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Pathogenesis of domestic pigs submitted to mycobacterial sensitizations previous to experimental infection with *Mycobacterium bovis*

María X. Cuerda[1*], María A. Colombatti[1*], María J. Gravisaco[1], María J. Marfil[2], Soledad Barandiaran[2], Iker A. Sevilla[3], Joseba M. Garrido[3], Roberto D. Moyano[1], Martín J. Zumárraga[1], María I. Romano[1], Ramón A. Juste[3] and María de la Paz Santangelo[1]

[1] *Institute of Agrobiotechnology and Molecular Biology (IABIMO), INTA-CONICET, Los Reseros y Nicolas Repetto, 1686, Hurlingham, Buenos Aires, Argentina.* [2] *School of Veterinary of Buenos Aires University, Chorroarín 280, 1427, Buenos Aires, Argentina.* [3] *NEIKER-Instituto Vasco de Investigación y Desarrollo Agrario. Animal Health Dept. Bizkaia Sci. & Technol. Park 812L. 48160 Derio (Bizkaia). Spain.*

** Both authors contributed equally to this work*

Abstract

Aim of study: To demonstrate the virulence of a *Mycobacterium bovis* local pig isolate in order to contribute to a better understanding of the pathological and immunological consequences of *M. bovis* infection in previous sensitized animals.

Area of study: Buenos Aires, Argentina

Material and methods: One group of ten pigs received two oral doses of killed *M. bovis* suspension and a comparative intradermal tuberculin test (CIT) (multiple sensitized) and then was infected with the *M. bovis* strain. Another group only received the CIT (single sensitized) and the infective dose. Humoral immune response was followed monthly, and gross pathology, histopathological and bacteriological analysis were performed at necropsy 100 days after infection.

Main results: M. bovis oral infection induced lesions and allowed bacterial growth in most of the animals. Previous sensitization with killed *M. bovis* suspension slightly raised the intensity of the response, as the multiple sensitized group showed higher lesion scores and humoral response.

Research highlights: Although the differences in lesion scores were not statistically significant, oral route infection after sensitization can modify the course of infections towards a fast development of lesions with a higher fibrotic component suggestive of increased resistance to infection in the right conditions.

Additional key words: tuberculosis; swine; pathogenicity; histopathology; infectious disease; humoral response

Abbreviations used: bTB (bovine tuberculosis); CIT (comparative intradermal test); cOD (corrected optical density); EI (ELISA index); HIMB (heat inactivated *Mycobacterium bovis.*);H/E (hematoxylin/eosin); LN (lymph nodes); MaLS (macroscopic lesion score); MB 894 (*M. bovis* strain 894); MiLS (microscopic lesion score); MS (multiple sensitized); MTBC (*Mycobacterium tuberculosis* complex); OD (optical density); PPD (purified protein derivative); SENASA (Servicio Nacional de Sanidad y Calidad Agroalimentaria); SS (single sensitized); TB (tuberculosis); TB-LL (tuberculosis like lesions); TMaLS (total macroscopic lesion score); TMiLS (total microscopic lesion score).

Authors' contributions: Performed the experimental design, controlled the health status and overall behavior of the animals daily, acquisition of data, analysis and interpretation of data: MXC and MAC. Contributed to the necropsy and sample collection: MJM and SB. Performed the spoligotyping and helped with the interpretation of the results: MJM and MJZ. Contributed to the analysis and interpretation of data: RDM and MJG. Conceived the idea and provided financial support: MPS and MIR. Designed the experiments and performed the analysis: MPS. Contributed with the experimental design, provided the HIMB and the commercial ELISA: IAS, JMG and RJ. Wrote the draft of manuscript: MPS, MXC and RAJ. Carried out the statistical analyses: RAJ. All authors critically revised the manuscript.

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Correspondence should be addressed to Maria X. Cuerda: cuerda.maria@inta.gob.ar or Ramón A. Juste: rjuste@neiker.eus (shared corresponding authors)

Introduction

Mycobacterium bovis is the main causative agent of bovine tuberculosis (bTB), a disease that significantly affects cattle, but that also can infect other farm animals such as goats, deer and pigs as well as wildlife species, and represents a public health concern as cause of zoonotic tuberculosis (TB) (Gortazar *et al.*, 2005, Neill *et al.*, 2005). TB produces a chronic disease, characterized by formation of nodular granulomas, most frequently observed in the lungs and lymph nodes (LNs) (particularly of the head and thorax); and in generalized TB also in intestines, spleen and liver (Palmer & Waters, 2006).

Mycobacterial diseases are a big challenge to veterinary medicine, but also to public and environmental health. Efforts have been made to control TB in developing countries, due to both the economic losses caused to the industry and the impact on public health. Since the livestock industry is particularly important in Argentina, there is a rising interest in improving the sanitary status of dairy and beef herds. The control strategy is one of test and cull based on the Caudal Fold Skin Test, mainly in dairy herds (SENASA, 2012). However, the success of the eradication programs is compromised by the persistence of the disease in wildlife and reservoirs hosts.

Although data on swine TB prevalence in Argentina is scarce, there are reports of macroscopic TB-like lesions (TB-LL) observed during the slaughterhouse inspection (Barandiaran *et al.*, 2011). The higher frequency of lesions in the mandibular or retropharyngeal LNs, is consistent with a predominantly oral route of infection (Corner *et al.*, 1981; Naranjo *et al.*, 2008). However, there are also more generalized forms, including thorax and abdomen locations (Martín-Hernando, 2007; Di Marco *et al.*, 2012), that suggests that both respiratory and food-borne transmission may occur (Bailey *et al.*, 2013).

Swine TB is not a big concern in intensive systems where biosecurity is high. However, in countries where more extensive production systems predominate, like Argentina, where bTB is endemic, *M. bovis* might have a substantial impact (Barandiaran *et al.*, 2011; 2015b). Between 1969 and 2005, the incidence of swine TB in Argentina decreased from 8.4 to 0.7% and reached 0.3%, according with bTB prevalence in cattle (Torres, 2016). This is consistent with a reduction of transmission to pigs and other species due to progress of bTB eradication programs in cattle (https://www.senasa.gob.ar).

Domestic pigs and wild boar can be maintenance hosts acting as an important source of *M. bovis* infection for domestic cattle (Martín-Hernando, 2007; Naranjo *et al.*, 2008; Santos *et al.*, 2009; Di Marco *et al.*, 2012), although more frequently considered as a spillover than a self-sustained reservoir (Nugent *et al.*, 2011, 2012; Di Marco *et al.*, 2012; Bailey *et al.*, 2013; Pesciaroli *et al.*, 2014). Spoligotyping and mycobacterial interspersed repetitive-unit-variable-number tandem-repeat characterization of swine isolates, has contributed to the knowledge of the epidemiology of the TB in pigs in our country showing its close relationship with cattle lineages (Barandiaran *et al.*, 2011; 2015a; 2021).

In order to contribute to the understanding of the pathogenesis of *M. bovis* in Argentina, we designed a study where we observed experimental infection development in pigs with a local strain by the presumably most frequent route, the oral route, depending on previous sensitization level.

Material and methods

Animals and experimental design

Twenty one-month-old piglets, *Sus scrofa domestica*, were obtained from a commercial farm known to be free of mycobacterial lesions at slaughterhouse, since no history of TB-LL was declared for this farm in the last five years. The animals were housed in class III bio-containment facilities, where they were randomly distributed in four separated rooms of 5 m × 6 m (5 animals/room) with environment enrichments such as balls, rolls, and other toys to guarantee the animals´ welfare. They were fed daily with corn-based food (ration according to the producer's specifications) and had *ad-libitum* water throughout the entire experience. Health status and overall behavior were checked daily.

After one week of adaptation to the new facilities, 10 piglets were sensitized with 2 mL of a heat inactivated *M. bovis* (HIMB) suspension in two oral administrations one month apart (Multiple Sensitized group, MS). Fifty-seven days later, both, the MS group and the other group (Single Sensitized animals, SS), were sensitized with a comparative intradermal test (CIT). Eleven days after reading the CIT, all animals were infected with a *M. bovis* 894 (MB 894) strain suspension containing a total of 10^5 colony forming units (CFU) per 5 mL, administered by the oropharyngeal route as described (Garrido *et al.*, 2011) and kept for 100 days more (Fig. 1). Handling procedures and sampling frequency were designed in accordance with relevant institutional and national guidelines and regulations to reduce stress, and the protocol was approved by the Animal Care and Use Committee and under the regulations of the Ethical Committee of the National Institute of Agricultural Technology (INTA) (CICUAE Permit Number: 05-2017).

Mycobacterium bovis culture and inoculum preparation

M. bovis strain MB 894 spolygotype SB0153 was selected from pigs´ isolates obtained from TB-LL at slaughterhouse, because of its potential interest for public health,

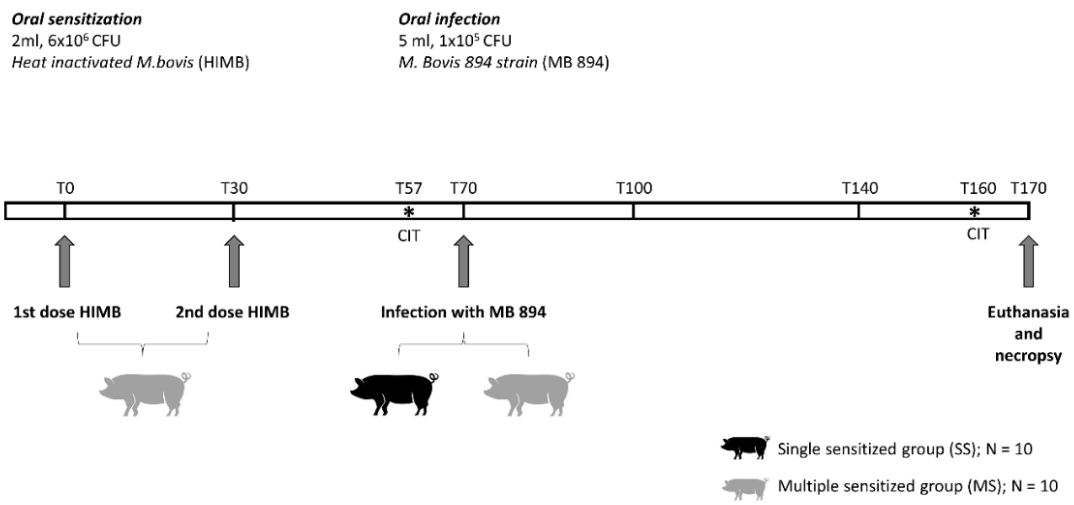

Figure 1. Experimental design. Twenty one-month-old piglets were randomly distributed into two equal groups. MS group (light gray pigs) was sensitized with two doses of HIMB suspension (T0 and T30). Twenty-seven days later (T57) both groups were submitted to comparative intradermal test (CIT). At T70, MS and SS (dark pigs) were orally infected with MB 894 strain. At T170 animals were euthanized and necropsied. Blood samples were taken at different time points (T0, T30, T70, T100, T140 and T170) for antibodies detection. CIT was also performed at the end of the experience. At necropsy, organs were inspected for visible tuberculosis-compatible lesions to determine macroscopic lesion score (MaLS) and bacteriological culture. Histopathological examination was performed for microscopic score determination (MiLS).

as explained in the discussion. Bacteria were propagated in Middlebrook 7H9 broth enriched with 0.4% pyruvate and 10% oleic acid-albumin-dextrose-catalase for 3-4 weeks. Cells were harvested by centrifugation at 2,500 x g for 20 min and washed twice in PBS. The bacterial pellet was re-suspended in PBS and declumped by 20 passages through a 25 G needle syringe. The concentration of this suspension was adjusted to an optical density of 2×10^4 bacteria/mL.

Intradermal skin test

The CIT was performed in each animal before *M. bovis* infection (T57) and at the end of the experiment (T160) (Fig. 1). Bovine and avian purified protein derivative (PPD, SENASA res.145/09) were inoculated intradermically in the base of the right and left ear, respectively. The reading was done 48hs later, and any reaction observed such as redness, inflammation or necrosis was considered positive.

ELISA assay

Whole blood samples were taken at six time points before and after infection (T0 and T30, T70 –day of infection–, T100, T140 and T170). Sera were tested for anti-*M. bovis* immunoglobulin G (IgG) antibodies by means of an in-house (Cuerda *et al.*, 2019) and a commercial indirect ELISA. In-house ELISA was performed according to Griffa *et al.* (2020) with modifications. Briefly, 96-well ELISA flat bottom plates (MaxiSorp, NUNC) were coated with a whole *M. bovis* AN5 lysate antigen (Griffa *et al.*, 2020) and incubated overnight at 4° C. Plates were washed three times with phosphate buffered saline solution containing 0.05% Tween 20 (PBST) and blocked for 1 hour at 37°C with 150 µL of 5% skim milk in saline solution. Plates were washed thrice, and sera were added directly on plate (100 µL/well) at a dilution of 1/200 in saline solution and incubated for 1 hour at 37°C. Samples, positive and negative controls, were tested in duplicate. Plates were then washed three times with PBST, and 100 µL of the anti-porcine IgG antibody conjugated to horseradish peroxidase (Sigma-Aldrich) at a dilution of 1/6000 in saline solution was added and incubated at 37° C for 1 hr. After washing, 100 µL of substrate (citrate buffer pH 5: 9.6 g/L citric acid, 16.2 g/L sodium citrate, 0.2% 3-ethylbenzothiazoline-6-sulfonic acid [ABTS, Sigma-Aldrich], 30% H_2O_2) were added. The colorimetric reaction was read at an optical density (OD) of 405 nm in a spectrophotometer after 15 min of incubation. The reactivity of each sample was expressed as a corrected optical density (cOD) determined as: cOD = OD_{405} of test serum – OD_{405} of buffer. Samples with cOD > 0.22 were considered positive (established cut off following the formula, cOD mean plus two standard deviations [XDOc + (2*DE)], after testing 228 negative sera). Since clear gaps were found between cOD values, two categorical variables were created in order to compare frequency distribution between sensitization groups.

Sera from T0 and T170, were simultaneously evaluated with a commercial ELISA (TB ELISA VK *RD-MAGRAMA SKU: PMBE Product Categories: ELISA,

Fabricante, Porcino, Vacunek Product, Spain), in order to compare the two tests. Commercial ELISA was performed following the manufacturer's instructions. The results were expressed as an ELISA index (EI). Samples with an EI over 0.2 were considered positive.

Necropsy and sample collection

One-hundred days after infection, pigs were anesthetized by intramuscular injection of xylazine/midazolam combination (1 mg/kg and 0.5 mg/kg respectively), stunned with a captive bolt pistol, and bled to death. A thorough postmortem examination was performed on 22 locations of each animal to detect the presence of macroscopic lesions. Tonsils, LNs, lung (each lobe considered separately), spleen, liver, and ileocecal valve were carefully sliced and inspected for visible TB-LL. The TB-LL locations were grouped into three regions: head (oropharyngeal tonsils, retropharyngeal, parotid and submaxillary LNs), thorax (lungs and mediastinal LNs), or abdomen (spleen, liver, mesenteric and gastro-hepatic LNs); and a macroscopic lesion score (MaLS), based on lesion type, size and number of granulomatous lesions was defined as previously described (Ballesteros *et al.*, 2009) (Table S1 [suppl]). The sum of the MaLS of each organ per animal resulted in the total MaLS (TMaLS).

Tissue samples were also collected and stored frozen until bacteriological culture was performed in order to confirm the presence of mycobacteria.

Histopathological examination

All samples from LNs, tonsils, lungs with or without visible TB-LL, were submitted to histopathological examination for microscopic score determination. Collected tissue samples were fixed in 10% neutral buffered formalin and routinely processed to obtain 4 µm paraffin-embedded histological sections, which were then stained with hematoxylin and eosin (H/E) and examined by light microscopy. Based on microscopic appearance, lesions were scored by the presence of necrosis, calcification, giant cells, fibrosis, and number of granulomas. The sum of all the lesion types determined in each organ resulted in the microscopic lesion score (MiLS) (Table S2 [suppl]). The total MiLS (TMiLS) was determined by the sum of the MiLS of all the organs for each animal.

Bacteriological culture and molecular typing

The samples were decontaminated using the Petroff´s method (Burdz *et al.*, 2003) and 1 mL of the suspension was seeded in Stonebrink (ST) medium, incubated at 37 ºC and checked weekly for up to 8 weeks if no colonies were observed. The DNA was prepared from colonies by suspending them in 200 µL of distilled water and boiling at 90° C for 20 min. The resulting suspension was then centrifuged at 12,000 rpm for 10 min, and 2 µL of the supernatant was used for PCR using primers IS*6110* for identifying *Mycobacterium tuberculosis* complex (MTBC) species (Hermans *et al.*, 1990). Spoligotyping was performed in other to confirm the genotype of the strain as described by Kamerbeek *et al.* (1997).

Statistical methods

Gross and microscopic lesion scores different from 0 for each location and type were transformed into their logarithms to better fit a normal distribution. Then, a mixed analysis of variance procedure was run in the SAS statistical package. Tissue type (lymphoid or lung) or region (head, thorax, or abdomen) and sensitization group (SS or MS) and their interactions as fixed effects, and individual pig as random independent effects and score logarithm for each type of lesion (MaLS, necrosis, calcification, giant cell abundance, fibrosis, number of granulomas and MiLS) as the dependent variable. This model provided the real statistical significance. However, in order to have a criterion to better discriminate potential sensitization effects, a fixed effects general linear model with the same variables but the random one was run with the GLM procedure. Level comparisons potential differences were estimated with the Student t test adjusted with the Tukey-Kramer multiple comparison correction.

Regarding the humoral immune response, a similar GLM procedure and means comparison was run with the independent variables positive/negative ELISA at T140 or T170, time point and treatment.

A Pearson correlation was calculated with the SAS proc CORR to determine associations between all quantitative and semiquantitative variables in the exploratory stage. Finally, only the correlation between humoral (cOD) and lesion score (MiLS) was retained.

Results

Gross pathology and histopathological analysis

All the infected animals were evaluated daily for health status and overall behavior, and none of the animals showed clinical signs or behavioral changes along the experience.

At the necropsy, 9/10 animals from the MS group and 8/10 from the SS group showed disseminated TB-LL, *i.e.*, they had TB-LL in head LNs, thorax, and abdomen (Fig. 2). As shown in Fig. 3A, such lesions most frequently affected head LNs, followed by thoracic and abdominal LNs and organs. Only three animals did not have gross lungs lesions

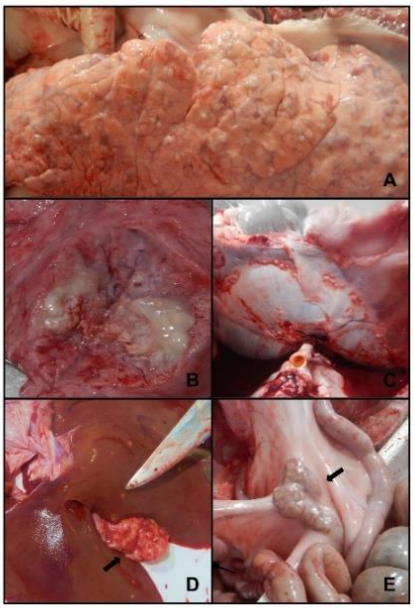

Figure 2. Gross lesions in different tissues representative of pigs, inspected at necropsy after sensitization and oral infection with *M. bovis* 894 strain. (A) Pig #11 (MS). Right lung with multiple granulomas distributed in the parenchyma, coincident with granulomatous pneumonia. (B) Pig #3 (SS). Cross section of the lung where the caseous necrotic-calcified tuberculosis like lesions are observed. (C) Pig #6 (MS). Miliary tuberculosis in pig's diaphragmatic pleura. (D) Pig #16 (SS). Liver with multiple granulomas distributed in the parenchyma and reactive gastro-hepatic lymph nodes (arrow). (E) Pig #14 (MS). Reactive mesenteric lymph nodes (arrow).

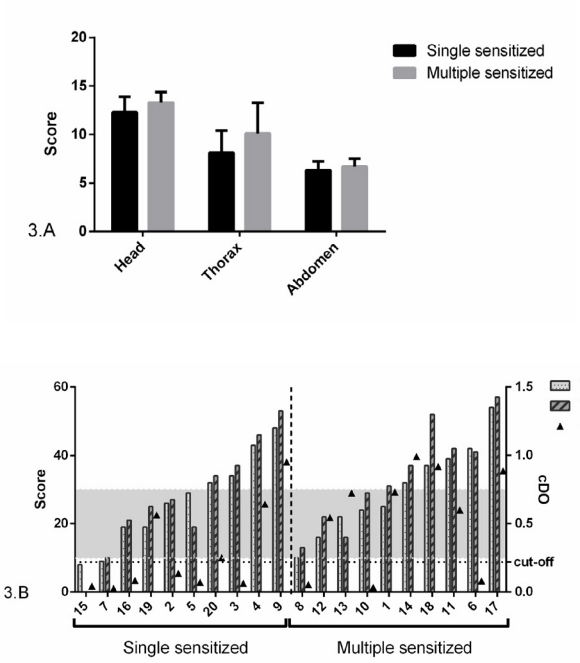

Figure 3. Macroscopic and microscopic lesion score from pigs after sensitization and infection with *M. bovis* 894 strain. (A) MaLS was calculated for each organ and grouped by region (head, thorax and abdomen). No statistically significant differences were observed between SS and MS. (B) TMaLS (light gray, dotted) and TMiLS (dark gray, striped) are represented along with cOD (triangles) for each individual of both groups. Animals were classified in three groups according to TMaLS: lower than 10, between 10 and 30 or higher than 30.

(#7, 12 and 15). The granulomatous inflammatory lesion in the lungs was characterized by a distribution exhibited by 60% of pigs having compromised both right and left cranial and caudal lobes, 55% had affected the accessory lobe, while 70% of the animals had affected the middle lobe.

All animals in both groups showed gross lesions, but no statistically significant differences were observed in the TMaLS score between SS and MS groups. However, the SS mean score was slightly lower and two SS animals (#7 and #15) had minimal gross lesions (TMaLS<10). Moreover, we neither find TB-LL in thorax nor recover *M. bovis* from cultures of animal 15. None of the individuals from the MS group had TMaLS<10. Four animals from the SS group (animals #2, 5, 16, 19) and 5 from the MS group (animals #1, 8, 10, 12, 13), showed TMaLS between 10 and 30 (10 < TMaLS < 30). The remaining four and five animals from each group, showed TMaLS higher than 30 (animals #3, 4, 9, 20 and animals #6, 11, 14, 18, 17, from the SS and MS groups, respectively) (Fig. 3B).

Similar results were obtained in the histopathological examination. The characteristic lesion was represented by the presence of numerous granulomas, extensive amorphous acidophilic areas with loss of tissue and cell detail, pyknotic nuclei, and some pyocytes (massive caseous necrosis) with marked calcification (abundant basophilic granulation) (Fig. 4A.). In general, there was a good correlation between macro and microscopic lesions (r=0.9384; p<0.0001) (Fig. 3B) that stood within each sensitization group (SS: r=0.9521; p<0.0001 and MS: r=0.9304; p<0.0001). As expected, animal #7 showed the lowest TMiLS (Fig. 3B) with initial granuloma composed of mononuclear inflammatory cells (especially lymphocytes) without a necrotic center, affected only one retropharyngeal LN, mediastinal and gastro-hepatic LNs, while no histopathological lesions were observed in animal #15. On the other hand, animals #4, 9, 18 and 17 displayed the highest TMiLS (Fig. 3B), finding granulomas with abundant calcifications occupying the entire necrotic area, and almost all the organs affected. In the middle, we found granulomas with a necrotic center surrounded by epithelioid cells and a variable number of multinucleated giant cells, macrophages, and lymphocytes; and more severe granulomas with a necrotic calcified center and peripheral fibroplasia (Figs. 4 B-C-D). Animal #9 was the only one from the SS group to have histopathology lesions in spleen, while in the MS group 4 animals (#1, 6, 11 and 17) had lesions in this organ, characterized by several granulomas, severe fibrosis, slight necrosis, and few calcification foci (Fig. 3B). Each lesion type in MS pigs had about 20% higher score than SS when they were evaluated independently. For example, mean necrosis value was 1.61 in the MS group *vs* 1.31 in the SS group. However, these differences were not statistically significant, except for fibrosis that had 44%

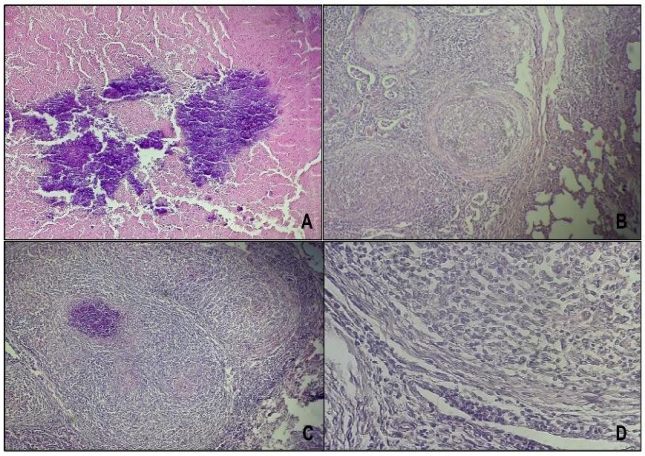

Figure 4. Histopathological lesions examined by light microscopy of representative samples stained with hematoxylin/eosin (H/E) stain, from pigs after sensitization and oral infection with *M. bovis* 894 strain. (A) Pig #9 (SS). Submaxillary lymph node 400x H/E: Granulomatous lymphadenitis with dystrophic calcification (central basophilic material) and caseous necrosis (external acidophilic material). (B) Pig #11 (MS). Lung 100x H/E: Granulomatous pneumonia. Observed multiple granulomas surrounded by thick fibrotic capsule. (C) Pig #9 (SS). Lung 100x H/E: Granulomas with the characteristic nodular appearance, with no capsule of fibrotic tissue delimiting them, tending to coalesce. A focus of necrosis with remains of pyknotic nuclei is observed. (D) Pig #2 (SS). Lung 400x H/E: Thin capsule showing few collagen fibers and some fibroblasts surrounding the granuloma composed of mononuclear cells (the image shows lymphocytes in the vicinity of the capsule and macrophages as epithelioid cells towards the center of the granuloma).

higher score (p=0.0031) (Table 1A). When we considered the mean lesion score, differences were statistically significant between groups, being higher in the MS group (1.11 *vs* 0.84; p=0.0045).

Culture isolation and molecular detection of *M. bovis*

M. bovis was isolated from 9 out of 10 animals from the SS group and 7 out of 10 animals from the MS group, and further confirmed by PCR IS*6110*. As mentioned, we were not able to recover *M. bovis* from animal #15, neither from animals #1, 6 and 10 from the MS group. Between 5 and 9 samples of each animal were cultured, including head LNs, mediastinal and mesenteric LNs. We recovered the bacteria most frequently from head LNs (12/20) and mediastinal LNs (11/20). Molecular typing confirmed that all isolates belonged to SB0153, the strain used for infection.

Comparative intradermal test

All animals tested negative for both bovine-PPD and avian-PPD before the infection (T57). By the end of the experience (T160), the test was performed in 10 animals (5 from each group). All of them showed positive reactions 48 hours after the test was performed, for both PPD tests (data not shown).

Humoral response

Anti-*M. bovis* antibodies were detected using an in-house ELISA. No positive values were obtained before experimental infection with MB 894 in any of the treatment groups. After infection, all MS animals showed a slight cOD increase and, by T140, two were considered positive (cOD≥0.22) among the SS, and another two among the MS. By day T170, the SS group had statistically significant higher values compared to initial cOD reading (p=0.0023). By this time (T170), the MS group cOD values increased significantly, both from its pre-sensitization values (T0) (p>0.0001) and from SS group cOD values at that time (T170) (p=0.0006). By the end of the experience, four animals from the SS and seven from the MS group, had positive results. Mean value for the MS group was significantly higher than that for the SS group (Table 1B).

The commercial ELISA did not detect any positive result before infection, but it did after infection in 4 animals from the SS group and 8 from the MS one (data not shown).

All the animals that showed positive in-house ELISA cOD values had MaLS and MiLS higher than 10. However, not all the animals with intermediate and high TLS (>10) had antibody titers detected by ELISA. There were good correlations between the serological results at T170 and all lesion scores, particularly for the microscopic score if both groups were taken together (p=0.0033). When separately analyzed, both groups had less significant correlations with the MiLS (p=0.0119 for SS and p=0.1871 for the MS group) (Fig. 5). Analysis of variance lesion score for ELISA positivity confirmed significantly different overall gross and microscopic lesion score. Means comparison showed that positive animals at T140 (MiLS: 47.00 *vs* negative animals MiLS: 25.13; p=0.0027 – MaLS: 43.20 *vs* 23.47; p=0.0011) and T170 (MaLS: 37.72 *vs* 21.89; p=0.0168 – MaLS: 33.36 *vs* 22.33; p=0.0569) had significantly (p<0.05) or nearly significantly (p=0.0569) higher mean score than negative ones.

Discussion

The results presented here confirm the virulence of a local strain of *M. bovis*, isolated from pigs at slaughterhouse, with spoligotype SB0153 that was the most frequent *M. bovis* spoligotype isolated from humans in Argentina. *M. bovis* infection induced TB-LL in all

Table 1. Gross pathology, histopathology, and immunology. A) Lesion scores and frequency of cultures recovered from TB-LL. Statistically significant differences were observed between groups in the fibrosis values (p=0.0031) and in the mean lesion scores (p=0.0045). B) Anti *M. bovis* antibodies detected by ELISA at different time points (mean cOD values). Statistically significant differences were observed at T170 respect to T0 values in both groups (SS, p=0.0023; MS, p=0.0001); and also, between groups (p=0.0006)

A)	Pathology and isolation								
	Gross lesions	**Microscopic lesions**	**Isolation**	**Necrosis**	**Calcification**	**Giant cells**	**Fibrosis**	**Granulomas**	**Mean lesion score**
SS	26.7	27.2	0.90	1.31	1.05	0.37	0.92	1.54	0.84
MS	30.1	34.0	0.70	1.61	1.32	0.40	1.30	1.89	1.11
SEM	4.19	4.85	0.13	0.14	0.14	0.05	0.09	0.15	0.06
p	0.5734	0.3342	0.2878	0.1351	0.1716	0.6684	0.0031	0.0968	0.0045

B)	In-house ELISA[1]						Commercial ELISA[2]	
	T0	**T30**	**T70**	**T100**	**T140**	**T170**	**T0**	**T170**
SS	0.043	0.081	0.040	0.056	0.187	0.284	0.083	0.409
MS	0.036	0.063	0.043	0.059	0.164	0.557	0.037	0.781
SEM			0.055				0.119	
p (SS) ^	0.9280	0.8162	0.9650	0.9711	0.7625	0.0006	0.7855	0.0336
p (T0) SS >	-	0.6237	0.9655	0.8687	0.0645	0.0023	-	0.0605
p (T0) MS >	-	0.7274	0.9274	0.7704	0.1008	<0.0001	-	<0.0001

SS: single sensitized. MS: multiple sensitized. SEM: standard error of the mean. [1] Values are expressed as cOD. [2] Values are expressed as EI. *p* (SS) ^: probability of the MS group mean cOD being different from to the SS group mean cOD at each time point. *p* (T0) SS >: probability of the corresponding time point SS group mean cOD being different from the T0 mean cOD. *p* (T0) MS >: probability of the corresponding time point MS group mean cOD being different from the T0 mean cOD.

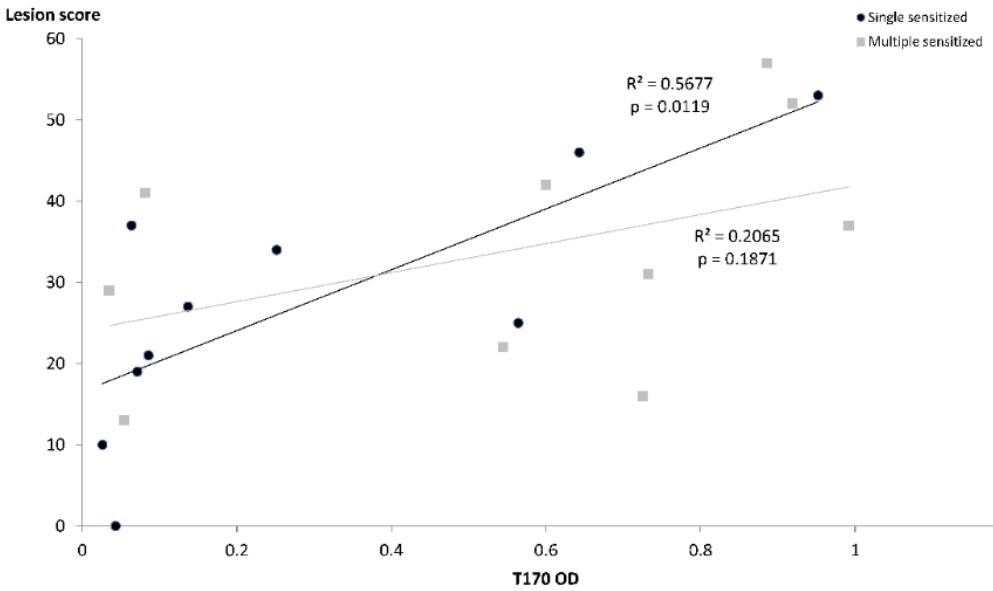

Figure 5. Individual correlation between ELISA cOD_{405} at T170 and MiLS according to sensitization group. Conventional significant linear correlation was observed only in the SS group (black circles) (Pearson correlation, p=0.0119). The MS group showed higher variability and therefore a p value (p=0.1871) above the standard (p<0.05) significance level. Improvement of p for both groups joint assessment (p=0.0033) suggested that lack of significance was a group size problem. This correlation seems to correspond to the clustering of individuals of both groups into two areas: low ELISA-low lesion, and high ELISA-high lesion groups confirmed by the means comparison (positive: 37.72 *vs* negative: 21.89; p=0.0168).

animals but we recovered bacterial growth in 80% of the animals. It was not possible to recover the bacteria from all the samples analyzed due to the strong decontamination process that the samples must undergo, a low bacillary burden in the lesions or even a regression or resolution of the infection (Bollo *et al.*, 2000; Nugent *et al.*, 2015). Previous sensitization with a killed *M. bovis* suspension slightly raised the intensity of the response, as the groups thus treated showed somewhat higher lesion scores and significantly higher humoral immune response by the end of the experiment. It is worth mentioning that there was an increase in the fibrotic response that could represent a slight resistance to infection (Santos *et al.*, 2009).

Different scenarios define the role of pigs in the epidemiology of TB. For instance, in Australia and New Zealand, the low prevalence of generalized lesions and in most cases self-limiting in feral pigs, combined with management and genetic findings, suggested that feral pigs are spillover hosts rather than sources of infection (Corner *et al.*, 1981; Corner, 2006; Nugent *et al.*, 2011). Conversely, in Spain and Portugal (Aranaz *et al.*, 2004; Martín-Hernando, 2007; Naranjo *et al.*, 2008; Santos *et al.*, 2009), the role of the closely related wild boar in the epidemiology of TB supports the theory of a reservoir or maintenance host (Bailey *et al.*, 2013; Pesciaroli *et al.*, 2014). Other authors observed that domestic pigs act as a true reservoir of MTBC in Spain and Sicily (Di Marco *et al.*, 2012; Cano-Terriza *et al.*, 2018).

In Argentina, bTB is endemic, and thus *M. bovis* is the main cause of TB-LL in pigs Barandiaran *et al*., 2011; 2015a; 2015b). The most frequent spoligotype present among the *M. bovis* isolates (SB0140), is also the most frequent type in bovine isolates in Argentina (Barandiaran *et al.*, 2011). It is also one of the most frequent spoligotypes reported worldwide, especially in Europe, and a predominant spoligotype in the British Islands and countries where British Cattle breeds have been introduced (Smith *et al.*, 2006; Smith, 2012; Zumarraga *et al.*, 2013). We focused our attention in one isolate with spoligotype SB0153. Interestingly, the spoligotype SB0153, has been previously detected in only 2.7% of total *M. bovis* isolates in pigs from Argentina and represents 34% of all the *M. bovis* human isolates (Barandiaran *et al.*, 2011; Zumarraga *et al.*, 2013). For this reason, we chose to characterize the *M. bovis* 894 (MB 894) strain in an experimental infection.

Twenty pigs were infected with this isolate by the oral route, since it is considered the most important route of *M. bovis* infection in domestic pigs. To our knowledge, this is the first experimental assay performed in domestic pigs in Argentina. The distribution of the granulomatous lesion in lungs allows to confirm the tropism of *M. bovis* for the respiratory tract, suggestive of an adaptation to this route of spread from infected individuals to the environment.

Histologically, lesions were similar to those observed in a slow generalization process. This corresponds to a lympho-hematic dissemination of few bacilli, which occurs successively, giving rise to polymorphous lesions in various organs, from incipient nodular granulomas (*i.e.*, #7) to ones with caseous and even caseo-calcareous necrosis and peripheral fibromatous reaction (*i.e.*, #9 and #17). Initial granulomas were predominant in cases of localized TB, whereas advanced granulomas were more prevalent in cases of generalized TB. This pattern is consistent with disease progression and thus also with the presence of active infection.

Lesion in LNs is a constant in TB infections, regardless of the entry route making TB a predominantly lymphoid disease. However, predominance of head LNs lesions confirms the effectiveness of the oral route for TB models in pigs (Pesciaroli *et al.*, 2014).

When we evaluated antibodies response, we detected four animals from the SS group, and seven from the MS group, with positive cOD values detected by in-house ELISA. A strong correlation was observed when we compared with the commercial test, supporting the results given by the in-house ELISA. These results are in accordance with (Cardoso-Toset *et al.*, 2015), who suggested the use of the antibody ELISA for monitoring the TB status of domestic pigs at the herd level, and not in individual pigs, due to a limited sensitivity of the technique. Additionally, it has been proposed that ELISA may be used not only for prevalence studies, but also for classifying infected animals as showing a less or more advanced disease (Garrido *et al.*, 2011). Regarding this, higher significance differences in the lesion scores between positive and negative ELISA animals is very interesting to discuss. We would expect larger differences as the infection progress and lesions expand. At T140 only 5 animals out of 20 (3 from MS and 2 from SS) allowed the bacteria to grow in an amount enough to trigger a detectable humoral immune response. At T170, the longer time instead, that would allow enough antigen exposure to induce a humoral response, only 11 animals (7 from MS and 4 from SS) resulted positive to ELISA with lesion extension and associated bacterial load. Still, we cannot discard the possibility that the animals with no humoral response, would evolve to a positive status in a longer experimental time, considering that bTB is a chronic disease. MS animals did react strongly, with seven animals exhibiting positive cOD values at the end of the experiment, all of them with TMaLS and TMiLS over 10. In summary, antibody levels could be a good marker of infection progress, but due to the low sensitivity of this technique we were not able to detect all the infected animals. Moreover, the lack of antibodies in SS animals #5,16, 2 and 3, and in MS #8, 10 and 6, also belonging to the intermediate and high TMaLS (>10) groups, could be due to a delay in the progress of the infection of each individual.

As it can be seen in Fig. 5, individuals of both groups seem to cluster at high and low antibody levels with a 30% - 70% splitting each with significantly different mean lesion scores. All this led us to speculate that polarized anti-inflammatory or pro-inflammatory predominant individual responses might occur in each cluster. Within these clusters, 70% of the SS individuals were located in the low-level antibody group sharing the lowest lesion scores, and even showing the two lowest values. On the other hand, 70% of the individuals of the MS group were situated in the high-level lesion/ antibody cluster, including the animal with the highest value. These observations suggest the effect of a factor that was not initially considered of importance in the experimental design and that would be acting in the MS group: the immune stimulation through the CIT after first sensitization. The MS group might have experienced a tolerating reaction as a consequence of an excess of exposure boosted right before the infection.

Mycobacterial sensitization induced higher responses against experimental infection with a local *M. bovis* isolate, but no statistically significant differences between MS and SS groups were observed regarding gross and microscopic score lesions. However, fibrosis had a twofold increase compared to other microscopic lesions, which is suggestive of a potentially beneficial bias towards lesion demarcation (Bollo *et al.*, 2000; Gortazar *et al.*, 2003; Santos *et al.*, 2009). Serological follow-up brought up a specific antibody response already noticeable by T140 and statistically significant between groups by T170, and for both groups compared with pre-infection T0. Animals in this study received one or multiple inoculations with *M. bovis* antigens, one with standard bovine tuberculin and, in the case of MS group, additionally with a suspension of killed whole cells in a short period of time. Since experiments with killed mycobacteria have shown some degree of protection (Garrido *et al.*, 2011; Beltran-Beck *et al.*, 2014; Nol *et al.*, 2020), it is possible to think that the less efficient protection observed in this study, could be attributed to a shift in the immune response towards a more humoral and less efficient one. The killed mycobacteria could have acted as a tolerating agent rather than as a boost. These results indicate that the oral route of infection with previous sensitization can modify the course of infections towards a fast development of lesions with a higher fibrotic component, suggestive of an increased resistance to infection (Nugent *et al.*, 2015). If a lower infectious dose, *i.e.* more representative of a natural exposure had been used, it might have provided some protection.

These results would lead to the conclusion that further investigation would be necessary to more precisely calibrate the stimuli necessary to cause one or another immune response shift. These situations have been previously recognized in humans with BCG vaccination (Buddle *et al.*, 2013). Moreover, studies using HIMB in different species have yielded different results. While in wild boar results have been encouraging, generally with good protection both in experimental and in natural infections (Garrido *et al.*, 2011; Diez-Delgado *et al.*, 2017; Arrieta-Villegas *et al.*, 2018), their domestic counterparts seem to have yielded worse results (Nol *et al.*, 2020). Overall, in this study we evaluated the virulence of a local strain of *M. bovis* in an oral experimental infection trial and demonstrated that sensitization with a killed *M. bovis* suspension slightly raised the intensity of the response, with a higher fibrotic response that could represent a slight resistance to infection. Further research is needed to obtain more consistent and precise protective effects and to avoid vaccination failure or negative effects when designing mycobacterial vaccines.

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