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1 Short communication

3 Experimental infection of Eurasian wild boar with *Mycobacterium*
4 *avium* subsp. *avium*5 J.M. Garrido ^a, J. Vicente ^b, R. Carrasco-García ^b, R.C. Galindo ^b, E. Minguijón ^a, C. Ballesteros ^b,
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ABSTRACT

The Eurasian wild boar (*Sus scrofa*) is increasingly relevant as a host for several pathogenic mycobacteria. We aimed to characterize the first experimental *Mycobacterium avium* subsp. *avium* (MAA) infection in wild boar in order to describe the lesions and the immune response as compared to uninfected controls. Twelve 1–4-month-old wild boar piglets were housed in class III bio-containment facilities. Four concentrations of MAA suspension were used: 10, 10² and 10⁴ mycobacteria (2 animals each, oropharyngeal route) and 2.5 × 10⁶ mycobacteria (2 animals each by the oropharyngeal and nasal routes). No clinical signs were observed and pathology evidenced a low pathogenicity of this MAA strain for this particular host. Bacteriological and pathological evidence of successful infection after experimental inoculation was found for the group challenged with 2.5 × 10⁶ mycobacteria. These four wild boar showed a positive IFN-γ response to the avian PPD and the real-time RT-PCR data revealed that three genes, complement component C3, IFN-γ and RANTES, were significantly down regulated in infected animals. These results were similar to those found in naturally and experimentally *M. bovis*-infected wild boar and may constitute biomarkers of mycobacterial infection in this species.

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1. Introduction

14 Mycobacterial diseases are re-emerging worldwide.
15 Among wildlife, the Eurasian wild boar (*Sus scrofa*) is
16 increasingly relevant as a host for several pathogenic
17 mycobacteria (Trcka et al., 2006; Gortazar et al., 2008;
18 Naranjo et al., 2008). Members of the *Mycobacterium avium*
19 complex (MAC) cause swine mycobacteriosis and oppor-
20 tunistic human infections (Thorel et al., 2001; Tirkkonen

et al., 2007). Within them, *Mycobacterium avium avium*
(MAA), a common pathogen of birds, occasionally affects
mammals (Mijs et al., 2002). MAA has been isolated in a 4%
of tuberculous lesions from wild boar in Spain (Parra et al.,
2006) and in a recent study this agent has been found
involved in 11% of tuberculous lesions of swine samples
from Portuguese abattoirs (Domingos et al., 2009).

Several experimental mycobacterial infections of pigs
with MAC subspecies are found in the literature (e.g.
Jorgensen, 1978; Ellsworth et al., 1980; Oliveira et al.,
1996; Ray et al., 1972). The aim of this study was to
describe the lesions and to monitor and characterize the
immune response of experimentally MAA-infected wild
boar in comparison to uninfected controls. To the best of

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our knowledge, this is the first report on wild boar experimental infection (EI) with *M. avium avium*.

2. Materials and methods

2.1. Animals

Eight 1-3-month-old wild boar piglets for the experiment 1 were captured in two different regions in Northern Spain where no tuberculosis compatible lesions have been reported in wildlife. Also, four 3-4-month-old wild boar piglets for the experiment 2 were bought in a commercial farm known to be free of mycobacterial lesions at slaughter and with a fully negative ELISA test (Modified from Aurtinetxe et al., 2008) in nearly 100% of the reproductive stock. The animals of both experiments were housed in class III bio-containment facilities where they had *ad libitum* food and water. All experiments were carried out following European, National and Regional Law and Ethics Committee regulations.

2.2. Experimental design

Culture and identification of the MAA isolate (Mijs et al., 2002) and preparation of the inocula was performed following standard bacteriological methods (see online supplementary file). We performed two experiments. In the first experiment, eight 1-3-month-old wild boar were anaesthetized with 50 mg of a 1:1 solution of zolacepan and tiletilamine (Zoletil 100, Virbac, 06511 Carros Cedex, France) and 5 ml of mycobacterial suspension were administered by the oropharyngeal route by emptying a needleless syringe in the back of the tongue. Three concentrations (10 , 10^2 and 10^4 mycobacteria) of MAA suspension were used. Two wild boar were used for each dose and two additional wild boar were maintained as uninfected controls. Due to absence of lesions in these animals an additional challenge with higher dose of mycobacteria and a different route of infection was attempted. In the second experiment, 10 ml of a suspension containing 2.5×10^6 mycobacteria were administered to two 4-month-old wild boar each by the oropharyngeal and the nasal routes, respectively, using the same animal handling protocol. For the nasal inoculation, the tips of the syringes were fitted with disposable punctured rubber caps that formed a spray that was delivered within the first 3 cm of the nasal cavity. Blood samples were taken at 0, 4.5, 11 and 15 weeks post-infection (wpi).

2.3. Necropsy, sample collection, histopathology and microbiology

Wild boar were anesthetized by intramuscular injection of zoletil as described in this section, and euthanized by captive bolt 15 wpi. A thorough postmortem examination was done to detect the presence of macroscopic lesions. Samples for culture were immediately processed and copies frozen at -80°C for mRNA isolation. After taking out pieces for mRNA, all main lymph nodes and the tonsils were serially sliced into 1-2 mm thick slices and carefully inspected for visible TB-compatible lesions and

sampled for histopathology and culture. Individual tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned at $4\ \mu\text{m}$, and stained with hematoxylin-eosin (HE) by use of standard procedures. An additional section of those tissues with lesions indicative of tuberculosis were stained by Ziehl-Neelsen (ZN) procedure to detect the presence of acid-fast organisms. Microscopic lesions were classified as previously described for naturally *M. bovis*-infected wild boar (Martin-Hernando et al., 2007). Tissue samples with gross lesions and pooled samples were cultured as described in the online supplementary file.

2.4. Gamma interferon (IFN- γ) test

Blood samples were collected into tubes with lithium heparin before challenge and at times 4.5, 11, and 15 wpi and shipped to the laboratory at room temperature. Stimulation of whole blood with PBS (nil control), and the avian and bovine purified protein derivative (PPD; CZ Veterinaria, Porriño, Spain) was performed within 8 h of collection as described for other species (Rothel et al., 1990; Liebana et al., 1998; Gormley et al., 2006). Detection of IFN- γ in supernatant was performed using a quantitative ELISA (Pierce Endogen, Rockford, IL, USA) following manufacturer's recommendations.

2.5. Selection of immunoregulatory genes and real-time RT-PCR analysis

Immunoregulatory genes were selected based on their putative role during mycobacterial infection and their association with *M. bovis* infection in European wild boar (Naranjo et al., 2006a,b; Perez de la Lastra et al., 2008). The genes analyzed herein included interferon gamma (IFN- γ), Regulated on Activation, Normal T Expressed and Secreted cytokine (RANTES, also known as CCL5), complement component 3 (C3), and interleukin 4 (IL-4).

Total RNA was extracted from peripheral white blood cell samples of animals in experiment 2 inoculated with 2.5×10^6 mycobacteria using TriReagent (Sigma, Madrid, Spain) following manufacturer's recommendations. The RNA yield and quality were assessed using the Experion Bioanalyzer (Bio-Rad, Madrid, Spain). Gene-specific oligonucleotide primers were designed and used for real-time RT-PCR as described (de la Lastra et al., 2009; Table S-1). The real-time RT-PCR was performed in 25 μl reaction volumes with 12.5 μl SYBR Green iScript[®] (Bio-Rad). Amplification conditions consisted of 95°C for 1 min, followed by 40 cycles of 95°C for 15 s and 55°C for 60 s. A dissociation curve was run at the end of the reaction to ensure that only one amplicon was formed and that the amplicon denatured consistently in the same temperature range for every sample (Ririe et al., 1997). All reactions were performed in duplicate. Oligonucleotide primers were used to amplify the cyclophilin gene transcript as a control gene to normalize expression data (Pérez de la Lastra et al., 2009). The mRNA values were normalized against *S. scrofa* cyclophilin (Genbank accession number AY008846) gene expression using the $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen, 2001). The normalized relative expression of each mRNA transcript was calculated as the

Table 1
MAA doses, routes, gross and microscopic lesions of tuberculosis and culture results for 12 wild boar used in the infection experiments.

Exp. #	Anim. #	MAA cfu and route	Gross lesions	Microscopic lesions	Acid-fast bacilli	Culture
1	1	10 oropharyngeal	—	—	ND	—
1	2	10 oropharyngeal	—	—	ND	+
1	3	10 ² oropharyngeal	—	—	ND	—
1	4	10 ² oropharyngeal	—	—	ND	—
1	5	10 ⁴ oropharyngeal	—	—	ND	—
1	6	10 ⁴ oropharyngeal	—	—	ND	—
1	7	Control	—	—	ND	—
1	8	Control	—	—	ND	—
2	9	2.5 × 10 ⁶ oropharyngeal	L mLN	L mLN (type 2)	—	+
2	10	2.5 × 10 ⁶ oropharyngeal	L mLN	L mLN (type 2 and 3)	—	—
2	11	2.5 × 10 ⁶ nasal	L tbLN	L tbLN (type 2)	+	—
2	12	2.5 × 10 ⁶ nasal	—	—	ND	—

Exp.: experiment; Anim.: animal; cfu: colony forming units; L: left; R: right; tb: tracheobronchial; m: mandibular; LN: lymph node. Tuberculous microscopic lesions classified as 1–3 according to Martin-Hernando et al. (2007). ND: not done.

ratio of the value from *M. avium*-infected animals to the average value from controls. Normalized Ct values were compared between infected and control samples by Student's *t*-test ($P = 0.05$).

3. Results

3.1. Clinical signs, pathology and microbiology

Clinical signs were observed neither in experiment 1 nor in experiment 2. None of the wild boar of experiment 1 showed either visible or microscopic TB-compatible lesions (Table 1). Experiment 2, using a higher dose, yielded gross and histopathological lesions of tuberculosis at postmortem examination. All visible lesions were less than 10 mm in diameter (type A following Martin-Hernando et al., 2007).

Histopathology revealed typical TB granulomas in three of the four wild boar in experiment 2. Granulomas consisted of a caseous necrotic centre, variably mineralized, surrounded by histiocytes, lymphocytes, neutrophils, eosinophils and occasional multinucleated giant cells surrounded by fibrous tissue. Scant acid-fast bacilli were detected by ZN staining of affected tissues in the necrotic centre of a type 2 granuloma in the left tracheobronchial lymphnode of one animal. According to the classification of bTB granulomas in wild boar by Martin-Hernando et al. (2007), the tuberculous granulomas observed in the lymph nodes ranged from type 2 to type 3 (Table 1).

Microbiological data are shown in Table 1.

3.2. IFN- γ response

The four wild boar challenged with 2.5×10^6 mycobacteria were positive at the IFN- γ test when their blood samples were stimulated with avian PPD (Fig. 1). This response was always at least twice the response to the bovine PPD control. In the animals with nasal EI, the mean OD of the sample stimulated with avian PPD was higher than its PBS control in the three samplings post-infection (with minimum difference values of 0.141 and 0.391) and was maintained throughout the experiment. However, response in animals with oropharyngeal EI was slower. It

was not observed at the first control but was strong at the second one and decreased thereafter.

3.3. Gene expression analyses

The mRNA levels of C3, IFN- γ , IL-4 and RANTES were analyzed in wild boar of the experiment 2. The RT-PCR data revealed that three genes, C3, IFN- γ and RANTES were significantly down regulated in infected animals ($P < 0.05$; Fig. 2). Differences were not observed between animals inoculated using oropharyngeal or nasal routes.

4. Discussion

This study provides valuable information on doses and routes, and raises new questions concerning natural MAA infection in wild boar.

Absence of clinical signs in the experimentally MAA-infected wild boar was no surprise and had been previously reported in *M. avium*-infected pigs (Ellsworth et al., 1979; Jensen 1977, 1978a,b). In pigs, MAA produces an almost asymptomatic chronic infection (Thorel et al., 2001). In fact, while MAA has repeatedly been reported in wild boar, reports on clinical infection or even mortality are lacking (reviewed by Machackova et al., 2003).

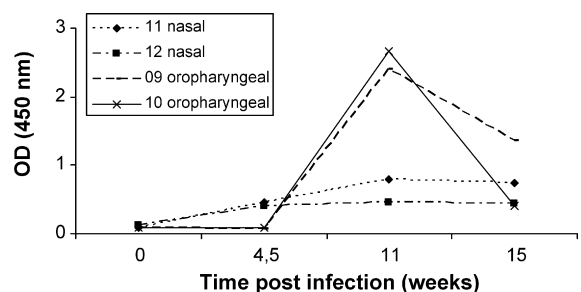


Fig. 1. Kinetics of the IFN- γ results of four wild boar infected with MAA by the oropharyngeal (#9 and #10) and nasal route (#11 and #12; experiment 2). Blood samples were stimulated with avian PPD in vitro overnight; data show OD of sample stimulated with avian PPD minus the nil sample (PBS).

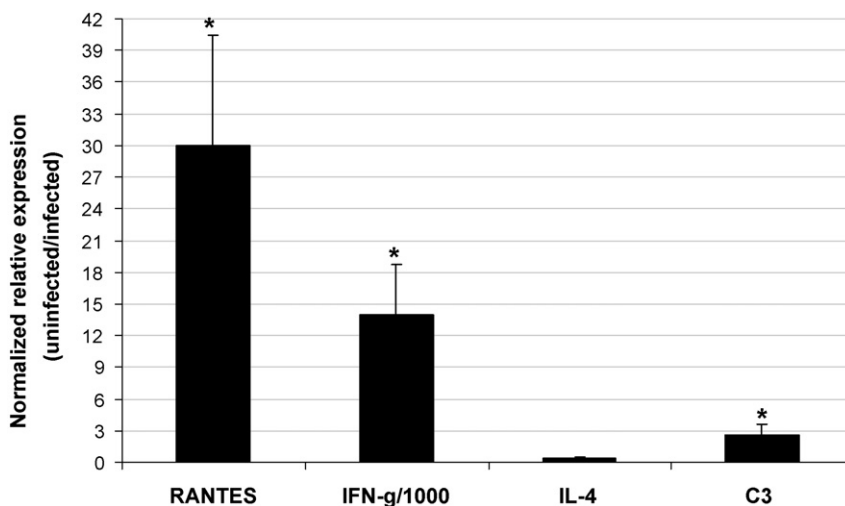


Fig. 2. Quantitative gene expression analysis in peripheral white blood cell samples of *M. avium*-infected and uninfected control European wild boar using real-time RT-PCR analysis. The mRNA levels of C3, IFN- γ , IL-4 and RANTES were analyzed in wild boar inoculated with 2.5×10^6 cfu (experiment 2; $N = 4$). Three experimental replicates were done for each analysis with similar results. The mRNA levels were normalized against *S. scrofa* cyclophilin. Bars show uninfected to infected average \pm S.E. mRNA ratios. Asterisks (*) indicate significant differences in mRNA levels between uninfected and infected animals ($P < 0.05$, Student's *t*-test).

206 The first striking finding was the fact that no lesion was
207 produced and almost no infection confirmed at doses up to
208 10^4 MAA mycobacteria in the first experiment. Moreover,
209 only limited lesions were produced even at very high doses
210 in experiment 2. Several reports in pigs indicate that high
211 doses of MAA are required to achieve consistent develop-
212 ment of lesions and these were grossly visible from 47 dpi
213 (e.g. Jorgensen, 1978). This suggests a low pathogenicity of
214 MAA for wild boar or at least of this particular strain.

215 In experiment 2, the higher dose of 2.5×10^6 myco-
216 bacteria administered by the oropharyngeal route resulted
217 in grossly visible lesions in the head lymphnodes. This
218 finding is in concordance with other reports on wild boar
219 naturally infected by MAA that showed lesions more
220 frequently in head and mesenteric lymphnodes (Biet et al.,
221 2005; Machackova et al., 2003). In experimentally
222 (Jorgensen, 1978) and naturally infected pigs (Hibiya et al.,
223 in press) these organs were found to be commonly
224 affected. Otherwise, lesions found in wild boar infected
225 by the nasal route were either lacking (#12) or limited to
226 the left bronchial lymph node (#11). However, some cases
227 involving lesions exclusively located in bronchial LNs have
228 been observed in swine (Di Guardo et al., 1991, cited in
229 Thorel et al., 1997). Hence, the oropharyngeal route seems
230 appropriate for future infection experiments and might
231 indicate that this is the most common route of natural
232 infection in wild boar.

233 No lung lesions were detected and no MAA was isolated
234 from the lungs in the present study. This is in agreement
235 with data from pigs (Ray et al., 1972; Ellsworth et al., 1979;
236 Jorgensen, 1978; Oliveira et al., 1996; Mores et al., 2006). In
237 contrast to findings reported herein, naturally *M. bovis*-
238 infected wild boar consistently develop mandibular
239 lymphnode lesions. Tracheobronchial lymphnode lesions
240 are visible in over one third of the cases (Martin-Hernando
241 et al., 2007; Ballesteros et al., 2009). Microscopically, the

tubercles were similar to those described in EI pigs 242
(Jorgensen, 1978; Hibiya et al., 2008). 243

244 Low culture sensitivity has repeatedly been reported for
245 MAC infected pigs (Thoen, 1992; Cavirani et al., 1996). This
246 may be due to a low number of organisms in the lesions,
247 partly loosing viability during the decontamination pro-
248 tocol (Prichard et al., 1977; Silva, 1998). The failure to
249 detect acid-fast organisms by Ziehl-Neelsen staining in
250 two out of three animals with tuberculous lesions in
251 experiment 2 confirmed that only small numbers of acid-
252 fast organisms were present. Previous oral MAA EI in pigs
253 resulted in a reduction in the number of tissues with macro-
254 or microscopic lesions and viable acid-fast organisms from
255 days 65 to 177 that might indicate that pigs were
256 overcoming the infection (Jorgensen, 1978b). In the
257 present study all wild boar were killed at 15 weeks after
258 exposure so no information regarding evolution of the
259 disease in this species can be inferred.

260 The IFN- γ assay successfully detected the immune
261 response of the MAA-infected wild boar. The four animals
262 showed a specific response to avian PPD, which was always
263 higher than those obtained with bovine PPD. The
264 magnitude and dynamics of the response to the IFN- γ
265 assay was apparently more associated to the route of
266 infection than to the actual outcome of the EI. Indeed, even
267 though one of the nasally infected animals showed lesions
268 and had visible acid-fast bacilli, both showed a similar
269 pattern of cellular immune response. In a field situation,
270 this could mean that infection acquired by close contact
271 with other animals (likely to be similar to the nasal EI) or
272 infection through consumption of infected carcasses
273 (oropharyngeal EI) would modify the development of
274 the immune response and thus, this could have an effect on
275 the potential outcome of the mycobacterial infection. An
276 additional interpretation of the different immunopatho-
277 logical pattern according to the route of infection is that

the nasal route could develop at a slower pace, and that in the short timespan of the current experiment it had not spread enough to be detectable by our microbiological and histopathological sampling procedure. As discussed above, this is rather unlikely since the type of lesions associated to this route is very rare in natural infections.

Gene expression studies of host response to infection can provide a powerful tool for understanding the interactions between pathogens and the host immune system and may be particularly powerful for identifying specific molecules or pathways that are involved in protective response to infection or have been targeted by pathogens for immune evasion. The analysis of mRNA levels of selected genes by RT-PCR revealed that three genes, C3, IFN- γ and RANTES were significantly down regulated in infected animals when compared to controls. These results were similar to those found in naturally and experimentally *M. bovis*-infected wild boar and may constitute biomarkers of mycobacterial infection in this species (Pérez de la Lastra et al., 2009; Ballesteros et al., 2009).

In conclusion, the infection with MAA of wild boar by different routes and infective doses has been characterized.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.vetmic.2009.12.041](https://doi.org/10.1016/j.vetmic.2009.12.041).

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