CVI Accepts, published online ahead of print on 31 August 2011
Clin. Vaccine Immunol. doi:10.1128/CVI.05323-11

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

Bernat Pérez de Val[1,*], Sergio López-Soria[1], Miquel Nofrarías[1], Maite Martín[1], Martin H. Vordermeier[2], Bernardo Villarreal-Ramos[2], Nadine Romera[3], Manel Escobar[3], David Solanes[1], Pere-Joan Cardona[4] and Mariano Domingo[1,5]

[1]Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra, Catalonia, Spain

[2]TB Research Group, Veterinary Laboratories Agency (VLA)-Weybridge, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom

[3]Centro Médico Teknon, Radiology Service, 08022 Barcelona, Catalonia, Spain.

[4]Unitat de Tuberculosi Experimental, Fundació Institut per a la Investigació en Ciències de la Salut Germans Trias i Pujol, Universitat Autònoma de Barcelona, 08916 Badalona, Catalonia, Spain

[5]Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Catalonia, Spain

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

Institution at which the work was performed:

Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra, Catalonia, Spain.

Formatted: Numbering: Continuous

***Corresponding author**:

Bernat Pérez de Val

Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra, Catalonia, Spain.

Tel.: +34 93 5813284

Fax: +34 93 5814490

E-mail: bernat.perez@cresa.uab.cat

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 x 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*.

128

129 ***Mycobacterium caprae*** **cultures and experimental infection**

130 The *M. caprae* SB0416 (www.mbovis.org) field strain used as inoculum was originally
131 isolated from a tuberculous goat from Catalonia. The isolate was subcultured in
132 Middlebrook 7H11 solid media (BD Diagnostics, Spark, USA) and bacteria were
133 resuspended in Brain Heart Infusion broth with 20% of glycerol at a concentration of 2
134 x 10^6 colony forming units (cfu)/ml (calculated by plating dilutions on Middlebrook
135 7H11 media). The suspension was stored at -80 ºC in 0.5 ml aliquots. The inoculum was
136 prepared to the required final concentration by diluting the suspension with sterile
137 phosphate buffered saline (PBS).

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

151 **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 ºC for mycobacteria inactivation and stored at -80 ºC 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% CO_2 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 OD_{450}. Specific reaction was expressed as ΔOD_{450} (OD_{450} of antigen-stimulated sample minus OD_{450} of non-stimulated control). A sample was classified as positive when the PPD-B ΔOD_{450} was higher than 0.05 and OD_{450} of PPD-B was higher than OD_{450} 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 ΔOD_{450} was calculated as sample OD_{450} minus background OD_{450} (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 ºC 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'-TCAGCCGCGTCCACGCCGCCA-3') (28) adding another reverse primer for the second reaction (IS-R2: 5'-CTCGTCCAGCGCCGCTTCGG-3') (16). These primers are specific for the MTC-IS*6110* 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 (log_{10} cfu/g) between pulmonary LN, as well as comparison of IgG or IFN-γ ELISA absorbance values (ΔOD_{450}) between antigens within the infected group, were analyzed by non-parametric Friedman test with the post-hoc Mann-Withney-Wilcoxon test. Correlation between MDCT and direct visual measure of macroscopic lesions was performed by non-parametric Spearman rank test. Immune responses and log_{10}-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

294 at 12 wpi. The mean increase of skin-fold thickness 72h after PPD-B and PPD-A
295 application were 21.3 mm (19.4-23.2, 95% C.I.) and 10.9 mm (9-12.7, 95% C.I)
296 respectively. All infected goats were positive to PPD-B according to the official
297 interpretation criterion described above.
298 Cell-mediated immunity (CMI) was measured throughout the course of the experiment
299 by the release of IFN-γ from whole blood stimulated with PPD-B, PPD-A, ESAT-
300 6/CFP-10 or Rv3615c (see Fig. 1). According to the standard interpretation of the
301 Bovigam assay (considering the PPD-B stimulated well), all infected goats were
302 negative from the day of infection to the 2nd wpi, but all goats became positive at 4 wpi
303 (individual data not shown) and remained positive throughout the experiment with the
304 exception of two goats which were negative at 10 wpi (and turned to be again positive
305 at 12 and 14 wpi). Peak mean value of PPD-B stimulation was reached at 8 wpi, and
306 from that point onwards, a progressive decrease on the mean PPD-B absorbance was
307 observed until the end of the experiment (Fig. 1A). Uninfected goats remained negative
308 through the experiment (individual data not shown). Production of IFN-γ in blood
309 cultures in response to stimulation with PPD-A was also first detectable at 4 wpi and it
310 was maintained until 14 wpi, however the mean ΔOD_{450} was significantly lower to that
311 observed in cultures stimulated with PPD-B at 4 wpi ($p < 0.005$) (Fig. 1A). As expected,
312 no avian reactors (Bovigam readout) were observed at any time point.
313 The release of IFN-γ to both ESAT-6/CFP-10 and Rv3615c peptide cocktails followed
314 similar kinetics but was also significantly weaker than to PPD-B at 10 wpi and 14 wpi
315 ($p < 0.05$) (Fig. 1B). If the standard cut-off point for positivity of the Bovigam assay is
316 used for these antigens (OD_{450} of stimulated sample – OD_{450} of unstimulated control >
317 0.05), all goats reacted positive from the 4 wpi onward, with the exception of the same
318 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 ΔOD_{450} 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 areshown 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 mediastinial LN in all goats, from tracheobronchial LN of 10/11 goats, and from cranial mediastinial 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 (3-3.6, 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 respiratory 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, $p = 0.028$).

393 Humoral immune responses to MPB83 at 14 wpi correlated positively with both
394 bacterial load in LN (Pearson r = 0.775, $p = 0.001$) and volume of gross lesions in lungs
395 determined by MDCT (Pearson r = 0.685, $p = 0.007$), whereas IgG responses to PPD-B
396 did not correlate significantly with bacterial load (Spearman rho = 0.322, $p = 0.141$) and
397 were slightly positively correlated with volume of gross lesions in lungs (Spearman rho
398 = 0.481, $p = 0.048$).
399
400 **DISCUSSION**

401 Recent interest on development of TB vaccines in domestic ruminants and wildlife, as
402 badgers and wild boar, has driven research to standardize infection models in domestic
403 animals like ruminants (7) and pigs (18). Modelling TB in goats may be of great value
404 to increase our knowledge of infection in this species, and at the same time the model
405 can be used for research of TB in cattle. With these aims we have established an
406 efficient experimental goat model of TB, with slight clinical signs (coughing at the end
407 of the experiment) and a relatively fast progression of lesions, very similar to natural
408 disease. Gross TB lesions were reproduced in all the infected goats, which is an
409 advantage over the previous existing model in adult goats (3). It is well known from
410 experiments in calves and other models that the route and dose of challenge can be very
411 relevant for the pathological outcome of infection (see (29) for a review). A high
412 challenge dose (higher than 10^6 cfu), by non-natural routes (as intravenous or
413 subcutaneous) may lead to systemic dissemination of infectionwith lesions that are not
414 representative of natural field cases (40). Using a relative low challenge dose of $1.5 \times$
415 10^3 cfu by the endobronchial route we have been able to reproduce typical
416 granulomatous caseous-necrotizing lesions in lung and lung-associated LN in 11 out of
417 11 experimentally infected goats, resembling those observed in naturally-infected goats

418 (12, 31), and as seen sometimes in natural cases, the majority of our goats had
419 liquefactive necrosis and cavernous lung lesions, which is a feature of tuberculosis in
420 humans. In a previous study in goats experimentally infected with *M. caprae* (3), adult
421 goats were infected transthoracically with 2-3 × 10^3 cfu, achieving infection in all 6
422 infected goats (as demonstrated by mycobacterial culture), but with absence of
423 macroscopic lesions in lung parenchyma in two goats, in spite of a much longer
424 duration of the infection (nine months). This difference could be due to the use of 6-
425 month-old goats in our study compared to adult animals, and is clearly an advantage
426 over a model with adult goats. Extension of the infection with production of gross
427 lesions in extrapulmonary sites is often included in scoring systems to assess vaccine
428 efficacy, and therefore, an inoculation route that conveys the challenge dose to a
429 circumscribed area, mimicking natural infection, should be preferred to models that
430 disperse mycobacteria into different systems or mucosal surfaces. In this respect,
431 transthoracic inoculation drops inoculated mycobacteria directly in the lung
432 parenchyma, but may cause also pleuritis (according to our own personal observation),
433 and local infection of the thoracic wall at inoculation point, with mycobacteria draining
434 to regional LN like the axillary nodes, thus complicating the assessment of
435 extrapulmonary dissemination. In our study four animals had extension of the infection
436 from thoracic primary focus to extrapulmonary tissues, like medial retropharyngeal or
437 mesenteric lymph nodes, and to the spleen (one case, indicative of systemic circulation
438 of mycobacteria). Probably, pulmonary lesions allow the dissemination to upper
439 respiratory/head and mesenteric lymph nodes, by mycobacterial shedding in
440 tracheobronchial secretion and its subsequent ingestion. Similarly to results in calves
441 inoculated with a dose of 10^4 cfu of *M. bovis* (26) nasal shedding of *M. caprae* was not
442 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

467 to express severity of lung lesions in a quantitative way, to allow better comparisons
468 between treatment groups and different experiments. The use of high-resolution 64-slice
469 Multi-Detector Computed Tomography (MDCT) can resolve the whole burden of lung
470 lesions to a volume, and the ratio of affected lung can be calculated. Additionally we
471 made an effort to compare results obtained with MDCT with a conventional visual
472 inspection of sliced lung, photography and calculation of area of lesions in each picture
473 by image analysis. If sections of lung are similar in thickness, an approximate volume
474 of lesions can be obtained by adding volumes of lesions in each slice. This is a time-
475 consuming task, although it also provides with an approximate total volume of lesion
476 per lung. We have shown that results of visual inspection had a strong correlation with
477 those obtained by MDCT, although in general were lower. Interestingly, MDCT seems
478 to have the capacity to detect small changes in density patterns due to inflammatory
479 reactions around the granuloma that may be not visible by direct macroscopic
480 observation. This, together with error introduced by the use for calculation of the same
481 thickness for all lung slices, could explain the slightly higher but homogenous animal-
482 to-animal volume values obtained by using MDCT in respect to direct observation
483 measures. Therefore, the MDCT can be a far more precise method, in comparison to the
484 usually applied pathology scoring systems, to assess the severity of lesions or their
485 reduction in future vaccine efficacy assays. A similar approach to the measurement of
486 lung lesions was followed recently by Sharpe et al. in macaques (34). These authors
487 measured the total volume of lung lesion in relation to the whole lung volume after
488 fixation by immersion in formalin by using magnetic resonance imaging (MRI)
489 stereology. They concluded that the ratio of lung lesion to whole volume was superior
490 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

515 mycobacterial dose usually showed a significant specific IFN-γ response at 2 wpi,
516 especially in samples stimulated with PPD-B (6, 37, 39). Nevertheless, the appearance
517 of detectable levels of IFN-γ a week later has been also reported in cattle infected with a
518 low dose of *M. bovis* (13). Unexpectedly, a decrease of IFN-γ responses seemed to
519 occur in infected goats at 10 wpi, whereas in a long-term cattle infection these responses
520 were maintained in their intensity for at least 20 weeks (6). This phenomenon, if
521 confirmed in further long-term studies, could correlate to the fast progression of
522 infection in our goat model as deduced from the extent of lesions observed, coincident
523 with a decline of activity of effector IFN-γ producing cells.
524 Peptide cocktails ESAT-6/CFP-10 and Rv3615c are being considered as new DIVA
525 reagents for use in cattle (36, 38). The usefulness of ESAT-6/CFP-10 has been
526 successfully demonstrated in the field, showing high sensitivity and specificity in
527 comparison to tuberculins in cattle that have been naturally infected with *M. bovis* (8)
528 and more recently in goats infected naturally with *M. caprae* (2). Interestingly, the
529 sensitivity obtained in our study for the two peptide cocktails (88 %) would increase to
530 91 % if combining the results obtained for the two cocktails, the same theoretic
531 sensitivity that was reported previously for cattle infected with *M. bovis* (36). Moreover,
532 the IFN-γ response to ESAT-6/CFP-10 but not to Rv3615c correlates positively with the
533 bacterial burden in LN, although an even higher correlation has been described
534 previously for IFN-γ responses to ESAT-6 and bacterial burden in *M. bovis*-infected
535 cattle (24). These findings are consistent with the concept that bacterial load in infected
536 tissues is proportional to host IFN-γ responses against antigens secreted by active
537 growing mycobacteria such ESAT-6 and CFP-10 (27), but these responses get lower at
538 the end of the experiment, so the correlation should be considered at each stage of the
539 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

564 can also benefits from large animal models different from non-human primates, due to
565 the similarities with the human disease, and its lower cost (39, 42).
566 Also, reports of caprine TB have increased in recent years, and studies are needed to
567 validate whether control measures used in cattle can be applied to goat herds.
568 Vaccination based on BCG has been developed for use in wild species that act as
569 reservoirs of *M. bovis* and could represent a control tool for caprine TB and to limit its
570 transmission to cattle and humans.

571

572 **COMPETING INTERESTS**

573 The authors declare that they have no competing interests.

574

575 **AUTHORS CONTRIBUTIONS**

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

583

584 **ACKNOWLEDGMENTS**

585 This study was founded by European Union projects NADIR (FP7-INFRA-2008-1.1.2,
586 no. 228394) and TB-STEP (FP7-KBBE-2007-1.3.04, no. 212414).
587 We would like to thank to Radiology Service of the Centro Médico Teknon for
588 providing its facilities for performing the MDCT scan analysis.

We want to give special thanks to Félix García, Ana Andaluz and Xavier Moll from the Department of Animal Medicine and Surgery of the Universitat Autònoma de Barcelona for their help with the experimental mycobacterial inoculation. We also thank to Xavier Abad and Raúl Núñez for their assistance in *M. caprae* inoculum preparation and in bacterial cultures.

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

MTC peptides were kindly supplied by Veterinary Laboratories Agency.

REFERENCES

1. **Aranaz, A., D. Cousins, A. Mateos, and L. Dominguez.** 2003. Elevation of *Mycobacterium tuberculosis* subsp. *caprae* Aranaz et al. 1999 to species rank as *Mycobacterium caprae* comb. nov., sp. nov. Int. J. Syst. Evol. Microbiol. **53:**1785-1789.

2. **Bezos, J., J. Álvarez, L. de Juan, B. Romero, S. Rodríguez, I. G. Fernández-de-Mera, R. G. Hewinson, M. Vordermeier, A. Mateos, L. Domínguez, and A. Aranaz.** 2011. Assessment of in vivo and in vitro tuberculosis diagnostic tests in *Mycobacterium caprae* naturally infected caprine flocks. Prev. Vet. Med. **In Press, Corrected Proof:**.

3. **Bezos, J., L. de Juan, B. Romero, J. Álvarez, F. Mazzucchelli, A. Mateos, L. Domínguez, and A. Aranaz.** 2010. Experimental infection with *Mycobacterium caprae* in goats and evaluation of immunological status in tuberculosis and paratuberculosis co-infected animals. Vet. Immunol. Immunopathol. **133:**275.

611 4. **Buddle, B. M., F. E. Aldwell, A. Pfeffer, and G. W. de Lisle.** 1994. Experimental
612 *Mycobacterium bovis* infection in the brushtail possum (*Trichosurus vulpecula*):
613 pathology, haematology and lymphocyte stimulation responses. Vet. Microbiol. **38:**241-
614 254.

615 5. **Buddle, B. M., D. N. Wedlock, and M. Denis.** 2006. Progress in the development of
616 tuberculosis vaccines for cattle and wildlife. Vet. Microbiol. **112:**191-200.

617 6. **Buddle, B. M., D. N. Wedlock, N. A. Parlane, L. A. L. Corner, G. W. de Lisle,**
618 **and M. A. Skinner.** 2003. Revaccination of neonatal calves with *Mycobacterium bovis*
619 BCG reduces the level of protection against bovine tuberculosis induced by a single
620 vaccination. Infect. Immun. **71:**6411-6419.

621 7. **Buddle, B., M. Skinner, D. Neil Wedlock, G. de Lisle, H. Martin Vordermeier,**
622 **and R. Glyn Hewinson.** 2005. Cattle as a model for development of vaccines against
623 human tuberculosis. Tuberculosis (Edinb). **85:**19-24.

624 8. **Cockle, P. J., S. V. Gordon, R. G. Hewinson, and H. M. Vordermeier.** 2006. Field
625 evaluation of a novel differential diagnostic reagent for detection of *Mycobacterium*
626 *bovis* in cattle. Clin. Vaccine Immunol. **13:**1119-1124.

627 9. **Corner, L. A., and A. C. Trajstman.** 1988. An evaluation of 1-hexadecylpyridinium
628 chloride as a decontaminant in the primary isolation of *Mycobacterium bovis* from
629 bovine lesions. Vet. Microbiol. **18:**127-134.

630 10. **Corner, L. A. L., E. Costello, S. Lesellier, D. O'Meara, D. P. Sleeman, and E.**
631 **Gormley.** 2007. Experimental tuberculosis in the European badger (*Meles meles*) after

632 endobronchial inoculation of *Mycobacterium bovis*: I. Pathology and bacteriology. Res.
633 Vet. Sci. **83:**53-62.

634 11. **Cvetnic, Z., V. Katalinic-Jankovic, B. Sostaric, V. Katalinic-Jankovic, M.**
635 **Obrovac, S. Marjanovic, M. Benic, B. K. Kirin, and V. Katalinic-Jankovic.** 2007.
636 *Mycobacterium caprae* in cattle and humans in Croatia. Int. J. Tuberc. Lung Dis.
637 **11:**658.

638 12. **Daniel, R., H. Evans, S. Rolfe, R. de la Rua-Domenech, T. Crawshaw, R. J.**
639 **Higgins, A. Schock, and R. Clifton-Hadley.** 2009. Outbreak of tuberculosis caused by
640 *Mycobacterium bovis* in golden Guernsey goats in Great Britain. Vet. Rec. **165:**335-
641 342.

642 13. **Dean, G. S., S. G. Rhodes, M. Coad, A. O. Whelan, P. J. Cockle, D. J. Clifford,**
643 **R. G. Hewinson, and H. M. Vordermeier.** 2005. Minimum infective dose of
644 *Mycobacterium bovis* in cattle. Infect. Immun. **73:**6467-6471.

645 14. **Domingo, M., O. Gil, E. Serrano, E. Guirado, M. Nofrarias, M. Grassa, N.**
646 **Cáceres, B. Pérez, C. Vilaplana, and P. J. Cardona.** 2009. Effectiveness and safety of
647 a treatment regimen based on isoniazid plus vaccination with *Mycobacterium*
648 *tuberculosis* cells' fragments: field-study with naturally *Mycobacterium caprae*-infected
649 goats. Scand. J. Immunol. **69:**500-507.

650 15. **Duarte, E. L., M. Domingos, A. Amado, and A. Botelho.** 2008. Spoligotype
651 diversity of *Mycobacterium bovis* and *Mycobacterium caprae* animal isolates. Vet.
652 Microbiol. **130:**415-421.

653 16. **Eisenach, K. D., M. Donald Cave, J. H. Bates, and J. T. Crawford.** 1990.
654 Polymerase chain reaction amplification of a repetitive DNA sequence specific for
655 *Mycobacterium tuberculosis*. J. Infect. Dis. **161:**977-981.

656 17. **Erler, W., G. Martin, K. Sachse, L. Naumann, D. Kahlau, J. Beer, M. Bartos,**
657 **G. Nagy, Z. Cvetnic, M. Zolnir-Dovc, and I. Pavlik.** 2004. Molecular fingerprinting
658 of *Mycobacterium bovis* subsp. *caprae* isolates from Central Europe. J. Clin. Microbiol.
659 **42:**2234-2238.

660 18. **Gil, O., I. Díaz, C. Vilaplana, G. Tapia, J. Díaz, M. Fort, N. Cáceres, S. Pinto, J.**
661 **Caylà, L. Corner, M. Domingo, and P. J. Cardona.** 2010. Granuloma encapsulation
662 is a key factor for containing tuberculosis infection in minipigs. PLoS ONE. **5:**e10030.

663 19. **Gutiérrez, M., J. Tellechea, and J. F. García Marín.** 1998. Evaluation of cellular
664 and serological diagnostic tests for the detection of *Mycobacterium bovis*-infected goats.
665 Vet. Microbiol. **62:**281-290.

666 20. **Jassal, M., G. Nedeltchev, J. Osborne, and W. Bishai.** 2011. A modified scoring
667 system to describe gross pathology in the rabbit model of tuberculosis. BMC Microbiol.
668 **11:**49.

669 21. **Kubica, T., S. Rusch-Gerdes, and S. Niemann.** 2003. *Mycobacterium bovis* subsp.
670 *caprae* caused one-third of human *M. bovis*-associated tuberculosis cases reported in
671 Germany between 1999 and 2001. J. Clin. Microbiol. **41:**3070-3077.

672 22. **Liebana, E., A. Aranaz, J. J. Urquía, A. Mateos, and L. Dominguez.** 1998.
673 Evaluation of the gamma-interferon assay for eradication of tuberculosis in a goat herd.
674 Aust. Vet. J. **76:**50-53.

675 23. **Lin, P. L., M. Rodgers, L. Smith, M. Bigbee, A. Myers, C. Bigbee, I. Chiosea, S.**
676 **V. Capuano, C. Fuhrman, E. Klein, and J. L. Flynn.** 2009. Quantitative comparison
677 of active and latent tuberculosis in the Cynomolgus macaque model. Infect. Immun.
678 **77:**4631-4642.

679 24. **Lyashchenko, K., A. O. Whelan, R. Greenwald, J. M. Pollock, P. Andersen, R.**
680 **G. Hewinson, and H. M. Vordermeier.** 2004. Association of tuberculin-boosted
681 antibody responses with pathology and cell-mediated immunity in cattle vaccinated
682 with *Mycobacterium bovis* BCG and infected with *M. bovis*. Infect. Immun. **72:**2462-
683 2467.

684 25. **Lyashchenko, K. P., R. Greenwald, J. Esfandiari, M. A. Chambers, J. Vicente,**
685 **C. Gortazar, N. Santos, M. Correia-Neves, B. M. Buddle, R. Jackson, D. J.**
686 **O'Brien, S. Schmitt, M. V. Palmer, R. J. Delahay, and W. R. Waters.** 2008. Animal-
687 side serologic assay for rapid detection of *Mycobacterium bovis* infection in multiple
688 species of free-ranging wildlife. Vet. Microbiol. **132:**283-292.

689 26. **McCorry, T., A. O. Whelan, M. D. Welsh, J. McNair, E. Walton, D. G. Bryson,**
690 **R. G. Hewinson, H. M. Vordermeier, and J. M. Pollock.** 2005. Shedding of
691 *Mycobacterium bovis* in the nasal mucus of cattle infected experimentally with
692 tuberculosis by the intranasal and intratracheal routes. Vet. Rec. **157:**613-618.

693 27. **Pai, M., L. W. Riley, and J. Colford John M.** 2004. Interferon-γ assays in the
694 immunodiagnosis of tuberculosis: a systematic review. Lancet Infect. Dis. **4:**761-776.

695 28. **Plikaytis, B. B., K. D. Eisenach, J. T. Crawford, and T. M. Shinnick.** 1991.
696 Differentiation of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG by a
697 polymerase chain reaction assay. Mol. Cell. Probes. **5:**215-219.

698 29. **Pollock, J. M., J. D. Rodgers, M. D. Welsh, and J. McNair.** 2006. Pathogenesis
699 of bovine tuberculosis: The role of experimental models of infection. Vet. Microbiol.
700 **112:**141-150.

701 30. **Prodinger, W. M., A. Brandstatter, L. Naumann, M. Pacciarini, T. Kubica, M.**
702 **L. Boschiroli, A. Aranaz, G. Nagy, Z. Cvetnic, M. Ocepek, A. Skrypnyk, W. Erler,**
703 **S. Niemann, I. Pavlik, and I. Moser.** 2005. Characterization of *Mycobacterium caprae*
704 isolates from Europe by mycobacterial interspersed repetitive unit genotyping. J. Clin.
705 Microbiol. **43:**4984-4992.

706 31. **Quintas, H., J. Reis, I. Pires, and N. Alegria.** 2010. Tuberculosis in goats. Vet.
707 Rec. **166:**437-a-438.

708 32. **Rodríguez, E., L. P. Sánchez, S. Pérez, L. Herrera, M. S. Jiménez, S. Samper,**
709 **and M. J. Iglesias.** 2009. Human tuberculosis due to *Mycobacterium bovis* and *M.*
710 *caprae* in Spain, 2004-2007. Int. J.Tuberc. Lung Dis. **13:**1536-1541.

711 33. **Rodriguez, S., J. Bezos, B. Romero, L. de Juan, J. Alvarez, E. Castellanos, N.**
712 **Moya, F. Lozano, M. T. Javed, J. L. Saez-Llorente, E. Liebana, A. Mateos, L.**
713 **Dominguez, A. Aranaz, and The Spanish Network on Surveillance and Monitoring**
714 **of Animal Tuberculosis.** 2011. *Mycobacterium caprae* infection in livestock and
715 wildlife, Spain. Emerg. Infect. Dis. **17:**532-535.

716 34. **Sharpe, S. A., E. Eschelbach, R. J. Basaraba, F. Gleeson, G. A. Hall, A.**
717 **McIntyre, A. Williams, S. L. Kraft, S. Clark, K. Gooch, G. Hatch, I. M. Orme, P.**
718 **D. Marsh, and M. J. Dennis.** 2009. Determination of lesion volume by MRI and
719 stereology in a macaque model of tuberculosis. Tuberculosis (Edinb). **89:**405-416.

720 35. **Sharpe, S. A., H. McShane, M. J. Dennis, R. J. Basaraba, F. Gleeson, G. Hall,**
721 **A. McIntyre, K. Gooch, S. Clark, N. E. R. Beveridge, E. Nuth, A. White, A.**
722 **Marriott, S. Dowall, A. V. S. Hill, A. Williams, and P. D. Marsh.** 2010.
723 Establishment of an aerosol challenge model of tuberculosis in Rhesus macaques and an
724 evaluation of endpoints for vaccine testing. Clin. Vaccine Immunol. **17:**1170-1182.

725 36. **Sidders, B., C. Pirson, P. J. Hogarth, R. G. Hewinson, N. G. Stoker, H. M.**
726 **Vordermeier, and K. Ewer.** 2008. Screening of highly expressed mycobacterial genes
727 identifies Rv3615c as a useful differential diagnostic antigen for the *Mycobacterium*
728 *tuberculosis* complex. Infect. Immun. **76:**3932-3939.

729 37. **Vordermeier, H. M., M. A. Chambers, P. J. Cockle, A. O. Whelan, J. Simmons,**
730 **and R. G. Hewinson.** 2002. Correlation of ESAT-6-specific gamma interferon
731 production with pathology in cattle following *Mycobacterium bovis* BCG vaccination
732 against experimental bovine tuberculosis. Infect. Immun. **70:**3026-3032.

733 38. **Vordermeier, H. M., A. Whelan, P. J. Cockle, L. Farrant, N. Palmer, and R. G.**
734 **Hewinson.** 2001. Use of synthetic peptides derived from the antigens ESAT-6 and
735 CFP-10 for differential diagnosis of bovine tuberculosis in cattle. Clin. Diagn. Lab.
736 Immunol. **8:**571-578.

737 39. **Vordermeier, H. M., B. Villarreal-Ramos, P. J. Cockle, M. McAulay, S. G.**
738 **Rhodes, T. Thacker, S. C. Gilbert, H. McShane, A. V. S. Hill, Z. Xing, and R. G.**
739 **Hewinson.** 2009. Viral booster vaccines improve *Mycobacterium bovis* BCG-induced
740 protection against bovine tuberculosis. Infect. Immun. **77:**3364-3373.

741 40. **Waddington, F. G., and D. C. Ellwood.** 1972. An experiment to challenge the
742 resistance to tuberculosis in B.C.G. vaccinated cattle in Malawi. Br. Vet. J. **128:**541-
743 552.

744 41. **Waters, W. R., M. V. Palmer, T. C. Thacker, J. P. Bannantine, H. M.**
745 **Vordermeier, R. G. Hewinson, R. Greenwald, J. Esfandiari, J. McNair, J. M.**
746 **Pollock, P. Andersen, and K. P. Lyashchenko.** 2006. Early antibody responses to
747 experimental *Mycobacterium bovis* infection of cattle. Clin. Vaccine Immunol. **13:**648-
748 654.

749 42. **Young, D.** 2009. Animal models of tuberculosis. Eur. J. Immunol. **39:**2011-2014.

750
751
752
753
754
755
756
757
758
759

760 **FIGURES**

761 **Figure 1**: Kinetics of IFN-γ responses in infected goats. The release of IFN-γ was
762 measured by ELISA after in vitro stimulation of whole blood with different antigens.
763 Results are expressed as mean ΔOD_{450} responses with 95 % CI. Dashed horizontal line,
764 cut-off point for positivity. (A) PPD-B (closed circles) and PPD-A (open circles). (**) p
765 < 0.005, significant differences determined by non-parametric Mann-Withney-
766 Wilcoxon test. (B) PPD-B (closed circles), ESAT-6/CFP-10 (open circles) and Rv3615c
767 (open squares). (*) $p < 0.05$, significant differences determined by non-parametric
768 Friedman test with post-hoc Mann-Withney-Wilcoxon test.

769

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

776

777 **Figure 3**: Gross pathology analysis of a goat case. (A) MDTC-3D representation of the
778 whole lung after excluding air and TAC's table (H, head; F, foot, L, left, R, right). The
779 total volume of lung is calculated in cm^3 and is showed in red-dashed box at the bottom.
780 (B) Volume rendering image of the lung showing different tissue densities
781 discriminated by colour: water in grey, air in black and calcium in white. The volume of
782 affected lung is also showed. (C) Pathological areas identified by segmentation in axial
783 (see at the top; A, anterior; P, posterior), coronal and sagital planes (see at the bottom).
784 (D) Formalin fixed, 5 mm-sections of left diaphragmatic lobe which showing a large

785 cavitary lesion. Cranial to caudal sections are represented as bottom-up and left-right in
786 the picture. Bar = 3 cm.
787
788 **Figure 4**: Correlation between volumes of lesions in lungs measured by two
789 quantitative methods. Visible lesions in lungs calculated by Multi-Detector Computed
790 Tomography (MDCT) and by image analysis of photographs of lung sections (direct
791 observation) in infected goats (n = 11). Statistical analysis conducted with non-
792 parametric Spearman rank test.
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814

815 **TABLES**

816 **Table 1**: Detection of antibodies to MPB83 in plasma of goats infected or not with *M.*
817 *caprae***.** ELISA results at different time points post-infection (Week 0 represents day of
818 infection) are expressed according to the cut-off described above as: +, positive or -,
819 negative. Goats were subjected to SICCT test 13 days before the blood sampling at 14
820 wpi.

		Weeks post-infection							
Group	Goat	0	2	4	6	8	10	12	14
Infected	572	-	-	-	-	-	-	-	+
	605	-	-	-	+	+	+	-	+
	571	-	-	-	-	+	+	-	+
	567	-	-	-	-	-	-	+	+
	607	-	-	-	+	+	-	-	+
	563	-	-	-	-	+	+	+	+
	597	-	-	-	+	+	+	+	+
	568	-	+	-	-	-	-	+	+
	562	-	-	-	-	-	+	+	+
	565	-	-	-	+	+	+	+	+
	577	-	-	+	+	+	+	+	+
Non-infected	162	-	-	-	-	-	-	-	-
	187	-	-	-	-	-	-	-	-

821

822

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.

Goat	64-MDCT		Direct visual observation	
	Volume of lesions in lung (cm^3)	Volume ratio lesion/lung (%)	Volume of lesions in lung (cm^3)	No. of lobes with lesions
572	26.8	1.8	5.4	1/7
605	97.1	8.3	39.5	2/7
571	35.5	3.8	17.2	2/7
567	100	10.6	108.9	6/7
607	59.3	5	25	4/7
563	22.3	1.9	3.2	1/7
597	24.1	2.5	9.8	3/7
568	66.3	6.5	58.3	6/7
562	182	14.3	104.8	6/7
565	31	1.1	9.3	4/7
577	18.8	2.4	0.9	2/7
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)

829 **Table 3**: Pathological findings and bacterial load in pulmonary lymph nodes (LN) of infected goats.

Goat	Bacterial load (Log_{10} cfu)								Volume of lesions (cm^3)			
	crm[a] LN		tb[b] LN		cdm[c] LN		Total		crm LN	tb LN	cdmLN	Total
	g[d]	LN[e]	g	LN	g	LN	g	LN				
572	-	-	2.4	3.2	3	4.1	3.1	4.2	0	0.3	5.8	6.1
605	3.2	3.8	2.1	2.7	3.1	4.4	3.5	4.5	3.6	0.003	50.1	53.7
571	2.5	3.2	3	4	3.3	4.2	3.5	4.4	0.3	1.7	4.7	6.8
567	-	-	2.9	4.3	4	5.3	4	5.3	0	4.6	15.7	20.2
607	-	-	2.8	4	2.5	3.6	3	4.1	0.05	0	2.5	2.5
563	-	-	-	-	2.8	3.8	2.8	3.8	0	0.001	1.3	1.3
597	3.3	3.6	2.4	3.3	3.6	4.6	3.8	4.6	0.07	0.9	2.8	3.8
568	3.3	3.9	2.6	3.5	3	4.1	3.5	4.4	0.08	0.6	3.4	4.1
562	2.9	3.4	2.5	3.4	3.4	4.8	3.6	4.8	0.2	1.2	57.5	58.9
565	3.4	4.3	3.2	4.2	3.3	4.5	3.8	4.8	1.2	6.8	8	16
577	3	3.3	2.4	3.2	4.1	5.2	4.1	5.2	0.1	0.1	4.2	4.5
Mean (95% C.I.)	2 (1-2.9)	2.3 (1.2-3.4)	2.4 (1.9-2.9)	3.3 (2.6-4)	3.3 (3-3.6)	4.4 (4.1-4.7)	3.5 (3.3-3.8)	4.6 (4.3-4.8)	0.5 (0-1.2)	1.5 (0.2-2.8)	14.2 (2.3-26)	16.2 (3.9-28.4)

[a]crm: cranial mediastinal
[b]tb: tracheobronchial
[c]cdm: caudal mediastinal
[d]bacterial count per gram of tissue
[e]bacterial count in the whole lymph node

830

831

Formatted: Numbering: Continuous