrongylus* spp., have shown higher probabilities of suffering from severe TB lesions (Risco et al. 2014). In this scenario, sanitary measures focused on controlling these concomitant pathogens, like vaccination against PCV2 or periodic anthelmintic treatments, could be useful to reduce TB prevalence and TB severity in the wild boar.

The aim of this study was to explore whether a single-dose vaccination against PCV2 targeting young animals affects TB prevalence and TB severity in wild boar.

Methods

Study Area

The study was conducted on a game estate with a well-known history of TB (prevalence of 52% in December of 2012) and located in Oropesa (Toledo, central Spain, 40°00′41.2″N, 5°09′14.3″W). This area has a continental thermal Mediterranean climate, with hot dry summers (26–28°C) and mild and moderately wet winters (7–10°C). The vegetation consists mainly of scrublands of *Cistus* spp. and arbutus (*Arbutus unedo*) and evergreen oak forests (*Quercus suber*). The estate, which covers 2000 Ha, is completely surrounded by a fence to prevent the dispersion of the approximately 350 wild boar that live there, and to avoid the entrance of new animals. Wild boar are continuously fed with a specific fodder (Jabalí Familia, Mercoguadiana S.A., Spain) that is supplied in six feeders located throughout the estate. All of these feeders are surrounded by a fence with gates of different sizes allowing the selection of the size of the animals that will be fed. In addition, these gates are provided with a locking system allowing the entrance of animals but not their exit when it is activated, acting as a selective feeding trap.

Experimental Design

The aim of the experimental design was to generate two groups of animals (a control group and a PCV2 vaccinated group), which were living in the same estate and under the same conditions. To do this, three different capturing events were conducted in the study area in August of 2013, 2014 and 2015. The selective feeding traps were used to carry out the captures allowing the entrance of wild boar ranging approximately from 4 to 8 months of age. Captures were scheduled in August since this is the month when the natural feed offered by the estate is scarcest, ensuring that most of the wild boar population attended the fodders (Ballesteros et al. 2009).

Captured animals were individually identified using a microchip (Pet link, Datamars SA, Switzerland) applied subcutaneously just behind the right ear and were vaccinated against PCV2 with one dose of a commercial vaccine (Suvaxyn PCV2, Zoetis, Spain). The animals were then immediately released and allowed to return to the rest of the population. In addition, from a proportion of captured wild boar (animals showing a null or low stress level during the manipulation), blood samples were taken by puncturing the ophthalmic sinus in order to assess the presence of antibodies against PCV2 at the moment of the vaccination.

Captured animals (electronically identified and vaccinated) formed the vaccinated group in this experiment, whereas animals of similar age, which were not captured but were also living on the estate, formed the control group. Animals belonging to both groups lived together in the same TB epidemiological scenario until they were hunted.

Sampling Procedure

Animals included in this study were sampled during hunting events in December of 2013, 2014 and 2015. To analyse only wild boar that could have been vaccinated (vaccinated and control groups), sampled animals were chosen according to their ages and taking into account the year of the experiment. Thus, animals between 8 months and 1 year of age were sampled in 2013; animals ranging

between 8 months and 2 years in 2014; and finally, animals between 8 months and 3 years in 2015. The age of the hunted wild boar was assessed according to teeth replacement patterns, following previously described protocols (Boitani and Mattei 1992).

Once the animals included in the study were selected, they were examined and sampled, and the presence of a microchip behind the right ear was checked using an appropriate reader. Animals with transponders were included in the vaccinated group, whereas animals without transponders formed the control group. Blood samples were collected from studied animals by puncturing the retro-orbital cavernous sinus (Arenas-Montes et al. 2013).

TB Diagnosis

TB diagnosis was based on the presence of TB-like lesions and the microbiological isolation of *M. bovis*, following previously described procedures (Risco et al. 2016). Briefly, a post-mortem examination to detect visible TB-like lesions was performed targeting the thoracic and abdominal organs, and the main lymph nodes: submandibular, retropharyngeal, mediastinal and mesenteric. The lungs and cervical lymph nodes (submandibular and/or retropharyngeal) were extracted from all the studied animals and stored at 4°C for further procedures in the laboratory.

The presence of *M. bovis* in sampled tissues was evaluated by microbiological isolation following standard culture procedures (Corner and Trajstman 1988), confirmed by PCR and typed by spoligotyping (Cousins et al. 1991; Kamerbeek et al. 1997). Positive wild boar were classified as animals with a 'localized TB pattern' if *M. bovis* was confirmed in only one organ or lymph node, whereas those in which *M. bovis* was confirmed in more than one organ or lymph node were classified as 'generalized TB pattern' cases. Furthermore, a pathological score based on the extent of TB lesions and ranging between 0 (no lesions) and 5 (extensive lesions) was used to assess the severity of TB in the studied wild boar (Menin et al. 2013).

PCV2 ELISA and PCV2 DNA Detection

In order to assess whether PCV2 vaccination produced a serological response in vaccinated animals, antibody titre against PCV2 was estimated in blood samples collected from capture animals (pre-vaccination) and from hunted wild boar (post-vaccination) using a commercial indirect ELISA (INGEZIM CIRCO IgG, INGENASA S.A., Spain) following manufacturer's recommendations.

Furthermore, to evaluate the effect of PCV2 vaccination on PCV2 prevalence and viral load, a PCV2 diagnosis was carried out in some of the studied animals. DNA from their cervical lymph nodes was extracted using a commercial kit (QiAmp DNA Mini Kit®, Qiagen Ltd., UK) following the manufacturer's protocol. A specific real-time PCR assay was then conducted using a previously described pair of primers and a fluorescent probe (Brunborg et al. 2004). Duplicate reactions were run for template samples (500 ng of DNA extracted from cervical lymph nodes), standards and non-template controls. The number of DNA copies present in each sample was estimated based on the standard curve calculated using the thermocycler-specific software (Applied Biosystem 7300, Thermo Fisher Scientific Inc., USA). Results were expressed as numbers of PCV2 genome copies per 500 ng of DNA. Finally, to discount the presence of PCR inhibitors in nasal samples included in this study, a TaqMan® base real-time PCR to detect the β-actin gene was carried out using previously described primers, probes and conditions (Toussaint et al. 2007).

Due to immunological protection acquired through the PCV2 commercial vaccine being valid for 5 months, and the age of vaccinated animals ranging between 4 and 8 months at the moment of vaccination, PCV2 ELISA and PCV2 DNA detection used to confirm the efficacy of vaccination were only conducted on animals younger than 13 months at the time of sampling.

Statistical Analysis

Effect of PCV2 Vaccination on M. bovis Infection and TB Severity

To explore the effect of PCV2 vaccination on TB development in wild boar, we fitted two sets of independent generalized linear models (GLM) with binomial errors and logit link function, in which *M. bovis* infection (infected, non-infected) and TB severity (localized or generalized pattern) were explained by the single and the additive effects of vaccination against PCV2, age (in years) and their two way interaction. The effect of age was included as an explanatory covariate in these models because 1) it has been reported to have an effect on both TB prevalence and TB severity in wild boar (Vicente et al. 2006; Risco et al. 2013b); and 2) PCV2 vaccination produces a temporary immunological protection (5 months) and hence, the effect

of this treatment on TB development could be mild on older animals. Another set of GLMs with similar explanatory variables but using a Gaussian error distribution, were fitted to explain TB pathological score (1–5).

Among all the proposed models, the most parsimonious one was selected to explain each response variable following an information-theoretic approach based on the Akaike Information Criterion corrected for small sample sizes (AICc) (Burnham and Anderson 2003). The Akaike weight (*wi*), that is, the relative likelihood of the model given the data available, was also estimated. This statistical procedure was performed using the package 'stats', version 3.0.2 of the statistical software R (Team 2017).

Effect of PCV2 Vaccination on Viral Load and Antibody Titres

T test for paired samples was conducted to compare pre-vaccination and post-vaccination mean PCV2 antibody titre in the vaccinated group. Furthermore, ordinary *T* test was carried out to compare mean PCV2 antibody titre between vaccinated and control animals at the time of sampling.

The frequency of animals infected by PCV2 and the viral load were compared between vaccinated and non-vaccinated animals using Chi-square test and the Man Whitney *U* test, respectively.

Results

Captures and Hunting Events

A total of 70 animals between four and eight months of age were captured, identified, vaccinated and released, in the three captures carried out during this experiment (August of 2013, 2014 and 2015, see Table 1). Thirty-three of these captured animals were hunted-harvested during December of 2014 and 2015, forming the vaccinated group. Furthermore, another 36 animals of similar ages but without transponders were also hunted and included in the control group (see Table 1). No differences in mean age (1.41 years in both groups) or sex ratio were observed between control and vaccinated groups.

TB Diagnosis and Relationship with PCV2 Vaccination

M. bovis was isolated in 37 out of the 69 animals studied (53.62%). Among TB positive animals, 21 (56.75%) showed localized infection, whereas 16 showed generalized patterns (43.25%). The number of *M. bovis* infected animals, the percentage of TB generalized patterns and the mean TB pathological score found in each group (vaccinated and control) are summarized in Table 2.

According to our model selection procedure (see Table 3), the most parsimonious model to explain *M. bovis*

Table 1. Number of Animals Captured, Identified and Vaccinated During the Three Captures Carried Out in August of 2013, 2014 and 2015; and Number of Animals Hunted in Hunting Events Celebrated in December of 2013, 2014 and 2015 Which Were Included in this Study.

	2013	2014	2015
Captured animals (August)	33 (10 ♂/23 ♀)	18 (10 ♂/8 ♀)	19 (10 ♂/9 ♀)
Hunted animals (December)	Age < 1 year	Age < 2 years	Age < 3 years
Vaccinated	–	15 (7 ♂/8 ♀)	18 (7 ♂/11 ♀)
Control	–	10 (4 ♂/6 ♀)	26 (7 ♂/19 ♀)

Table 2. Results of TB Diagnosis, TB Severity Assessment and PCV2 Detection in the Animals Studied.

	M. bovis infection		TB lesion pattern		Score	PCV detection		Mean PCV copies
	Negative	Positive	Localized	Generalized		Positive	Negative	
Control group	17 (47.2%)	19 (52.7%)	7 (36.8%)	12 (63.2%)	2.42	12 (66.6%)	6 (33.4%)	1573.51 (SD = 6499.72)
Vaccinated group	15 (45.5%)	18 (54.5%)	14 (77.7%)	4 (22.3%)	1.55	8 (53.3%)	7 (46.7%)	85.71 (SD = 215.92)

Table 3. Model Selection to Explore the Influence of PCV2 Vaccination on the Probabilities of *M. bovis* Infection and TB Severity of Studied Wild Boar.

Biological models	*K*	AICc	Δ*i*	*wi*
Response variable: M. bovis infection				
Age	3	94.64	0.00	0.56
PCV vaccination + age	4	96.87	2.23	0.18
Mo	2	97.47	2.83	0.14
PCV vaccination × age	5	97.71	3.07	0.12
PCV vaccination	3	99.64	5.00	0.04611
Response variable: TB pattern				
PCV vaccination	3	48.80	0.00	0.56
PCV vaccination + age	4	51.17	2.37	0.17
PCV vaccination × age	5	51.18	2.38	0.17
Mo	2	52.97	4.16	0.06999
Age	3	54.97	6.16	0.02578
Response variable: TB pathological score				
PCV vaccination	3	126.45	0.00	0.56
PCV vaccination + age	4	127.75	1.30	0.29
Mo	2	128.45	2.01	0.21
PCV vaccination × age	5	129.73	3.28	0.10896
Age	3	130.16	3.71	0.08768

Generalized linear models with binomial (*M. bovis* infection and TB pattern) and Gaussian (TB pathological score) error structure have been used.
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.

infection in studied wild boar did not include PCV2 vaccination as explanatory variable. Model including age as the only explanatory variable was the most suitable option to explain TB infections ($w_{\text{Age}} = 0.56$, Deviance explained = 5.26%), showing a positive association between age and the probabilities of TB infection.

Conversely, models including PCV2 vaccination as the unique explanatory variable were the most parsimonious options to explain TB severity [$w_{\text{PCV2 Vaccination}} = 0.56$, Deviance explained = 12.91%, odds ratio = 6 (1.49–28.46)] and TB pathological score ($w_{\text{PCV2 Vaccination}} = 0.56$, Deviance explained = 11.17%, $\beta = 0.44$), showing that the presence of generalized TB pattern and TB pathological score were lesser in vaccinated animals (Fig. 1).

PCV2 ELISAs and PCV2 DNA Detection

PCV2 antibody titres found in vaccinated animals were higher at post-vaccination time-point (mean = 1527) than at pre-vaccination time-point (mean = 142.80, $t = 6.8654$, $P = 1.73e^{-5}$). Furthermore, vaccinated animals showed higher mean PCV2 antibody titre (1195.50) than the control group (mean = 664.78, $t = -2.62$, $P = 0.01$) at the time of sampling.

PCV2 DNA was detected in 58.49% ($n = 20$) of the animals checked ($n = 33$) with a mean viral load of 1428 copies of PCV2 genome/500 ng DNA. Among the vaccinated group, the percentage of positive animals was 53.3% (8/15) with a mean load of 31 copies of PCV2 genome/500 ng DNA, whereas in the control group, the percentage of positive animals was 66.6% (12/18) with a mean viral load of 2514 copies of PCV2 genome/500 ng (see Table 2). No statistically significant differences were found in the frequency of animals infected by PCV2 ($\chi^2 = 0.178$, *P* value = 0.67), nor in the PCV2 load ($W = 151$, *P* value = 0.33) between vaccinated and control animals.

Discussion

The results generated by this experiment showed an association between single-dose PCV2 vaccination and TB severity in wild boar. Animals vaccinated against PCV2 displayed less severe TB lesions than control animals, with a

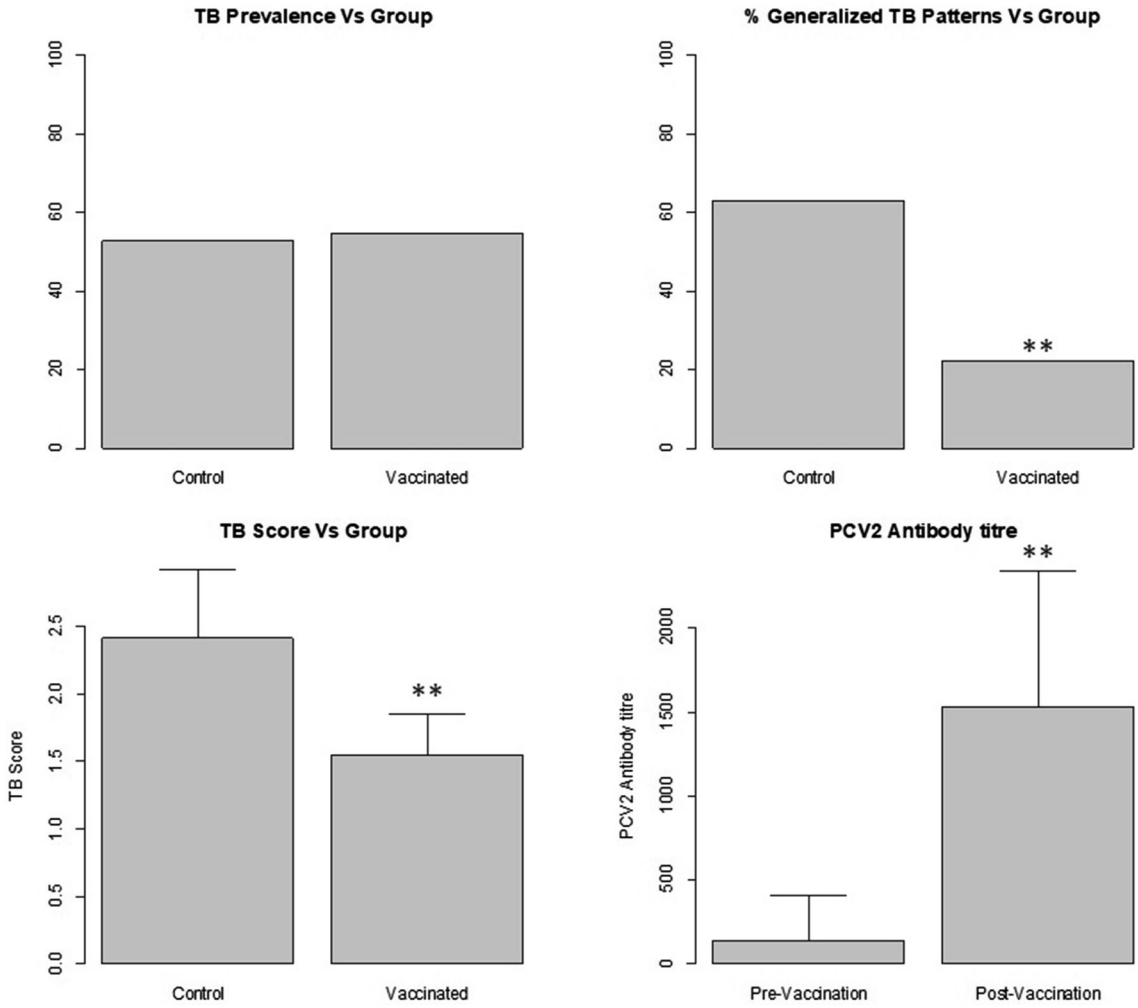

Fig. 1. TB prevalence, percentage of generalized TB patterns and TB pathological score, found in wild boar vaccinated against PCV2 and control group; and PCV2 antibody titres found in vaccinated animals at pre-vaccination and post-vaccination time-points.

60% reduction in the number of animals suffering from TB generalized patterns (63.2% in control group vs. 22.3% in vaccinated group). In addition, mean TB pathological score observed in vaccinated animals was 36% lower than mean score found in control group (1.55 vs. 2.42). These findings suggest that PCV2 vaccination targeting young animals may help reduce TB severity in wild boar populations.

PCV2 infections in TB-affected wild boar may trigger TB development, leading to more severe generalized TB patterns (Risco et al. 2013a; Risco et al. 2014). Temporary immunological protection (5 months) conferred by a single-dose PCV2 vaccination in young wild boar seems to limit the negative effects of this virus on TB development under field conditions. Although some of the wild boar (older than 13 months) were sampled when the immunological protection conferred by vaccination had finished, the association between PCV2 vaccination and TB severity did not depend on the age of the animals. In fact, models trying to explain TB severity (TB patterns and TB pathological score) in which age was included as explanatory variable were discarded following AICc criteria.

The significant increment of PCV2 antibody titres between pre-vaccination and post-vaccination time-points confirms a serological response produced by PCV2 vaccination. Furthermore, despite that the prevalence of PCV2 infections was similar in control and vaccinated animals, the latest showed higher antibody titres which may be also attributed to the effect of vaccination. Although not significant (perhaps due to the low number of animals and a large standard deviation, see Table 2), large differences

were also observed in mean viral load found in lymph nodes from the control (2514 copies/500 ng) and vaccinated groups (31 copies/500 ng), suggesting a positive effect of vaccination. In fact, animals with viral loads close to those considered to be pathological in PCV2 infections (10^7 copies/500 ng of DNA) (Brunborg et al. 2004) were only observed in animals from control group. These results are in agreement with the aim of the vaccine used in this study (Suvaxyn PCV, Zoetis, Spain) that is to reduce viral load and limit clinical signs, but not protect against new PCV2 infections (Zoetis 2009).

In contrast to TB severity, TB prevalence was not associated with PCV2 vaccination. A similar percentage of *M. bovis* infected animals was found in both control (54.5%) and vaccinated groups (52.7%). These results are in agreement with those obtained in a previous experiment which tried to modulate TB in buffalo using anthelmintic treatments (Ezenwa and Jolles 2015). In that case, treatment against parasites did not reduce TB prevalence in treated animals, but raised the likelihood of survival of TB-affected animals, suggesting a decrease in the severity of the disease.

Improvement of survival rates in TB-affected buffalos by anthelmintic treatment was related to a negative effect at the population level, increasing the likelihood of the spread microbial pathogens, including *M. bovis* (Ezenwa and Jolles 2015). However, in the case of the wild boar, the situation may be the opposite. PCV2 vaccination does not directly reduce the probabilities of *M. bovis* infection in wild boar but could reduce TB severity, which has been positively associated with TB prevalence (Risco et al. 2013a) and *M. bovis* shedding in the wild boar (Santos et al. 2015). Therefore, it would be expected that the reduction of generalized TB patterns through PCV2 vaccination could lead to a decrease in TB prevalence. More years of vaccination and greater vaccination pressure will likely be necessary to note a significant reduction in TB prevalence.

In conclusion, the results obtained in this work show that PCV2 vaccination using feeding traps could be a useful tool to reduce TB severity on wild boar game estates. Further research will be necessary to assess whether long-term PCV2 vaccination schedules are able to reduce TB prevalence and to evaluate the combination of this measure with other sanitary actions applicable to this types of estates, such as vitamin D supplementation (Risco et al. 2016) or TB vaccination (Díez-Delgado et al. 2016).

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