Experimental model of tuberculosis in the domestic goat after endobronchial infection with *Mycobacterium caprae*

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**Running title:** Experimental model of *M. caprae* infection in goats

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ABSTRACT

Caprine tuberculosis has increased in recent years, highlighting the need to address the problem this infection poses in goats. Moreover, goats may represent a cheaper alternative for testing of prototype vaccines in large ruminants and humans. With this aim, a *Mycobacterium caprae* infection model has been developed in goats. Eleven 6-month old-goats were infected by the endobronchial route with $1.5 \times 10^3$ cfu, and two other goats were kept as non-infected controls. Animals were monitored for clinical and immunological parameters throughout the experiment. After 14 weeks, goats were euthanized and detailed post-mortem analysis of lungs lesions performed by Multi-Detector Computed Tomography (MDCT) and direct observation. Respiratory lymph nodes were also evaluated and cultured for bacteriological analysis.

All infected animals were positive to single intradermal comparative cervical tuberculin (SICCT) test at 12 weeks post-infection (wpi). IFN-γ antigen-specific responses were detected from 4 wpi until the end of the experiment. Humoral response to MPB83 was especially strong at 14 wpi (13 days after SICCT-boost). All infected animals presented severe TB lesions in lung and associated lymph nodes. *M. caprae* was recovered from pulmonary lymph nodes in all inoculated goats. MDCT allowed a precise quantitative measure of TB lesions. Lesions in goats induced by *M. caprae* appeared to be more severe than those induced in cattle by *M. bovis* over a similar period of time. The present work poses a reliable new experimental animal model for a better understanding of caprine tuberculosis and future development of vaccine trials in this and other species.
INTRODUCTION

Tuberculosis (TB) in the domestic goat (Capra hircus), mainly caused by Mycobacterium caprae (1), is an endemic disease in the Iberian Peninsula. M. caprae is widespread in goat herds and is an emerging infectious agent in cattle (15, 33). Infected goat herds can constitute a reservoir of TB-inducing mycobacteria in the field, posing a risk of infection to cattle and wildlife (17, 33). Furthermore, caprine TB not only may hamper the eradication campaigns of bovine TB in affected areas, but may be also responsible for cases of TB in humans (11, 21, 30, 32).

In the last decade, interest on vaccines against bovine TB has been renewed, as a tool for controlling infection in cattle and in wildlife (5) in areas where eradication by the test and slaughter scheme alone is not considered feasible. Moreover, ruminant and porcine models of TB may be useful for screening prototype vaccines for humans, due to their similar lesional pattern and immunological responses to mycobacteria (7, 14, 18). Standardization of the goat as a model of TB would improve our understanding of TB in this species, which in turn could help develop new strategies to combat this disease in goat flocks. Similarly, it could be used as an animal model for TB vaccine development in humans.

Caprine and bovine TB are closely related in regards to the immune response and pathological characteristics. In natural infections, such as in cattle, TB in goats is primarily a lower respiratory tract disease, with lesions in the lungs and associated lymph nodes. Occasionally tuberculous lesions may also be found in the upper respiratory tract lymph nodes and other organs like spleen, liver or mesenteric lymph nodes (12, 31). Histologically, lesions are similar to those observed in cattle and humans. Typical tuberculous granulomatous necrotizing lesions are observed,
characterized by central caseous necrosis often with some mineralization, surrounded by macrophages, foamy macrophages, numerous giant cells, lymphocytes and a fibrotic capsule. Acid-fast bacilli are usually present inside the caseous necrosis, but in very low number (11).

Several TB diagnostic tests currently available for use in cattle, such as the tuberculin skin test or the interferon-gamma (IFN-γ) assay, can be also applied with minor modifications for diagnosis of TB in goats (19, 22). Refinement of specificity of these tests has been achieved in recent years for their use in humans, based on the detection in peripheral blood of effector T-cells reacting against antigens secreted by active growing bacilli, such ESAT-6 and CFP-10, which are not induced by BCG vaccination (27). As it has been observed previously in cattle (37), we have recently shown that an IFN-γ-ESAT-6 specific response also occurs in goats naturally infected with *M. caprae*, which is positively correlated with the severity of the pathological changes (14). A peptide cocktail containing ESAT-6 and CFP-10 has been also successfully used for diagnosis of TB in naturally infected goats (2). In cattle, it has been shown that the route of challenge can have a significant influence on infection outcome (29). The endobronchial route of inoculation has been used successfully in several experimental models of TB infection in cattle (13), brushtail possums (4) or European badgers (10) for its capacity to mimic the natural infection. In adult goats, an infection model of transthoracic inoculation of *M. caprae* has been previously described (3), demonstrating the potential of this species as a research model for TB.

Qualitative or semiquantitative scoring systems of gross lesions have been used to assess efficacy of vaccines, based on lesion distribution and extension. Improvement in this scoring system into a more precise quantitative system would be of benefit to allow better comparison between treatment groups and experiments. Recently, magnetic
resonance imaging (MRI) has been used in to measure disease burden in macaques experimentally infected with *M. tuberculosis* (34, 35), with promising results. The aim of the present work was to reproduce experimentally TB infection in young goats by inoculation with *M. caprae* through the endobronchial route, to characterize the immune response, and to standardize methods for quantifying pathological changes in target tissues, including the assessment of Multi-Detector Computed Tomography (MDCT) to measure the magnitude of lesions in pulmonary tuberculosis. To our knowledge, this is the first study aimed at comprehensively characterising the effect on endobronchial infection of goats with *M. caprae*.

**MATERIALS AND METHODS**

**Experimental animals**

Thirteen Murciano-Granadina female 6 months-old goats obtained from an officially TB-free herd were used. Goats were negative to the single intradermal comparative cervical tuberculin (SICCT) test and the IFN-γ assay (Bovigam™, Prionics, Schlieren, Switzerland) as well as seronegative for paratuberculosis (Paratub.Serum-S™, Institut Pourquier, Montpellier, France). The herd was not vaccinated against Paratuberculosis. Eleven goats were housed in appropriate containment accommodation for a week, prior to infection with *M. caprae*. Two additional goats were kept uninfected in an outdoor box throughout the experiment. All experimental procedures with animals were in agreement with the European Union Laws for protection of experimental animals and were approved by the Animal Welfare Committees of the *Universitat Autònoma de Barcelona* and the *Generalitat de Catalunya*. 
Mycobacterium caprae cultures and experimental infection

The *M. caprae* SB0416 (www.mbovis.org) field strain used as inoculum was originally isolated from a tuberculous goat from Catalonia. The isolate was subcultured in Middlebrook 7H11 solid media (BD Diagnostics, Spark, USA) and bacteria were resuspended in Brain Heart Infusion broth with 20% of glycerol at a concentration of 2 \( \times 10^6 \) colony forming units (cfu)/ml (calculated by plating dilutions on Middlebrook 7H11 media). The suspension was stored at -80 ºC in 0.5 ml aliquots. The inoculum was prepared to the required final concentration by diluting the suspension with sterile phosphate buffered saline (PBS).

For infection, goats were pre-anaesthetized with 0.05 mg/kg of acepromacin (Calmo Neosan®) and 0.2 mg/kg of butorphanol tartrate (Torbugesic®) co-administered by intramuscular injection; after 30 minutes a catheter was placed into the left cephalic vein and 4-6 mg/kg of propophol (Propofol Lipuro®) and 0.2 mg/kg of midazolam (Dormicum®) were both administered intravenously. Goats were then intubated with an endotracheal tube and were placed in right lateral decubitus. A plastic cannula (3.3 mm outer diameter) was passed through the endotracheal tube to the level of the carina. For inoculation a thinner cannula (2.1 mm outer diameter) was passed through the thicker one to a bronchus, and then 0.5 ml of *M. caprae* inoculum was injected into the inner cannula, followed by flushing with 5 ml of 0.9%-saline. The inoculum was titrated after the inoculation in duplicate by ten-fold serial dilution in Middlebrook 7H11 solid media; accordingly, each goat received 1.5 \( \times 10^3 \)cfu of *M. caprae*. The animals recovered from anaesthesia in sternal decubitus.

Clinical signs and sampling
Before and during the experimental infection goats were observed for clinical signs. Rectal temperature was measured weekly and weight every two weeks. Blood samples were collected every two weeks from the jugular vein in heparinized blood tubes for immunological studies and isolation of mycobacteria. Also, two nasal swabs were collected from each animal at the same time points, one was decontaminated and subsequently cultured and the other one was submerged in ultrapure water 1 hour at 75 °C for mycobacteria inactivation and stored at -80 °C until a specific *M. tuberculosis* complex (MTC)-PCR assay was performed.

**Antigens and peptides**

Bovine (PPD-B) and Avian (PPD-A) tuberculins were obtained from CZ Veterinaria (Porriño, Galicia, Spain). ESAT-6/CFP-10 and Rv3615c peptide cocktails synthesized as described earlier (36, 38) were received from Dr. H.M. Vordermeier, Veterinary Laboratories Agency (Weybridge, UK). Recombinant MPB83 was obtained from Lionex (Braunschweig, Germany). Phytohemagglutinin (PHA) (Sigma-Aldrich, Steinheim, Germany) was used as a positive control.

**Skin Test**

SICCT test was performed in all goats at 12 weeks post-infection (wpi) (2 weeks before sacrifice) by inoculating 0.1 ml of both PPD-B and PPD-A on the left and the right side of the neck respectively. The preinoculation skin-fold thickness was recorded before PPD injection, and the skin-fold thickness was measured again after 72h. The goats were considered positive if the increase of skin-fold thickness after PPD-B application was higher than 2 mm and higher than the increase after PPD-A application.

**Whole-blood IFN-γ assay**
Blood samples were collected at the time points described above, preserved at room temperature and processed in less than two hours after collection. One ml of whole blood was stimulated in 96-well cell culture plates with PPD-A, PPD-B and PHA at a final concentration of 10 µg/ml, or with peptide cocktails, each one at a final concentration of 5 µg/ml. PBS was used as non-stimulated control. Plasma supernatants were collected after 24h of culture at 37ºC and 5% CO₂ and were stored at -20ºC, and thawed just before performing the Bovigam IFN-γ enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions. ELISA results are reported as OD₄₅₀. Specific reaction was expressed as ΔOD₄₅₀ (OD₄₅₀ of antigen-stimulated sample minus OD₄₅₀ of non-stimulated control). A sample was classified as positive when the PPD-B ΔOD₄₅₀ was higher than 0.05 and OD₄₅₀ of PPD-B was higher than OD₄₅₀ of PPD-A, according to the manufacturer’s interpretation.

**Serology**

Plasma samples of all animals were analyzed in duplicate for antibodies to mycobacteria using ELISA, as described previously (18) with minor modifications. The 96-well plates were coated with PPD-B (2 µg/ml) or MPB83 (1 µg/ml) diluted in carbonate/bicarbonate buffer and incubated overnight at 4ºC. After blockade of 45 min. at 37ºC with PBS containing 0.05% Tween 20 (PBS-T20) with 0.5% casein, plasma samples (at 1/200 dilution in PBS-T20 with 1% casein) were added in duplicate and incubated 1h at 37ºC. After washing, a combination of Protein A and Protein G conjugated with peroxidase (Sigma-Aldrich, Steinheim, Germany) was added at a final concentration of 50 ng/ml and 100 ng/ml respectively. Plates were then read in a spectrophotometer and ΔOD₄₅₀ was calculated as sample OD₄₅₀ minus background OD₄₅₀ (unspecific absorbance in wells where antigen had not been added). A sample
was classified as positive when the ΔOD_{450} was higher than the cut-off point, calculated as the mean of background OD_{450} + 3SD.

**Post-mortem examination**

All goats were euthanized at 14 wpi by intravenous injection sodium pentobarbital and carefully examined in order to evaluate the extension of tuberculous lesions in lungs and respiratory lymph nodes (LN).

**A. Lungs**

Lung gross lesions were recorded first by palpation and external observation of the different lobes. LN were removed for bacteriological investigation, taking special care of not incising the pleural surface. The heart and pericardium were removed, and then, whole lungs were fixed with 10%-buffered formalin by pouring the fixative into the trachea while holding the lungs in a vertical position, until the trachea was filled up with fixative. After that, the trachea was tied, and formalin-flooded whole lungs were immersed into a container with formalin as previously described (18), for two months. After complete fixation, lungs were scanned by using a high-resolution 64-slice Multi-Detector Computed Tomography (MDCT) scanner (Brillance CT 64-channel, Philips Medical Systems, Cleveland, Ohio, USA). MDCT data were analyzed and post-processed on a workstation (Aquarius Station, TaraRecon, Foster City, California, USA). Tuberculous lesions were defined as the following 4 lesion-types in respect to their density patterns: calcified lesions, cavitary lesions, solid lesions and complex lesions. The total pulmonary volume and volume of lesions were measured. MDCT quantification of lesions was compared with conventional visual inspection with the aid of image analysis software. For that purpose, lungs were sliced at 4-5 mm width intervals. Each slice was photographed and gross lesions were subsequently quantified.
in pictures with the aid of an image analyzer (ImageJ 1.43u, National Institutes of Health, USA). Approximate volume of granulomas was calculated for each slice (area of lesion × slice thickness). Total volume of granulomas of each lobe was calculated adding slice-partial volumes. Data obtained by applying both the MDCT and the visual direct scoring were compared in order to evaluate the correspondence between the two methods. Representative sections of gross lesions were also processed for histopathological examination (haematoxylin-eosin staining and Ziehl–Neelsen staining for acid-fast bacilli) to confirm the tuberculous nature of the lesions.

B. Lymph nodes (LN)

The number and diameter of the macroscopic lesions were recorded in cranial mediastinal LN, tracheobronchial LN, caudal mediastinal LN as well as both right and left retropharyngeal LN. The approximate volumes of gross lesions were calculated as $4/3 \times \pi \times r^3$ assuming that most lesions showed fairly spherical morphology. The same pathologist performed all evaluations in order to ensure homogeneous application of the scoring criteria. After pathological measures each LN was processed entirely for bacterial enumeration.

Culture of M. caprae

A. Lymph nodes

To calculate the bacterial load (cfu/g) of each LN, the weight was recorded before homogenization. Then, the LN were mechanically sliced using dissection scissors and automatically homogenized in 10 ml of sterile distilled water in a Masticator (IUL Instruments, Barcelona, Catalonia, Spain). The homogenate was decontaminated with a final concentration of 0.35% w/v hexadecylpyridinium chloride (HPC) (9) for 15 min. in orbital shaking after which it was centrifuged at 2471×g for 30 min. The supernatant
was discarded and the pellet was resuspended in 10 ml of PBS containing 0.05% Tween 80. Viable bacterial enumeration was determined by plating 0.1 ml of ten-fold serial dilutions of LN homogenates on Middlebrook 7H11 agar and incubated at 37 °C for 28 days.

B. Peripheral blood

Whole blood (5 ml) from each goat was inoculated to BacT/ALERT MB flasks (Biomérieux España, Madrid, Spain) at the time points described above and incubated for 30 days before being considered negative, as recommended by the manufacturer.

C. Nasal swabs

Nasal swabs were decontaminated for 30 min. with 0.35% w/v HPC and subsequently cultured on Coletsos and pyruvate-enriched Löwenstein-Jensen media (Biomérieux España, Madrid, Spain); cultures were incubated for 60 days before being considered negative.

DNA amplification

The DNA from inactivated samples from nasal swabs was extracted using DNA purification kit (Promega Biotech Iberica, Madrid, Spain). A seminested-PCR was run under standard conditions. Two consecutive PCR reactions were performed using oligonucleotide primers described previously (IS-F: 5’-CCTGCGAGCGTAGGCGTCGG-3’, IS-R1: 5’-TCAGCCGCGTCCACGCCCA-3’) (28) adding another reverse primer for the second reaction (IS-R2: 5’-CTCGTCCAGCGCCGCTTCGG-3’) (16). These primers are specific for the MTC-IS6110 insertion sequence.

Data analysis
Differences in the mean rectal temperature between weekly measures were compared by employing analysis of variance (ANOVA) with Student-Newman-Keuls multiple comparison test and are reported with the 95% confidence interval (C.I.). Comparison of bacterial loads (log₁₀ cfu/g) between pulmonary LN, as well as comparison of IgG or IFN-γ ELISA absorbance values (ΔOD₄₅₀) between antigens within the infected group, were analyzed by non-parametric Friedman test with the post-hoc Mann-Whitney-Wilcoxon test. Correlation between MDCT and direct visual measure of macroscopic lesions was performed by non-parametric Spearman rank test. Immune responses and log₁₀-transformed pathological and bacteriological data were compared by applying linear regression, or by the non-parametric Spearman rank test depending on whether experimental units passed the Shapiro-Wilk normality test. Analysis of the data was performed using SPSS statistical package version 17.0.

RESULTS

Clinical observations

Few clinical signs were observed in goats infected with *M. caprae* throughout the experiment. A significant increase in mean rectal temperature was detected at 4 wpi (39.6 ºC, 39.5-39.7, 95% C.I.) compared with the rest of time points (mean of 39 ºC, 39.0-39.1, 95% C.I.) (p < 0.05). Coughing was observed in 3 out of 11 (27 %) infected goats at six wpi; the majority of goats (9/11) showed coughing at the end of experiment (14 wpi). One goat also showed tachypnea.

Immunological response

The immunological response to infection with *M. caprae* was characterized using both cell-mediated and humoral immunological tests. Goats were subjected to the SICCT test
at 12 wpi. The mean increase of skin-fold thickness 72h after PPD-B and PPD-A application were 21.3 mm (19.4-23.2, 95% C.I.) and 10.9 mm (9-12.7, 95% C.I.) respectively. All infected goats were positive to PPD-B according to the official interpretation criterion described above.

Cell-mediated immunity (CMI) was measured throughout the course of the experiment by the release of IFN-γ from whole blood stimulated with PPD-B, PPD-A, ESAT-6/CFP-10 or Rv3615c (see Fig. 1). According to the standard interpretation of the Bovigam assay (considering the PPD-B stimulated well), all infected goats were negative from the day of infection to the 2nd wpi, but all goats became positive at 4 wpi (individual data not shown) and remained positive throughout the experiment with the exception of two goats which were negative at 10 wpi (and turned to be again positive at 12 and 14 wpi). Peak mean value of PPD-B stimulation was reached at 8 wpi, and from that point onwards, a progressive decrease on the mean PPD-B absorbance was observed until the end of the experiment (Fig. 1A). Uninfected goats remained negative through the experiment (individual data not shown). Production of IFN-γ in blood cultures in response to stimulation with PPD-A was also first detectable at 4 wpi and it was maintained until 14 wpi, however the mean ΔOD₄₅₀ was significantly lower to that observed in cultures stimulated with PPD-B at 4 wpi (p < 0.005) (Fig. 1A). As expected, no avian reactors (Bovigam readout) were observed at any time point.

The release of IFN-γ to both ESAT-6/CFP-10 and Rv3615c peptide cocktails followed similar kinetics but was also significantly weaker than to PPD-B at 10 wpi and 14 wpi (p < 0.05) (Fig. 1B). If the standard cut-off point for positivity of the Bovigam assay is used for these antigens (OD₄₅₀ of stimulated sample – OD₄₅₀ of unstimulated control > 0.05), all goats reacted positive from the 4 wpi onward, with the exception of the same two goats which were negative for PPD-B at 10 wpi, that resulted also negative at the
same time point using both peptide cocktails. Moreover, one of these goats was also negative at 14 wpi and, in addition, another goat was negative at 14 wpi using Rv3615c (individual data not shown). Therefore, considering the three time points analyzed between 6-14 wpi 29/33 samples from infected goats were positive for the two peptide cocktails (sensitivity of 88 %), whereas 31/33 samples were positive for PPD-B at the same time points (sensitivity of 94 %). The two uninfected goats remained negative to all IFN-γ tests during the trial (data not shown).

To analyze the IgG response to infection with M. caprae, plasma samples of all goats were tested by ELISA every two weeks after infection, in plates coated with PPD-B or the mycobacterial antigen MPB83. All goats were seronegative to both antigens before M. caprae infection, and uninfected goats remained seronegative through the experiment. After infection, goats remained seronegative to PPD-B at all time points before the tuberculin-boost at 12 wpi, but all seroconverted at 14 wpi (at 13 days after SICCT test) (individual data not shown). By contrast, seropositivity to MBPB83 after infection appeared earlier in some animals, but it was weak and inconstant, with a total of 7/11 goats positive at 8, 10 or 12 wpi (Table 1). All infected goats showed strong responses to MBPB83 after SICCT test, indeed pronounced differences of mean ΔOD450 were found between both antigens used, with much higher IgG responses to MBPB83 than to PPD-B (p < 0.005) (Fig. 2).

Pathology

The pathological findings were mainly restricted to thoracic cavity. All the infected goats showed granulomatous caseous-necrotizing lesions in the lungs and in lung-associated lymph nodes. With few exceptions, the majority of lung lesions were located at right lobes, being the right diaphragmatic lobe affected in most goats. Seven out of eleven goats showed well-developed cavitary lesions. MDCT scan technology allowed a
3-D representation of the lungs, and a cross-sectional visualization and analysis of lesions (Fig. 3). The comparison of the volume of granulomatous-necrotizing lesions in the lungs measured by MDCT and by image analysis of photographs of lung sections (direct observation) is shown in Table 2. By MDCT, volume of TB lesions for each goat ranged from 18.8 to 182 cm$^3$, with a mean of 60 cm$^3$. The MDCT allowed calculation of percentage of lung volume occupied by TB lesions (Table 2), which ranged from 1.1 to 14.3% (mean value of 5.3%). The extension of lesions in the lung was also measured by recording number of affected lung lobes in each infected goat (visual inspection), and these values are also shown in Table 2.

Significant positive correlation was observed between volume values obtained by MDCT and by direct observation (Spearman rho = 0.955, $p < 0.001$) (Fig. 4) although volume values were higher for MDCT data (see Table 2).

Pulmonary LN involvement was also extensive. All infected animals presented gross lesions in caudal mediastinal LN, whereas 10/11 and 8/11 goats presented lesions in tracheobronchial and cranial mediastinal LN respectively. Also, two animals showed lesions in retropharyngeal LN. TB lesions in mesenteric LN were also recorded in other two animals, one of them also showing TB lesions in spleen. In total, 4/11 goats showed extrapulmonary TB-lesions. The volumes of gross lesions in LN as well as the number of affected LN in each goat are shown in Table 3.

**Bacteriology**

*M. caprae* was isolated from post-mortem tissue samples from all inoculated animals, but it was not detected by PCR nor mycobacterial isolation from any of the nasal swabs or blood samples taken during the experiment.

Mycobacteria were recovered from caudal mediastinal LN in all goats, from tracheobronchial LN of 10/11 goats, and from cranial mediastinal LN of 7/11 goats. In
contrast, mycobacteria were isolated from retropharyngeal LN only in two goats. *M. caprae* was also isolated in all TB lesions observed in extra-respiratory organs. The bacterial load of *M. caprae* per gram of cultured pulmonary LN tissues and the total bacterial load of each LN are also shown in Table 3.

The mean bacterial load (log$_{10}$ cfu/g) in the cultured pulmonary LN was 3.5 log$_{10}$ cfu/g (3.3-3.8, 95% C.I.), with a range between animals of 2.8 to 4.1 log$_{10}$ cfu/g. Some differences were also found among bacterial load of each pulmonary LN. In caudal mediastinal LN bacterial load was of 3.3 log$_{10}$ cfu/g (3.3-3.6, 95% C.I.), significantly higher than in cranial mediastinal LN (2 log$_{10}$ cfu/g, 1-2.9, 95% C.I.) ($p < 0.05$) and in tracheobronchial LN (2.4 log$_{10}$ cfu/g, 1.9-2.9, 95% C.I.) ($p < 0.01$). When the total bacterial count of respiritory LN was considered, this value ranged from 3.8 to 5.3 log$_{10}$ cfu, with a mean value of 4.6 (4.3-4.8, 95% C.I.). Also, whole bacterial load was higher for the caudal mediastinal LN than for other LN (Table 3).

**Cross-sectional analysis**

Association between cellular and humoral immune responses, pathology and bacteriology were evaluated transversally combining data obtained from all experimental goats (n = 13). In LN, a positive correlation was found between pathology (volume of lesions as log$_{10}$ mm$^3$) and bacterial load (log$_{10}$ cfu/g) (Pearson r = 0.858, $p < 0.001$). Positive correlations were also found between bacterial load in LN and IFN-γ specific responses to PPD-B (Pearson r = 0.528, $p = 0.032$) and to ESAT-6/CFP-10 (Pearson r = 0.579, $p = 0.019$), but not to Rv3615c (Spearman rho = 0.296, $p = 0.163$), at 14 wpi. However, only IFN-γ responses to PPD-B at 14 wpi were correlated significantly with volume of gross lesions in lungs determined by MDCT (Pearson r = 0.540, $p = 0.028$).
Humoral immune responses to MPB83 at 14 wpi correlated positively with both bacterial load in LN (Pearson \( r = 0.775, p = 0.001 \)) and volume of gross lesions in lungs determined by MDCT (Pearson \( r = 0.685, p = 0.007 \)), whereas IgG responses to PPD-B did not correlate significantly with bacterial load (Spearman rho = 0.322, \( p = 0.141 \)) and were slightly positively correlated with volume of gross lesions in lungs (Spearman rho = 0.481, \( p = 0.048 \)).

**DISCUSSION**

Recent interest on development of TB vaccines in domestic ruminants and wildlife, as badgers and wild boar, has driven research to standardize infection models in domestic animals like ruminants (7) and pigs (18). Modelling TB in goats may be of great value to increase our knowledge of infection in this species, and at the same time the model can be used for research of TB in cattle. With these aims we have established an efficient experimental goat model of TB, with slight clinical signs (coughing at the end of the experiment) and a relatively fast progression of lesions, very similar to natural disease. Gross TB lesions were reproduced in all the infected goats, which is an advantage over the previous existing model in adult goats (3). It is well known from experiments in calves and other models that the route and dose of challenge can be very relevant for the pathological outcome of infection (see (29) for a review). A high challenge dose (higher than \( 10^6 \) cfu), by non-natural routes (as intravenous or subcutaneous) may lead to systemic dissemination of infection with lesions that are not representative of natural field cases (40). Using a relative low challenge dose of \( 1.5 \times 10^3 \) cfu by the endobronchial route we have been able to reproduce typical granulomatous caseous-necrotizing lesions in lung and lung-associated LN in 11 out of 11 experimentally infected goats, resembling those observed in naturally-infected goats.
liquefactive necrosis and cavernous lung lesions, which is a feature of tuberculosis in humans. In a previous study in goats experimentally infected with *M. caprae* (3), adult goats were infected transthoracically with $2-3 \times 10^3$ cfu, achieving infection in all 6 infected goats (as demonstrated by mycobacterial culture), but with absence of macroscopic lesions in lung parenchyma in two goats, in spite of a much longer duration of the infection (nine months). This difference could be due to the use of 6-month-old goats in our study compared to adult animals, and is clearly an advantage over a model with adult goats. Extension of the infection with production of gross lesions in extrapulmonary sites is often included in scoring systems to assess vaccine efficacy, and therefore, an inoculation route that conveys the challenge dose to a circumscribed area, mimicking natural infection, should be preferred to models that disperse mycobacteria into different systems or mucosal surfaces. In this respect, transthoracic inoculation drops inoculated mycobacteria directly in the lung parenchyma, but may cause also pleuritis (according to our own personal observation), and local infection of the thoracic wall at inoculation point, with mycobacteria draining to regional LN like the axillary nodes, thus complicating the assessment of extrapulmonary dissemination. In our study four animals had extension of the infection from thoracic primary focus to extrapulmonary tissues, like medial retropharyngeal or mesenteric lymph nodes, and to the spleen (one case, indicative of systemic circulation of mycobacteria). Probably, pulmonary lesions allow the dissemination to upper respiratory/head and mesenteric lymph nodes, by mycobacterial shedding in tracheobronchial secretion and its subsequent ingestion. Similarly to results in calves inoculated with a dose of $10^4$ cfu of *M. bovis* (26) nasal shedding of *M. caprae* was not detected in our study, showing that, if nasal shedding occurs, it should happen at a very
low load, or intermittently. As expected, blood culture was also negative through the whole experiment in all goats, indicating that bacteremia is not a feature of TB in goats, at least in the early phase of the infection.

The data obtained here strengthen the hypothesis that young goats seem to be highly susceptible to infection by *M. caprae*. Pathological and bacteriological findings point to a fast progression of lesions, which reached relatively large size in some animals (more than 5% of lung affected). In natural cases of TB, such large lesions with liquefactive necrosis and caverna formation are usually associated to a long period of lesion progression in herds not subjected to eradication (12, 14, 31). In contrast, in trials carried out in other species like badgers (10) and calves (39) using the endobronchial route of infection, lesions progressed slowly, resembling what is observed in naturally cases of TB in these species. Particularly, in the low challenge dose experiments in calves, big coalescent lesions were not usually found, in contrast our model appears to be faster in the progression of lesions, which can be considered an advantage.

Assessment of vaccine efficacy in experimental trials by non-immunological parameters has used semiquantitative scoring systems based in number of pulmonary lobes affected and size of lesions in lung and pulmonary LN, as well as bacterial load in LN in cattle (37), in rabbits (20), and in macaques (23). A drawback of these scoring systems is that the intrapulmonary extension of lesions to one or more lobes may be strongly influenced by the inoculation procedure, and consequently, this may also influence the extension to lymph nodes (which depends of the drainage of the lobes affected). A clear evidence of this is in our study the direction of the inoculum to the right lung by inoculation of the goats in the right decubitus position. To avoid this drawback and increase the usefulness of the pathological assessment, in our study we have attempted
to express severity of lung lesions in a quantitative way, to allow better comparisons
between treatment groups and different experiments. The use of high-resolution 64-slice
Multi-Detector Computed Tomography (MDCT) can resolve the whole burden of lung
lesions to a volume, and the ratio of affected lung can be calculated. Additionally we
made an effort to compare results obtained with MDCT with a conventional visual
inspection of sliced lung, photography and calculation of area of lesions in each picture
by image analysis. If sections of lung are similar in thickness, an approximate volume
of lesions can be obtained by adding volumes of lesions in each slice. This is a time-
consuming task, although it also provides with an approximate total volume of lesion
per lung. We have shown that results of visual inspection had a strong correlation with
those obtained by MDCT, although in general were lower. Interestingly, MDCT seems
to have the capacity to detect small changes in density patterns due to inflammatory
reactions around the granuloma that may be not visible by direct macroscopic
observation. This, together with error introduced by the use for calculation of the same
thickness for all lung slices, could explain the slightly higher but homogenous animal-
to-animal volume values obtained by using MDCT in respect to direct observation
measures. Therefore, the MDCT can be a far more precise method, in comparison to the
usually applied pathology scoring systems, to assess the severity of lesions or their
reduction in future vaccine efficacy assays. A similar approach to the measurement of
lung lesions was followed recently by Sharpe et al. in macaques (34). These authors
measured the total volume of lung lesion in relation to the whole lung volume after
fixation by immersion in formalin by using magnetic resonance imaging (MRI)
stereology. They concluded that the ratio of lung lesion to whole volume was superior
to thoracic radiography or pathology scores for measuring disease burden. Also, in their
aerosol model of infection, the total volume of lesions accurately reflected differences in challenge dose in different groups.

Methodologically, irrespective of whether MRI or MDCT technologies are used, it is worth stressing the importance of insufflation of lung with formalin to distend the lung to approximately the same volume as they would have in the pulmonary cavity. This renders the ratio of lesion volume to total lung volume comparable between different experiments and research groups. The use of this ratio corrects also for slight differences that could exist in size of the animals and of the lungs, even in age matched animals. We believe that this very precise quantitative data set offers the possibility of a better assessment of vaccine efficacy in TB studies. The same conclusion has been drawn by Sharpe et al. (34), who stressed the benefits of MRI stereology as an accurate and quantifiable assessment, easy to standardize and comparable between laboratories, suggesting that it will be an essential component of pathology assessment in vaccine efficacy studies.

Our experimental model may be useful for assessing the performance of diagnosing techniques in caprine TB. The infection was detected satisfactorily at 12 wpi with SICCT test, the official ante-mortem diagnostic tests currently used for bovine TB eradication campaigns, and all infected goats were also positive to the standard IFN-γ assay from 4 wpi, confirming the usefulness of these techniques also for diagnose of caprine TB as described previously by others (19, 22).

Intriguingly, the kinetics of cell-mediated immune responses to infection with M. caprae, measured as anamnestic IFN-γ secretion, was slightly different to described previously in the calf model. In goats, for all antigens used the levels of specific IFN-γ were unappreciable until 4 wpi while experimental infections in cattle with similar
mycobacterial dose usually showed a significant specific IFN-γ response at 2 wpi, especially in samples stimulated with PPD-B (6, 37, 39). Nevertheless, the appearance of detectable levels of IFN-γ a week later has been also reported in cattle infected with a low dose of *M. bovis* (13). Unexpectedly, a decrease of IFN-γ responses seemed to occur in infected goats at 10 wpi, whereas in a long-term cattle infection these responses were maintained in their intensity for at least 20 weeks (6). This phenomenon, if confirmed in further long-term studies, could correlate to the fast progression of infection in our goat model as deduced from the extent of lesions observed, coincident with a decline of activity of effector IFN-γ producing cells.

Peptide cocktails ESAT-6/CFP-10 and Rv3615c are being considered as new DIVA reagents for use in cattle (36, 38). The usefulness of ESAT-6/CFP-10 has been successfully demonstrated in the field, showing high sensitivity and specificity in comparison to tuberculins in cattle that have been naturally infected with *M. bovis* (8) and more recently in goats infected naturally with *M. caprae* (2). Interestingly, the sensitivity obtained in our study for the two peptide cocktails (88 %) would increase to 91 % if combining the results obtained for the two cocktails, the same theoretic sensitivity that was reported previously for cattle infected with *M. bovis* (36). Moreover, the IFN-γ response to ESAT-6/CFP-10 but not to Rv3615c correlates positively with the bacterial burden in LN, although an even higher correlation has been described previously for IFN-γ responses to ESAT-6 and bacterial burden in *M. bovis*-infected cattle (24). These findings are consistent with the concept that bacterial load in infected tissues is proportional to host IFN-γ responses against antigens secreted by active growing mycobacteria such ESAT-6 and CFP-10 (27), but these responses get lower at the end of the experiment, so the correlation should be considered at each stage of the disease. The capacity of ESAT-6/CFP-10 to predict the disease status, the increment of...
sensitivity when the two peptide cocktails are used and their DIVA capability in animals vaccinated with *M. bovis* bacillus Calmette-Guérin (BCG), could make them a useful tool for vaccine trials to distinguish vaccinated-protected and infected animals.

Serology is another important tool for assessing infection or exposure to mycobacteria and could be another useful biomarker to determine disease status; although it is not yet clear whether antibody responses play a role in controlling TB. In recent years serological tests have been assessed in trials in cattle and wild mammals, and most of them have concluded that MPB70, and especially MPB83 are serodominant, being recognised in early stages of infection (24, 25, 41). The serodominance of MPB83 described in other species is also consistent with our findings, as most of the goats (10/11) were seropositive at least at one time point before the boost effect of the SICCT test (12 wpi). Moreover, two weeks after boosting with PPDs, all animals reacted strongly increasing dramatically the sensitivity of the ELISA as has been shown in cattle (39). In contrast, the IgG-ELISA with PPD-B as antigen failed to detect any positive animals before the boost effect of the SICCT test, after which, antibody responses were positive, although very weak in comparison to MPB83 IgG-ELISA. This result suggests that serology to MPB83 could be a useful tool to detect infected animals in farms, as well as to monitor the progression of the infection in experimental trials.

Summarizing, our goat TB infection model may be useful in TB research for the understanding of pathogenesis of TB in goats and for testing of therapeutic and immunoprophylactic treatments and of new diagnostic tools. The use of MDCT for quantification of volume of lesions and their ratio to the whole lung volume may serve for a quantitative evaluation of pathology in vaccination trials. Research in human TB
can also benefit from large animal models different from non-human primates, due to the similarities with the human disease, and its lower cost (39, 42).

Also, reports of caprine TB have increased in recent years, and studies are needed to validate whether control measures used in cattle can be applied to goat herds. Vaccination based on BCG has been developed for use in wild species that act as reservoirs of *M. bovis* and could represent a control tool for caprine TB and to limit its transmission to cattle and humans.

**COMPETING INTERESTS**

The authors declare that they have no competing interests.

**AUTHORS CONTRIBUTIONS**

BPV and MD conceived and designed the experiments, analyzed the data and drafted the manuscript. Conduction of experiments: MD, SL-S, MN and DS performed the necropsy and pathological records; BPV and MM performed the immunological and bacteriological assays; DS performed the skin tests; NR and ME performed the MDCT and analyzed resulting data. MHV, BVR and P-JC contributed substantively in scientific discussion of the results. All authors have read and approved the final manuscript.

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We express our appreciation to the staff of Level 3 Biocontainment Unit of CReSA for their technical assistance.

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REFERENCES


**FIGURES**

**Figure 1**: Kinetics of IFN-γ responses in infected goats. The release of IFN-γ was measured by ELISA after in vitro stimulation of whole blood with different antigens. Results are expressed as mean ΔOD_{450} responses with 95 % CI. Dashed horizontal line, cut-off point for positivity. (A) PPD-B (closed circles) and PPD-A (open circles). (**) p < 0.005, significant differences determined by non-parametric Mann-Whitney-Wilcoxon test. (B) PPD-B (closed circles), ESAT-6/CFP-10 (open circles) and Rv3615c (open squares). (*) p < 0.05, significant differences determined by non-parametric Friedman test with post-hoc Mann-Whitney-Wilcoxon test.

**Figure 2**: Humoral responses to MBP83 and PPD-B at 14 weeks post-infection. OD_{450} absorbance of total IgG to MPB83 (circles) and PPD-B (triangles) from individual goats infected or not with *M. caprae*. Results are expressed as ΔOD_{450} (OD_{450} of antigen-stimulated wells minus OD_{450} of non-stimulated wells). Filled symbols, infected animals; open symbols, non-infected control animals; dashed horizontal line, cut-off point for positivity.

**Figure 3**: Gross pathology analysis of a goat case. (A) MDTC-3D representation of the whole lung after excluding air and TAC’s table (H, head; F, foot, L, left, R, right). The total volume of lung is calculated in cm³ and is showed in red-dashed box at the bottom. (B) Volume rendering image of the lung showing different tissue densities discriminated by colour: water in grey, air in black and calcium in white. The volume of affected lung is also showed. (C) Pathological areas identified by segmentation in axial (see at the top; A, anterior; P, posterior), coronal and sagittal planes (see at the bottom). (D) Formalin fixed, 5 mm-sections of left diaphragmatic lobe which showing a large...
cavitary lesion. Cranial to caudal sections are represented as bottom-up and left-right in
the picture. Bar = 3 cm.

**Figure 4:** Correlation between volumes of lesions in lungs measured by two
quantitative methods. Visible lesions in lungs calculated by Multi-Detector Computed
Tomography (MDCT) and by image analysis of photographs of lung sections (direct
observation) in infected goats (n = 11). Statistical analysis conducted with non-parametric Spearman rank test.
Table 1: Detection of antibodies to MPB83 in plasma of goats infected or not with *M. caprae*. ELISA results at different time points post-infection (Week 0 represents day of infection) are expressed according to the cut-off described above as: +, positive or -, negative. Goats were subjected to SICCT test 13 days before the blood sampling at 14 wpi.

<table>
<thead>
<tr>
<th>Group</th>
<th>Goat</th>
<th>Weeks post-infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>572</td>
<td>0 2 4 6 8 10 12 14</td>
</tr>
<tr>
<td>Infected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>605</td>
<td>-</td>
<td>- - - - - - + + +</td>
</tr>
<tr>
<td>571</td>
<td>-</td>
<td>- - - - - - - - +</td>
</tr>
<tr>
<td>567</td>
<td>-</td>
<td>- - - - - - - - +</td>
</tr>
<tr>
<td>607</td>
<td>-</td>
<td>- - - - - - - - +</td>
</tr>
<tr>
<td>Non-infected</td>
<td>162</td>
<td></td>
</tr>
<tr>
<td>187</td>
<td>-</td>
<td>- - - - - - - - -</td>
</tr>
</tbody>
</table>

- 36 -
Table 2: Quantification of gross lesions in lungs in goats infected with *M. caprae*. Total volume of lesions and percentage of affected lungs were calculated using 64-slice Multi-Detector Computed Tomography (MDCT) and were compared to direct visual quantification by slicing, photography, and image analysis.

<table>
<thead>
<tr>
<th>Goat</th>
<th>64-MDCT</th>
<th>Direct visual observation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Volume of lesions in lung (cm³)</td>
<td>Volume ratio lesion/lung (%)</td>
</tr>
<tr>
<td>572</td>
<td>26.8</td>
<td>1.8</td>
</tr>
<tr>
<td>605</td>
<td>97.1</td>
<td>8.3</td>
</tr>
<tr>
<td>571</td>
<td>35.5</td>
<td>3.8</td>
</tr>
<tr>
<td>567</td>
<td>100</td>
<td>10.6</td>
</tr>
<tr>
<td>607</td>
<td>59.3</td>
<td>5</td>
</tr>
<tr>
<td>563</td>
<td>22.3</td>
<td>1.9</td>
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<tr>
<td>597</td>
<td>24.1</td>
<td>2.5</td>
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<tr>
<td>568</td>
<td>66.3</td>
<td>6.5</td>
</tr>
<tr>
<td>562</td>
<td>182</td>
<td>14.3</td>
</tr>
<tr>
<td>565</td>
<td>31</td>
<td>1.1</td>
</tr>
<tr>
<td>577</td>
<td>18.8</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Mean (95% C.I.) 60.3 (30.8-89.8) 5.3 (2.8-7.8) 34.7 (11.4-58.1) 3.4/7 (2.3-4.4/7)
Table 3: Pathological findings and bacterial load in pulmonary lymph nodes (LN) of infected goats.

<table>
<thead>
<tr>
<th>Goat</th>
<th>Bacterial load (Log$_{10}$ cfu)</th>
<th>Volume of lesions (cm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>crm LN</td>
<td>tb LN</td>
</tr>
<tr>
<td>572</td>
<td>3.2</td>
<td>3.8</td>
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<td>4.3</td>
</tr>
<tr>
<td>577</td>
<td>3.3</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Mean (95% C.I.)

crm: cranial mediastinal
tb: tracheobronchial
cdm: caudal mediastinal

*a* bacterial count per gram of tissue

*b* bacterial count in the whole lymph node
(A) 

![Graph A]

(B) 

![Graph B]