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# Oral administration of heat-inactivated *Mycobacterium bovis* reduces the response of farmed red deer to avian and bovine tuberculin

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ABSTRACT

Orally delivered mycobacterial antigens may not sensitize the immunized animals causing a positive tuberculin skin test response. As the first step to address this critical issue, we characterized the response of farmed red deer (*Cervus elaphus*) to orally delivered heat-inactivated *Mycobacterium bovis*. Thirty-two adult red deer hinds from a farm known to be free of tuberculosis (TB) were randomly assigned to two different treatment groups, immunized ($n = 24$) and control ($n = 8$). Immunized hinds were dosed orally with 2 ml of PBS containing $6 \times 10^6$ heat-inactivated *M. bovis*. The mean skin test response of immunized deer to both avian purified protein derivative (aPPD) and bovine PPD (bPPD) was consistently lower in immunized than in control hinds. One year after immunization, immunized hinds had a significant reduction in the skin test response to aPPD and in the ELISA antibody levels against both aPPD and bPPD (24–36% reduction; $P < 0.05$). By contrast, no significant change was observed in the skin test response to phytohaemagglutinin, or in the ELISA antibody levels against the *M. bovis* specific antigen MPB70. The mRNA levels for C3, IFN-γ and IL-1β and serum protein levels for IFN-γ and IL-1β did not vary between immunized and control deer. However, serum C3 protein levels were significantly higher ($P = 0.001$) in immunized than in control deer six months after immunization. These results confirm that oral heat-inactivated *M. bovis* does not sensitize farmed red deer and therefore does not cause false-positive responses in the tuberculin skin test. The absence of sensitization in orally immunized deer opens the possibility of testing the vaccine in deer and possibly other ruminants without the risk of causing false-positive reactions in TB-tests. This study also provided the first evidence that orally-delivered inactivated mycobacterial antigens elicit some kind of immune response in a ruminant.



## 1. Introduction

Red deer (*Cervus elaphus*) farming is a significant economic activity in many countries worldwide. Mycobacterial infections such as those caused by *Mycobacterium bovis* and closely related members of the *Mycobacterium tuberculosis* complex (animal tuberculosis, TB) and those caused by members of the *Mycobacterium avium* complex (such as paratuberculosis, PTB, due to *M. avium paratuberculosis*) are among the main constraints of the deer farming industry because of the direct losses and the movement restrictions imposed on infected herds (Mackintosh et al., 2004; Thoen et al., 2014).

In order to control TB and PTB, farmed deer are skin tested. As in cattle, a skin test consists in injecting intradermally purified protein derivatives from *M. bovis* (bPPD) and eventually from *M. avium* (aPPD; comparative test), measuring the increase of the skinfold thickness 72 h after injection. The test works because during early and intermediate phases of the infection, mycobacteria trigger a Th1 lymphocyte mediated immunity. Memory T cells recruit other cells producing local inflammation and secreting cytokines for

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activation of macrophages to eliminate the antigen (Thom et al., 2004). However, several factors can modulate the individual skin test responsiveness. In cattle, site of injection, breed, age and PPD quality may cause variability in skin test responsiveness (Casal et al., 2015). In deer, farm or wild origin, sex, age, body condition and season can cause variability in the skin test response (Fernández-de-Mera et al., 2011, 2009; Queiros et al., 2012). Cross-reactions with environmental mycobacteria can also trigger false-positive skin test responses (Mackintosh et al., 2004; Queiros et al., 2012).

Moreover, observational and experimental evidence showed that co-infection with the liver fluke *Fasciola hepatica* reduces the skin test responsiveness of *M. bovis* infected cattle (Claridge et al., 2012). This might be due to a helminthes infection-mediated suppression of T-cell and IFN-γ responses to PPD, or to a protective effect of co-infections (Flynn et al., 2009). Whatever the mechanism, this finding is relevant because it could cause interference with the TB eradication campaigns (Claridge et al., 2012).

In suids, oral vaccination with heat-inactivated *M. bovis* has shown significant protection against challenge with virulent *M. bovis* (Beltrán-Beck et al., 2014; Garrido et al., 2011). Complement component 3 (C3) and other molecules are involved in the resistance to mycobaterial infection (Naranjo et al., 2006a,b). Moreover, in heat-inactivated *M. bovis* vaccine C3 and cytokines like interleukin 1 beta (IL-1β) are associated with a protective response by a toll-like receptor (Beltrán-Beck et al., 2014). Furthermore, experiments are planned to test the efficacy of the heat-inactivated *M. bovis* vaccine in ruminants such as cattle, goats and deer. However, in ruminants it is of paramount importance to assess, prior to the expensive vaccination and challenge experiments, if orally delivered mycobacterial antigens sensitize the immunized animals causing a positive skin test response (Palmer et al., 2014). This is an issue in deer farms because skin testing is statutory in some countries. In Spain, negative skin tests are required prior to allowing transports of deer (Royal Decree 1082/2009). Therefore, vaccines must not induce reaction. The goal of this study was assessing the response of farmed red deer to orally delivered heat-inactivated *M. bovis*.

## 2. Material and methods

### 2.1. Ethics statement

Handling procedures and sampling frequency were designed to reduce stress and health risks for subjects, according to European (86/609) and Spanish laws (R.D. 223/1988, R.D. 1021/2005). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Regional Agriculture Authority (Diputación Foral de Vizcaya, Permit Number: NEIKER-OEBA-2015-010).

### 2.2. Animals and experimental design

Thirty-two adult red deer hinds were selected from a farm known to be free of TB but not for *M. avium* complex (Queiros et al., 2012). The animals were kept in a fenced area inside the farm. Individuals were randomly assigned to two different treatment groups, immunized ($n = 24$) and control ($n = 8$). Treatment hinds were dosed orally with 2 ml of PBS containing 6 × 106 heat-inactivated *M. bovis* field isolate Neiker 1403 (spoligotype SB0339) obtained from a naturally infected wild boar, *Sus scrofa* (Garrido et al., 2011). Control hinds were dosed orally with 2 ml of PBS.

### 2.3. Sample collection and skin test

Blood samples were collected from the jugular vein into sterile tubes with EDTA, lithium heparin, and without anticoagulant at time points 0 (January 2014), 7 (August 2014) and 12 months after vaccination (January 2015). Serum and peripheral blood mononuclear cells (PBMC) were separated by centrifugation and stored at −20 and −80 °C respectively until used for serology and RNA studies. Standardized comparative cervical skin tests were performed (Fernández-de-Mera et al., 2009). Animals were handled twice during the test, at time 0 and 72 h. Deer were captured and then immobilized by physical restraint in a hydraulic crush. Three areas of 3 cm × 3 cm were shaved in the animal's neck, and three times repeated measurements of skin fold thickness were taken using a manual caliper. Then, 0.1 ml containing 2.500 IU of each antigen, avian PPD (CZ Veterinaria, Porriño, Spain), and bovine PPD (CZ Veterinaria), were injected intradermally. The positive control phytohaemoagglutinin (PHA; 0.1 ml containing 250 mg PHA diluted in PBS; Sigma, Madrid, Spain) was injected intradermally to confirm skin test responsiveness. At 72 h each animal was immobilized again by physical restraint, identified, and the skin fold thickness was measured again at each injection site three times. Immunized and control animals were compared for each antigen by Generalized Mixed Models, where skin fold thickness was the response variable. As explanatory variables we included treatment (immunized vs controls) and time of sampling (categorical). The individual was included as a random factor ($p = 0.05$). We used a normal error and an identity link. The statistical analyses were carried using SPSS software version 19.0.

### 2.4. Serological analyses

#### 2.4.1. Antibody response

Serum samples were tested for antibodies against bPPD, aPPD, paratuberculosis protoplasmatic antigen (PPA) and *M. bovis* antigen MPB70 using an in-house ELISA as previously described (Boadella et al., 2011). Results were expressed as ELISA percentage (*E*%) that was calculated using the formula *E*% = (mean sample OD/2 × mean negative control OD) × 100 (Boadella et al., 2011).

#### 2.4.2. Serum protein levels

For the quantitative analysis of Complement component 3 (C3), Interferon gamma (IFN-γ) and Interleukin-1 beta (IL-1β) in a subsample of 10 immunized and 7 control sera, commercially available sandwich ELISA tests were used (Bovine ELISA Assay Kit for C3, Cloud-Clone Corp., Houston, TX, USA; Bovine IFN-gamma ELISA Kit, RayBiotech Inc., Norcross, GA, USA and Bovine Interleukin-1 beta IL-1 β, Novatein Biosciences, Woburn, MA, USA). Samples and standards were analyzed following the manufacturer's protocols. Data was linearized by a standard curve and regression analysis was used to determine sample protein concentrations in ng/ml. Immunized and control hinds were compared for each antigen and protein by Generalized Mixed Models, where the measurement of *E*% or ng/ml was the response variable, respectively. As explanatory variables we included treatment (immunized vs controls) and time of sampling (categorical). The individual was included as a random factor ($p = 0.05$). We used a normal error and a identity link. The statistical analysis was carried using SPSS software version 19.0.

### 2.5. Gene expression analysis by real time RT-PCR

Total RNA from PBMC was extracted from five immunized and three control animals (the only ones with available samples for all three time points), using a QIAamp RNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) according to manufacturer's instructions. Gene expression profiles from selected genes involved in the innate immunity (*C3*, *IFN-γ* and *IL-1β*) were analyzed in response to heat-inactivated *M. bovis*. Real Time RT-PCR was performed with gene-specific primers and conditions (Table 1) using a Quantitect SYBR Green RT-PCR Kit and a Rotor Gene Q thermocycler (Qiagen, Inc. Valencia, CA, USA) following manufacturer's recommendations. A