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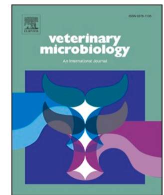

Experimental infection of goats with *Mycobacterium microti* induces subclinical pulmonary tuberculosis and mild responses to tuberculin skin tests

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A R T I C L E I N F O

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A B S T R A C T

Mycobacterium microti is a member of the *Mycobacterium tuberculosis* complex that seldom causes disease in livestock and humans. This study evaluated the effects on immunodiagnosis and the pathological findings in goats after experimental exposure by different routes and doses to *M. microti*. In a first experiment goats were challenged orally (PO, n = 7) or intranasally (IN, n = 7) with 10^4 CFU. In a second experiment, the endobronchial route was assessed, with a low dose of 10^2 CFU (EB-LD, n = 7) and a high dose of 10^5 CFU (EB-HD, n = 7) as well as the subcutaneous route (SC, n = 5). Temperature, body weight, clinical signs and immunological responses were monitored. Pathological evaluation was carried out and samples were processed for mycobacterial detection.

Results: demonstrated the induction of a subclinical pulmonary infection in all the EB-HD challenged animals. Infection was also confirmed in one animal of the SC group, but not in the EB-LD, PO or IN groups. Two animals belonging to the EB-HD and SC groups, respectively, showed positive results to the single intradermal tuberculin test, and another two animals of the EB-HD and EB-LD groups showed doubtful (inconclusive) results, indicating that *M. microti* can induce mild responses to tuberculin skin testing. No positive results were observed when defined antigens absent in *M. microti* (ESAT-6 and CPF-10) were used.

Our results indicate that animals exposed to *M. microti* can yield positive results to the skin tests currently performed in livestock tuberculosis eradication campaigns and reinforce the need to use specific antigens in antemortem tests to avoid interference with *M. bovis*/*M. caprae* diagnosis.

1. Introduction

Mycobacterium microti, an organism included within the *Mycobacterium tuberculosis* complex (MTBC), was initially described as the causative agent of tuberculosis (TB) in wild voles (Wells and Oxon, 1937) but afterwards cases of infection by *M. microti* have also been described in domestic (Michelet et al., 2020; Peterhans et al., 2020) and wildlife species (Boniotti et al., 2014; Michelet et al., 2021; Pérez de Val

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et al., 2019). Besides, a few cases of *M. microti* infection have also been described in humans, usually associated to immunocompromised patients (Panteix et al., 2010).

Nowadays, *M. bovis*, *M. caprae* and *M. tuberculosis* are defined by the World Organisation for Animal Health (WOAH) as the main causative agents of mammalian TB. Infections by *M. microti* in livestock are sporadic and cause mild or only microscopic lesions (Michelet et al., 2016, 2017, 2020). Compared to other MTBC species, *M. microti* has a deletion in the RD1 chromosomic region ($RD1^{MIC}$) that includes genes encoding virulence-related antigens. This has been suggested as the reason for its apparently lower pathogenicity in large mammals (Brodin et al., 2002; Orgeur et al., 2021), although the pathogenicity of *M. microti* in ruminants remains unstudied. Moreover, cattle reactors to the tuberculin skin test have been found in areas where *M. microti* circulates in wildlife (Michelet et al., 2020; Pérez de Val et al., 2019) suggesting a possible interference on TB diagnosis of cattle exposed to *M. microti* that may constrain bovine TB eradication campaigns.

The aim of this study was to evaluate the effects on the diagnosis of TB in goats experimentally exposed to *M. microti* and to characterize its pathogenicity.

2. Materials and methods

2.1. Experimental schedule

Two sequential experiments were carried out.

2.1.1. Experiment 1

Fourteen Pyrenean breed 4–5 months old female goats, from an officially TB-free herd located in Catalonia (Spain) were transferred to the IRTA-CReSA Biosafety Level 3 (BSL-3) facility (Bellaterra, Barcelona, Spain) and housed in an experimental box.

Animals were divided into 2 experimental groups of 7 animals each and, after an acclimatation period of one week, were challenged with a *M. microti* field strain prepared as previously described (Vidal et al., 2022). The first group was orally (PO) challenged with 1 ml of 10^4 CFU/ml *M. microti* suspension and the second group also received 1 ml of 10^4 CFU/ml *M. microti* by the intranasal (IN) route, distributed 0.5 ml into each nostril, using a syringe-adapted device for atomized spraying with a flexible cannula (MADgic®, Wolfe Tory Medical, Inc., Salt lake City, USA) as previously described (Melgarejo et al., 2023).

2.1.2. Experiment 2

Fifteen Murciano-Granadina breed, 4–5 months old female goats, from another officially TB-free herd located in Catalonia, were divided into three experimental groups of five animals each: ten animals were sedated by intramuscular injection of acepromazine maleate (0.05 mg/kg) and butorphanol (0.2 mg/kg) and subsequently anesthetized with propofol (5 mg/kg) and midazolam (0.2 mg/kg), both intravenously administrated. Afterwards, these animals were endobronchially (EB) challenged as previously described (Pérez de Val et al., 2011) with 0.5 ml of the same *M. microti* field strain described above at low dose (2×10^2 CFU/ml, EB-LD) and high dose (2×10^5 CFU/ml, EB-HD). The remaining five animals were inoculated with 1 ml of 10^4 CFU/ml suspension of *M. microti* at the right axilla by the subcutaneous (SC) route.

In both experiments, the animals were monitored daily for clinical signs after challenge. Rectal temperature and body weight were measured every week. Heparinized blood samples were collected as following: All animals were bled (10 ml) at week 0 (before *M. microti* exposure), and the PO and IN groups were also bled at weeks 3, 5, 7 and 9 (end point), while the EB-HD, EB-LD and SC groups were bled at weeks 3, 6 and 8 (end point). Animals from experiment 1 and 2 were humanely euthanized at 9 and 8 weeks after challenge, respectively, with an intravenous overdose of sodium pentobarbital. A diagram of the experimental design is available in supplementary file 1.

2.2. Ethics statement

This experimental study was approved by the Institutional Animal Welfare Committee.

of the Institute of Agrifood Research and Technology (CEEA-IRTA) and by the Ethical.

Commission of Animal Experimentation of the Autonomous Government of Catalonia (Project Ref. #10795) and conducted by certified staff, in accordance with the European Union legislation for the protection of experimental animals (86/609/CEE, 91/628/EEC, 92/65/EEC and 90/425/EEC).

2.3. Antigens and reagents

M. bovis purified protein derivative (PPD-B, 2500 IU/ml) and *M. avium* PPD (PPD-A, 2500 IU/ml) tuberculins were obtained from CZ vaccines (Porriño, Spain). The MTBC-specific recombinant antigens ESAT-6, CFP-10 and MPB83 were obtained from Lionex (Braunschweig, Germany) at a concentration of 500 μg/ml each. ESAT-6 and CFP-10 were mixed 1:1 in an antigenic cocktail (E/C). The P22 antigenic complex was produced by immunopurification of PPD-B (CZ Vaccines) as described previously (Infantes-Lorenzo et al., 2017) and was supplied by the *Carlos III Research Institute* (Madrid, Spain), at a concentration of 500 μg/ml.

2.4. Whole blood IFN

2.4.1. -γ release assay (IGRA)

Whole blood samples were collected at the above-mentioned time points from the jugular vein using heparinized tubes and subsequently stimulated in 96-well cell culture plates (Eppendorf, Hamburg, Germany) with PPD-B, PPD-A at final concentration of 20 μg/ml each, P22 at 10 μg/ml and E/C at 10 μm/ml each. Phosphate-buffered saline was used as an unstimulated control. The samples were incubated at 37 ºC and 5% CO_2 for 18 ± 2 h. Plasma supernatants were collected after centrifugation at 18 *g* for 10 min and the released IFN-γ was measured by ELISA according to the instructions of the ID.screen® Ruminant IFN-g kit (Innovative Diagnostics, Grabels, France). Optical densities (OD) were read at 450 nm using a spectrophotometer (Biotek Power Wave XS®, Agilent, dana Clara, USA). Antigen-specific IFN-γ responses were calculated as OD of the antigen-stimulated well minus OD of the unstimulated well (ΔOD). Interpretation of the tuberculin based IGRA results was performed according to the manufacture recommended cut-off point and the positivity criteria are described as follows: PPD-B OD – PBS OD ≥ 0.05, P22 OD - PBS OD ≥ 0.05 and E/C OD – PBS OD ≥ 0.05, respectively.

2.5. Antibody detection assays

Plasma samples from all experimental animals were analyzed in duplicate to detect IgG antibody responses to MPB83 antigen by indirect ELISA, as previously described (Melgarejo et al., 2023). MPB83-IgG levels were expressed as the mean of OD of the antigen-coated wells minus OD of the uncoated well (ΔOD). A sample was classified as positive when $\Delta OD \geq 0.5$.

2.6. Skin tests

At week 7 post challenge in experiment 1 and week 6 in experiment 2, all animals were inoculated with 0.1 ml of each reagent at the inoculation points using a Dermojet® syringe (Akra Deermojet, Pau, France). PPD-B was inoculated on the upper-right side of the neck whereas E/C was inoculated on the lower-right side. P22 was inoculated on the upper-left side of the neck and PPD-A on the lower-left side. The increase in skinfold thickness was measured just before inoculation and 72 h post inoculation. The results were obtained from the thickness difference

(Δmm) at 0 and 72 h after the inoculation of the antigens and interpreted as described in the manual of the Spanish bovine TB eradication program (MAPA, 2023). For the Single Intradermal Tuberculin (SIT) test an animal was positive when Δmm (PPD-B) $\geq$ 4 and/or presence of clinical signs at the inoculation site, negative when Δmm (PPD-B) $<$ 2 mm and doubtful when 4 $>$ Δmm (PPD-B) $\geq$ 2 mm. Single Intradermal Comparative Cervical Tuberculin (SICCT) test was positive when SIT was positive and Δmm (PPD-B) - Δmm (PPD-A) $>$ 4, negative when Δmm (PPD-B) - Δmm (PPD-A) $<$ 1, and doubtful when 4 $\geq$ Δmm (PPD-B) - Δmm (PPD-A) $\geq$ 1. The skin tests with P22 and E/C were interpreted using the same criteria than SIT.

2.7. Pathological examination

Necropsies were performed immediately after euthanasia, the retropharyngeal (right and left), mediastinal (cranial and caudal) and tracheobronchial lymph nodes (LN) were sampled from all animals. Lung lesions as well as other TB-like visible lesions in extrapulmonary sites were recorded and fixed in 10% buffered formalin to be confirmed as TB lesions by histopathology through hematoxylin and eosin staining and Ziehl - Neelsen staining for acid-fast bacilli (AFB) detection.

2.8. MTBC DNA detection

LN and lung samples were processed individually for culture and PCR. DNA samples were extracted from tissue homogenates using the ID Gene™ spin universal extraction kit (Innovative Diagnostics) and amplified with the *Mycobacterium tuberculosis* complex duplex kit (Innovative Diagnostics) following the manufacturer's instructions. The amplification was performed in a 7500 FAST real-time PCR system (Applied Biosystems, Walham, MA, USA). The amplification results were interpreted according to the manufacturer's instructions.

2.9. Mycobacterial culture

Tracheobronchial, mediastinal (caudal and cranial), and retropharyngeal (left and right) lymph nodes were sampled separately from all animals and two lung tissue samples (animals 12 and 5 of the EB-HD and EB-LD groups, respectively). The tissues were sectioned separately with sterile scissors and forceps, then homogenized in 17 ml of sterile distilled water using a homogenizer (Masticator, IUL Instruments, Barcelona, Spain). An aliquot of 1.5 ml of tissue homogenates was collected and inactivated for 1 h at 75 ºC for subsequent evaluation of the bacterial load by qPCR. Ten millilitres of the remaining tissue homogenates were decontaminated with 10 ml of 5% oxalic acid for 30 min and then neutralized by adding 5 ml of NaOH 1 M. Samples were centrifuged for 30 min at 2471g, the supernatants were discarded and the pellets were suspended in 1 ml of sterile PBS. The suspensions were cultured as follows: 500 µl were inoculated into BBL® tubes (BD diagnostics, Sparks, MD, USA) tubes and incubated in the BACTEC MGIT 320 system (BD diagnostics); a swab was immersed in the remaining suspension for culture in Löwenstein-Jensen with pyruvate and Coletsos solid media tubes (BD Diagnostics) and another swab was cultured in Middlebrook 7H11 plates (produced by IRTA-CReSA). A culture was considered negative when no growth was observed on Middlebrook 7H11 plates at 28 days, or BACTEC MGIT and solid media tubes at 90 days (Vidal et al., 2022). Growth in positive cultures was confirmed as MTBC by multiplex PCR (Wilton and Cousins, 1992).

3. Results

One intercurrent death unrelated to the experimental procedure occurred in an animal of the EB-HD group at week 1 of the experiment. The animal was excluded from the study.

3.1. Immunodiagnostic results

For skin testing, PPD-B, PPD-A, E/C and P22 were inoculated intradermally in all the animals of the 5 experimental groups, these results are presented in Table 1. All skin tests were negative in the animals of the PO and IN groups. Two animals, one of the EB-HD and another of the SC group, had a positive result to the SIT. These two positive animals showed doubtful results at SICCT. Doubtful results to the SIT were also found in one animal of the EB-HD group and another of the EB-LD group. The latter also had a doubtful result to the SICCT. With regard to the P22 skin test, a doubtful reaction was recorded in one animal of the EB-HD group. There were no positive nor doubtful skin test reactions to the E/C.

ELISA IgG response to the MPB83 MTBC-specific antigen was only detected in one experimental animal of the EB-HD group (supplementary file 1).

The whole blood IFN-γ release assay (IGRA) with PPD-B, PPD-Av, P22 and E/C was negative in all animals throughout the experiments (supplementary file 1).

3.2. Clinical and postmortem findings

A homogenous body weight gain was recorded among the experimental groups. No fever was detected in any experimental animal. Clinical signs such as cough, dyspnea, eye discharge, enlarged lymph nodes, nasal discharge, and anorexia were absent (supplementary file 1).

Postmortem results are summarized in Table 1. At necropsy one animal from the EB-HD group presented a small (~2 mm) macroscopic TB compatible lesion (TBCL) in the lung. No gross TBCL were observed in any animal of the EB-LD, SC, PO and IN groups. The lung TBCL was confirmed as such by histopathology: a non-encapsulated, discreet focus of granulomatous infiltrate admixed with abundant lymphoplasmacytic infiltrate forming follicles (likely hyperplastic bronchiolar associated lymphoid tissue) with the presence of multinucleated Langhans cells and a minimal area of necrosis was observed (Fig. 1A-B). Additionally, 1 animal of the SC group showed similar microscopic TBCL in the mesenteric lymph nodes and lung, and another animal from the EB-LD had lesions solely in the mesenteric lymph node. However, acid-fast bacilli were only detected in the lung TBCL of the animal of the EB-HD group, which were often arranged in likely intracellular clusters and free within necrotic debris (Fig. 1C).

MTBC was isolated from the mediastinal LNs of all animals of the EB-HD group (4/4). Three out of the four animals of this group were also positive to qPCR performed directly on homogenates from pulmonary LNs. MTBC was only isolated from the mediastinal and tracheobronchial LNs of one out of five animals of the SC group, even though this animal showed a negative result to direct qPCR of these samples. The animals belonging to EB-LD, PO and IN groups were negative both to culture and direct qPCR (Table 1).

4. Discussion

Domestic goats are highly susceptible to MTBC mycobacteria such as *M. bovis* or *M. caprae* and the experimental infection of goats with these pathogens has been proposed as a reliable animal model to assess livestock TB control strategies (Melgarejo et al., 2023; Pérez de Val et al., 2011). Sporadic cases of *M. microti* infection have been detected in livestock, causing diagnostic interferences with the immunodiagnosis of TB due to *M. bovis* or *M. caprae* (Michelet et al., 2020). Since *M. microti* may hamper the success of TB eradication campaigns in areas with high circulation of this bacterium in the wildlife-livestock interface (Pérez de Val et al., 2019; Tagliapietra et al., 2021), the present study aimed to use the experimental goat model to assess the pathogenicity of *M. microti* under laboratory conditions and determine whether the exposure to *M. microti* would induce positive results to the immunodiagnostic tests used in TB eradication campaigns.

Table 1
Results of antemortem and post-mortem diagnostic tests in all groups.

Animal ID	Group	Skin tests				Post-mortem tests		
		SIT (Δmm PPD-B)	SICCT [PPD-B - PPD-A (mm)]	E/C (Δmm)	P22 (Δmm)	Histopathology/ AFB (tissue)	Culture (tissue)	qPCR (Tissue)
3	EB-HD	- (1.41)	- (−2.26)	- (0.31)	- (0.01)	NL	+ (MD)	-
9	EB-HD	**+ (4.28)**	**d (3.44)**	- (0.20)	- (0.06)	NL	+ (MD)	+ (MD, TBR)
11	EB-HD	**d (3.16)**	- (1.92)	- (0.47)	**d (3.03)**	NL	+ (MD)	+ (MD)
12	EB-HD	- (0.39)	- (−0.34)	- (−0.05)	- (0.22)	**TBCL/ + (lung)**	+ (MD)	+ (MD)
5	EB-LD	- (0.21)	- (0.22)	- (0.05)	- (0.25)	NL	-	-
7	EB-LD	- (0.74)	- (0.68)	- (−0.01)	- (0.05)	NL	-	-
8	EB-LD	**d (3.61)**	**d (2.03)**	- (0.10)	- (0.04)	**TBCL/ - (MS)**	-	-
13	EB-LD	- (0.03)	- (0.01)	- (0.10)	- (0.01)	NL	-	-
14	EB-LD	- (0.71)	- (0.37)	- (0.10)	- (−0.02)	NL	-	-
1	SC	- (1.62)	- (0.51)	- (0.52)	- (0.04)	NL	-	-
2	SC	- (0.94)	- (−0.28)	- (0.05)	- (0.34)	NL	-	-
6	SC	- (1.52)	- (1.43)	- (0.26)	- (0.09)	NL	-	-
10	SC	- (0.58)	- (0.44)	- (0.28)	- (0.42)	NL	-	-
15	SC	**+ (4.38)**	**d (2.99)**	- (0.33)	- (1.40)	**TBCL/ - (lung, MS)**	+ (MD, TBR)	-
240	PO	- (0.42)	- (−0.25)	- (0.37)	- (0.52)	NL	-	-
164	PO	- (1.33)	- (0.25)	- (0.84)	- (0.43)	NL	-	-
197	PO	- (0.61)	- (−0.44)	- (1.14)	- (1.05)	NL	-	-
172	PO	- (1.19)	- (0.4)	- (1.14)	- (0.38)	NL	-	-
243	PO	- (0.76)	- (−1.03)	- (0.49)	- (1.11)	NL	-	-
228	PO	- (0.59)	- (−0.05)	- (0.14)	- (0.43)	NL	-	-
208	PO	- (1.35)	- (0.38)	- (0.26)	- (−0.09)	NL	-	-
271	IN	- (1.14)	- (−0.70)	- (0.39)	- (0.30)	NL	-	-
154	IN	- (1.57)	- (−0.60)	- (0.85)	- (1.13)	NL	-	-
238	IN	- (0.31)	- (−0.36)	-(0.02)	- (1.26)	NL	-	-
152	IN	- (0.47)	- (−0.90)	- (0.38)	- (1.26)	NL	-	-
168	IN	- (1.08)	- (0.77)	- (0.85)	- (0.62)	NL	-	-
206	IN	- (0.78)	- (−0.39)	- (0.38)	- (0.56)	NL	-	-
274	IN	- (1.11)	- (0.44)	- (0.67)	- (0.76)	NL	-	-

d: doubtful; NL: no lesions; TBCL: TB compatible lesión; MD: mediastinal lymph node; TBR: tracheobronchial lymph node; MS: mesenteric lymph node; SIT:Single Intradermal Tuberculin; SICCT:. Single Intradermal Comparative Cervical Tuberculin; PPD-B: M. bovis purified protein derivative; PPD-A: M. avium purified protein derivate; E/C: ESAT-6 and CFP-10; AFB: acid fast bacilli (evidenced by Ziehl Neelsen staining); (+): test positive; (-): test negative.

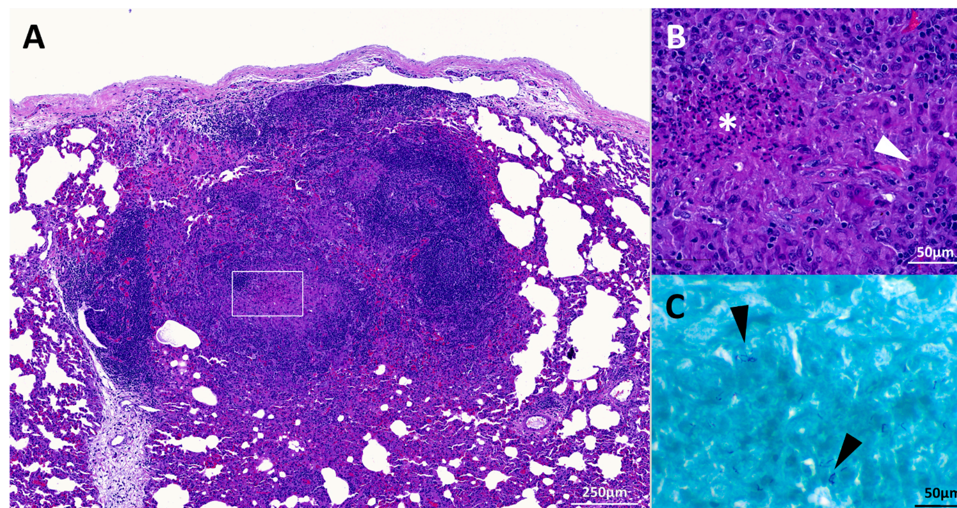

Fig. 1. Histopathological results in the lung of high dose *M. microti* endobronchially challenged goat number 12. (A) Subpleural, non-encapsulated, pulmonary granulomatous lesion surrounded by marked lymphoplasmacytic infiltrate with follicle formation and likely hyperplasia of bronchiolar associated lymphoid tissue. The lesion, within the white rectangular area, is magnified in (B) (Haematoxylin and Eosin staining, bar 250 µm). (B) Asterisk points to a minimal area of necrosis and degenerated neutrophils in the centre of the granuloma. A few Langhans-type multinucleated giant cells are present in the surrounding inflammatory infiltrate (white arrowhead) (Haematoxylin and Eosin staining, bar 50 µm). (C) Abundant acid-fast bacilli are observed within the lesion, often arranged in likely intracellular clusters (black arrowheads) and free within the necrotic debris (Ziehl Neelsen staining, bar 50 µm).

4.1. M. microti experimental challenge in goats can elicit a subclinical infection

The results of this study demonstrate that *M. microti* infection can be experimentally achieved in goats, especially by endobronchial and subcutaneous routes, although the disease course was subclinical and the pathological findings were very mild when compared to experimental infections of goats with *M. caprae* or *M. bovis* (Bezos et al., 2015; Pérez de Val et al., 2011).

Clinical parameters, especially body weight gain and rectal temperature, were not affected after challenge with *M. microti* in contrast to the loss of body weight gain and fever observed in other experimental

infections with *M. caprae* in the goat model (Arrieta-Villegas et al., 2018; Melgarejo et al., 2023). Only one animal of the EB-HD group showed minimal TBCL, which is consistent with findings in field cases of cattle and goats infected by *M. microti* in France (Michelet et al., 2016, 2017).

Significant differences were observed among infection routes, being the EB-HD the most efficient to induce pulmonary TB infection. Even though either the oral or intranasal intake of *M. microti* are the most likely routes of infection in a field context, a slower development of the disease may delay the appearence of gross TB lesions. These can explain the absence of TB lesions in a short period experiment such as the present one. Similarly, goats experimentally infected with *M. caprae* by the intranasal route (Melgarejo et al., 2023) induced lower degree of pulmonary lesions and bacterial replication compared to endobronchially infected goats with *M. caprae* (Melgarejo et al., 2023; Pérez de Val et al., 2011). Likewise, in cattle orally infected with *M. bovis* the development of lesions seemed to be slower and almost restricted to abdominal tissues compared to endotracheally infected cattle (Serrano et al., 2018).

Subclinical pulmonary infection was induced in all the EB-HD challenged animals. All were bacillary in pulmonary LN without visible or minimal TBCL. This finding plausibly be explained by the loss of the ability of the mycobacteria to escape from the phagosome and replicate in the cytosol causing cytotoxicity and the death of host cells due to the deletion in the RD1 genomic region including a large portion of the Esx-1 secretion system genes related to this translocation mechanism (Simeone et al., 2012). This is the main cause suggested for the lesser virulence and lower pathogenicity of *M. microti* compared to other MTBC members (Orgeur et al., 2021). The induction of bacillary but none to mild pathological lesions is consistent with negative to low immune responses.

4.2. M. microti infection can interfere with current tuberculin skin test protocols of M. bovis / M. caprae eradication programs

In the context of a TB outbreak investigation in a French goat herd, epidemiologically related with *M. bovis* infected cattle, three goats showed positive results to a skin test and one of them was subsequently confirmed to be infected with *M. microti* (Michelet et al., 2016). Similar findings in cattle show that these results could interfere with bovine TB eradication campaigns (Michelet et al., 2020). In our experiment, 4 out of 28 goats experimentally exposed to *M. microti* had positive or doubtful skin tests results. All of them showed TB lesions and/or positive cultures *postmortem*. This low number of low-intensity intradermal reactions and the absence of positivity to IGRA may be related with a early infection stage but also to a lower capacity of *M. microti* to induce tuberculin-based cell-mediated immune responses. The latter was confirmed in a recent study where guinea pigs challenged with *M. microti* induced lower delayed hypersensitivity reactions to PPD-B and P22 compared to other MTBC members (Fernández-Veiga et al., 2023). As expected, all animals were negative to E/C-based skin tests and IGRAs because genes encoding ESAT-6 and CFP10 proteins are not present in *M. microti* genome (Orgeur et al., 2021). Similarly, negative results to E/C-based skin tests or IGRAs were obtained in PPD-B and/or P22 positive animals after vaccination of goats with BCG, which also lacks genes encoding ESAT-6 protein family (Arrieta-Villegas et al., 2020; Pérez de Val et al., 2016).

In conclusion, our results evidence that goats experimentally exposed to *M. microti* can yield positive results to the skin tests currently performed in livestock TB eradication campaigns and they can also become infected and develop minor pulmonary TB lesions.

Also, these results underscore how challenging it is to demonstrate a recent *M. microti* infection through regular sampling procedures, since only one of the infected animals had a single minuscule visible lesion (∼2 mm) that would certainly go unnoticed in an abattoir setting. The absence of positive or doubtful reactions when using defined antigens absent or not expressed in *M. microti* strongly encourages the use of these antigens in antemortem tests in epidemiological contexts where the circulation of *M. microti* is suspected.

CRediT authorship contribution statement

Cobos Alex: Investigation, Writing – review & editing. **Melgarejo Cristian:** Formal analysis, Writing – original draft, Investigation. **Cantero Guillermo:** Investigation, Writing – review & editing. **Domingo Mariano:** Investigation, Writing – review & editing. **Boschiroli Maria Laura:** Funding acquisition, Methodology, Resources, Writing – review & editing. **Michelet Lorraine:** Methodology, Resources, Writing – review & editing. **Pérez de Val Bernat:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Writing – original draft. **Vidal Enric:** Conceptualization, Formal analysis, Investigation, Writing – original draft. **Garrido Joseba M.:** Methodology, Resources, Writing – review & editing. **Sevilla Iker A.:** Funding acquisition, Resources, Writing – review & editing, Methodology. **Moll Xavier:** Investigation, Writing – review & editing, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The databases generated and analysed during the current study are available from the corresponding author on a reasonable request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2024.110009.

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