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A high titer antibody response against P22 protein immunocomplex is not correlated with protection in naturally tuberculosis-infected goats

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ABSTRACT
Caprine livestock are significant reservoirs of the *Mycobacterium tuberculosis* complex (MTBC), contributing to tuberculosis (TB) transmission among animals and humans. The P22 protein immunocomplex (P22PI), derived from bovine tuberculin, shows immunostimulating capacity and is used for TB diagnosis. This study assessed the immunogenicity and protective efficacy of P22PI in two groups of goats: 24 naïve goats (12 immunised, 12 controls) from a TB-free herd, and 24 infected goats (12 immunised, 12 controls), referred to as pre-infected animals, from a *M. bovis*-infected herd. Both were exposed for 5 months to *M. bovis*-naturally infected goats. Reactors to single and comparative intradermal tuberculin (SIT and SCIT, respectively) tests and interferon-gamma release assay (IGRA) significantly increased ($p < 0.05$) in both groups 5 months' post-exposure, with no significant differences between immunised and control animals. However, immunised animals exhibited a significantly higher ($p < 0.05$) antibody response against P22PI. Most naïve animals (83.3%) and all pre-infected animals developed TB-compatible lesions, with extensive necrosis in the lungs and associated lymph nodes, compared to 50% and 83.3% of control animals, respectively. These findings suggest that while P22PI stimulates an intense antibody response under the conditions of the present study, it does not confer protection against TB and may exacerbate disease severity.

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1. Introduction

Caprine tuberculosis (TB) is a zoonosis with a significant public health and economic impact (Bezos et al. 2012; Napp et al. 2013). TB in goats is mainly caused by *Mycobacterium bovis* and *M. caprae* (Cadmus et al. 2009; Rodríguez et al. 2011). Goats are important TB reservoirs and have been described as the origin of cases of human TB (Martínez-Lirola et al. 2023) and outbreaks in other animal species, especially in cattle, thus making the eradication of the disease difficult (Napp et al. 2013). Although there is currently no TB eradication program for this species, its analysis is already considered in the context of the new Animal Health Law (Regulation EU 20162013/429, 2013). Moreover, in certain countries, such as Spain, TB eradication programs in goats are implemented in certain regions (MAPA 2024a) and testing is mandatory where goats coexist or are epidemiologically linked to cattle (MAPA 2024b). These specific programs are mainly based on a test-and-slaughter strategy using single and comparative intradermal tuberculin (SIT and SCIT, respectively) tests, the implementation of biosecurity measures on farms, control of movements, and passive surveillance in slaughterhouses (MAPA 2024a). Recently, TB vaccination studies in goats have been carried out suggesting that it could be a valuable control tool in the future, however there are aspects related to the immune response in this species to be elucidated (Arrieta-Villegas et al. 2018; Melgarejo et al. 2022; Roy et al. 2019).

The immune response against TB is complex and there are mechanisms related to protection and the latency phenomenon that are not well understood. It has been widely described that the cell-based immune response is essential for controlling infection, particularly during the early phases, and is the

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basis for official diagnostic tests (Bezos et al. 2012; Pollock and Neill 2002). Regarding the humoral response, studies in humans have suggested its limited significance in terms of protection against infection (Bitencourt et al. 2021; Dockrell and Smith 2017), even indicating a positive correlation between pathology and the high presence of antibodies in the serum of individuals with active infection (Bitencourt et al. 2021). However, other studies suggest that they could play a relevant role in reducing the likelihood of progressing from latent infection to disease (Li and Javid 2018). In animals, the role of antibodies in protecting against the disease has not been extensively studied. Previous studies on BCG vaccination, mostly conducted in cattle, reported a weak humoral response in contrast to that observed in animals infected with *M. bovis* (Biffar et al. 2020; Vidal et al. 2017), which some authors have associated with the observed protective effects (Tanner et al. 2019). In goats, the response to BCG vaccination using different antigens, such as ESAT-6, CFP-10, MPB70, MPB83, P22, and others, could differ depending on factors like age and the route of vaccine administration (Arrieta-Villegas et al. 2020; Pérez de Val et al. 2016; Roy et al. 2019; Vidal et al. 2017). Since the role of antibodies in protecting against TB is not well understood, it is crucial to determine if they significantly contribute to defense against the disease to assess the potential for new vaccine candidates or immunisation strategies to elicit this type of response (Achkar et al. 2015; Jacobs et al. 2016; Roy et al. 2019).

The P22 protein immunocomplex (P22PI), obtained through immunopurification from a commercial bovine purified protein derivative (PPD-B), has been widely used as the basis for TB serodiagnosis (Barral et al. 2022; Infantes-Lorenzo et al. 2017). This is attributed to its capacity to stimulate a potent humoral response, driven by its immunodominant antigens, including MPB70, MPB83, ESAT-6, and CFP-10 (Infantes-Lorenzo et al. 2017; Thomas et al. 2021). Its applicability has been studied in different animals, such as cattle (Barral et al. 2022; Infantes-Lorenzo et al. 2019), sheep (Infantes-Lorenzo et al. 2020, 2019), goats (Infantes-Lorenzo et al. 2019; Melgarejo et al. 2022), pigs (Infantes-Lorenzo et al. 2019; Thomas et al. 2019), alpacas (Barral et al. 2022), badgers (Balseiro et al. 2020; Barral et al. 2022), and wild boar (Barral et al. 2022).

The objective of this study was to determine the protective and the therapeutic effects of P22PI immunisation, an immunoprotein complex highly involved in the stimulation of humoral response, in preventing infection or reducing lesion severity in naturally TB-infected goats.

2. Material and methods

2.1. Design of the study

The present study was conducted using animals from two herds of Guadarrama-breed dairy goats located in central Spain: a TB-free herd ($n = 31$) and a TB-infected herd ($n = 220$). A pre-study assessment utilizing various cell-based and humoral tests, including SIT test, interferon-gamma release assay (IGRA), and the P22 ELISA, determined the immunological TB-status of the animals. Diagnostic confirmation relied on the combined results of these three tests to mitigate the risk of false positives, particularly considering the known specificity challenges associated with the P22 ELISA (Infantes-Lorenzo et al. 2019). Furthermore, the TB-infected herd was confirmed to be infected by environmental (feeders, drinkers, salt blocks, and milking parlours) and *post-mortem* (lung and local lymph nodes with lesions) bacteriological culture (*M. bovis* SB0121). Moreover, a vaccination program against *Mycobacterium avium* subsp. *paratuberculosis* (MAP) was implemented in the TB-infected herd, using the Gudair vaccine (CZ Vaccines, Porriño, Spain) in goats under 6 months of age. Conversely, the naïve animals came from a TB-free herd on a farm that did not implement vaccination against paratuberculosis.

Twenty-four animals from the TB-infected herd ($n = 220$), identified as positive for TB based on their reactivity to the IGRA and SIT/SCIT tests during the last herd evaluation prior to the study, were randomly selected and considered as pre-infected animals (range of study population: 1 to 3 years old) (Supplementary Table 1). Other 24 animals from the TB-free herd ($n = 31$) were confirmed to be negative to SIT/SCIT tests, IGRA and P22 ELISA in serum samples, and were considered as naïve animals (range of study population: 6 months to 1-year-old) (Supplementary Table 1). Subsequently, 12 out of 24 animals of each group were intramuscularly immunised with 100 µg of P22PI combined with Montanide PR01 at T0 (where T# refers to how many months after prime vaccination) and at T1 (one month after) (Figure 1). Once the immunisation protocol was completed (T1), animals from both naïve and pre-infected groups were exposed to TB-infected animals (donor goats; range of population: 1-6 years old) belonging to a high TB (*M. bovis* SB0121) prevalence herd ($n = 149$), according to the results of SIT test (74.49% of reactors), IGRA (48.99% of reactors) and P22 ELISA (79.86% of reactors) obtained previously, at T2 (Figure 1). The cellular and humoral responses were evaluated using SIT/SCIT tests, IGRA and P22 ELISA in serum samples during 5 months (Figure 1). These results were compared with those obtained from the other 12 non-immunised animals from each group, which were considered as controls. After the 5-month exposure period, all animals were sedated with xylazine at a dose of 10 mg/50 kg (2% Xilagesic, Calier SA, Barcelona, Spain), and then euthanized using an intravenous inoculation of T-61 at a dose of 5 ml/50 kg (Merck Sharp & Dohme Animal Health, S.L., Salamanca, Spain). Afterward, all goats underwent a systematic necropsy to assess the presence, size and extent of TB-compatible lesions. Small representative samples measuring 1.5 × 1 cm were collected from various organs for histopathological analysis, including the submandibular,

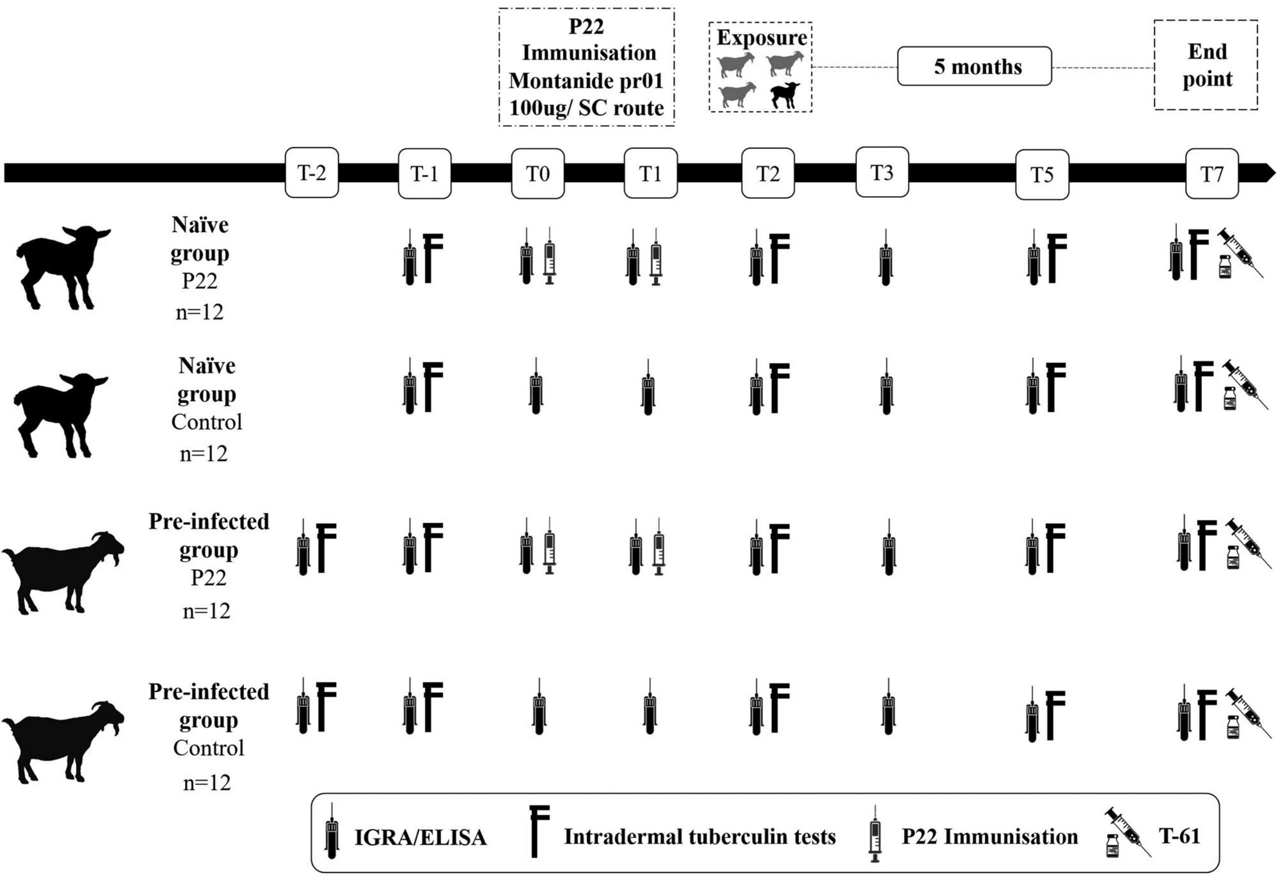

Figure 1. Summary of the experimental design. Silhouettes of black goats (pre-infected group) and kid goats (naïve group) represent the receptor animals, comprising both vaccinated and control subgroups. The grey goat silhouettes represent the donor goats infected with *M. bovis*.

retropharyngeal, tracheobronchial, mediastinal, and ileocecal lymph nodes (LNs), as well as all lung lobes. These samples underwent fixation in 10% neutral buffered formalin for 24 h, followed by dehydration through a graded ethanol series, immersion in xylene, and embedding in paraffin wax using an automated processor. Moreover, fresh samples of head and pulmonary LNs and lung lobes were chosen for bacteriological culture and isolation of bacteria in the laboratory (Diallo et al. 2016).

All handling, testing and sampling procedures were carried out by qualified veterinarians in compliance with European (Directive 2010/63/UE 2010) and Spanish (RD 53/2013) legislation. All procedures were authorised by an institutional ethical committee and approved by the local authorities (PROEX 245.8/20, Comunidad de Madrid).

2.2. P22PI immunisation

The P22PI was obtained from PPD-B (CZ Veterinaria, S.A.) through immunopurification with a monoclonal antibody (AcMo) as previously described (Infantes-Lorenzo et al. 2017). P22PI composition was analysed using nanoscale liquid chromatography-electrospray ionization tandem mass spectrometry, and the complex was found to consist of several immunodominant antigens, notably MPB70, MPB83, ESAT-6, and CFP-10. Animals were intramuscularly immunised twice at a 4-week interval with 1 ml of P22PI (100 µg) at a concentration of 0.54 mg/ml, adjuvanted with Montanide™ 01PR (SEPPIC).

2.3. Diagnostic methods

2.3.1. SIT/SCIT tests

The SIT/SCIT tests were carried out using the intradermal inoculation of 0.1 ml of avian PPD (PPD-A) and PPD-B (CZ Vaccines, Porriño, Spain) simultaneously on the left and right sides of the neck, respectively, using a Dermojet syringe (Akra Dermojet, Pau, France). Both tests were performed at T-2, T-1, T2, T5 and T7 (Figure 1). The reactions to the different intradermal tests were interpreted by the same veterinarian in all groups, and the interpretation of the results was carried out as described previously (Ortega et al. 2021). The SIT and SCIT tests were performed according to the protocol published by the European Union Reference Laboratory (EU-RL) for Bovine TB (European Union Reference Laboratory for Bovine Tuberculosis (EURL), 2021a). Briefly, one animal was considered a positive reactor to the SIT test when there was an increase of ≥ 4 mm in the skin fold thickness and/or the presence of clinical signs was observed. Regarding the SCIT test, an animal was considered a positive reactor if the bovine reaction was greater than the avian reaction by more than 4 mm; and/or there were clinical signs at the PPD-B inoculation site.

2.3.2. IGRA

In order to detect the IFN-γ production, blood samples were collected from the jugular vein by employing venipuncture, using evacuated tubes (BD Vacutainer Becton, Dickinson and Company, Franklin Lakes, USA) with lithium heparin at T-2, T-1, T0, T1, T2, T3, T5 and T7 (Figure 1). At the laboratory, blood samples were stimulated with PPD-B avian PPD (CZ Vaccines, Porriño, Spain), P22PI or ESAT-6/CFP10 (E/C, Lionex, Braunschweig, Germany) at a final concentration of 20 µg/ml each, and subsequently processed as described the protocol published by the EU-RL for Bovine TB (European Union Reference Laboratory for Bovine Tuberculosis (EURL), 2021b). IFN-γ production in plasma was measured using a commercial kit (Bovigam™ TB Kit, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions, and the results were interpreted as described elsewhere (Roy et al. 2019). Briefly, an animal was considered positive to the IGRA if the optical density (OD) of a sample stimulated with PPD-B (P22 or E/C) minus the OD of PBS was greater than 0.05 and greater than the OD of the sample stimulated with PPD-A minus the OD of PBS.

2.3.3. P22 ELISA

In order to evaluate the continuous evolution of the specific antibody levels against the *Mycobacterium tuberculosis* complex (MTBC), serum samples were analysed by employing an in-house indirect ELISA that detects antibodies against the P22PI, which is obtained by the immunopurification of PPD-B (CZ Vaccines, Porriño, Spain) (Infantes-Lorenzo et al. 2017). Blood samples were collected from the jugular vein using plastic serum tubes (BBD Vacutainer Becton, Dickinson and Company, Franklin Lakes, USA) at T-1, T0, T1, T2, T3, T5 and T7 (Figure 1). The ELISA was performed as described previously (Infantes-Lorenzo et al. 2019). The results were expressed as a P22 ELISA percentage (E%), which was calculated using the following formula: $E\% = [\text{mean sample OD}/(2 \times \text{mean of negative control OD})] \times 100$

According to the results of this formula, an animal was considered positive when the E% value of its serum sample was 100 or higher, as described elsewhere (Infantes-Lorenzo et al. 2019).

2.4. Pathology

A systematic examination of gross lesions was conducted on all organs of culled animals ($n = 48$) using two semi-quantitative systems: one was used for assessing the lungs, while another was employed for evaluating the LNs and the remaining organs. TB-compatible lesions were characterised by circumscribed to multifocal yellowish granulomatous inflammatory nodules, variably encapsulated by connective tissue, frequently containing central caseous necrosis and mineralization. The scoring system was based on the number, size, and distribution of TB-compatible lesions, including the percentage of the organ affected as was described previously (Roy et al. 2019). For the lung lobes, the following scoring system was applied: 0, no visible TB-compatible lesions; 1, < 25% of the lung lobe affected; 2, 25–50%; 3, > 50–75%; and 4, > 75%. An additional point was assigned to animals exhibiting pleural adhesions. Afterwards, the total lung score was calculated using the addition of the scores of the different lung lobes (left cranial, left caudal, right cranial, right middle, right accessory and right caudal) examined individually. Regarding LNs and the remaining organs, following the methodology described previously (Vordermeier et al. 2002), gross lesions were categorised into six groups as follows: 0, no visible lesions; 1, small lesions apparent on slicing; 2, ≤ 5 lesions of < 10 mm in diameter; 3, more than 5 lesions of < 10 mm in diameter or presence of one lesion > 10 mm in diameter; 4, presence of more than one lesion > 10 mm in diameter; and 5, coalescing lesions. Each individual LNs was examined separately, and the scores of the different LNs were summed to calculate the total LNs score. Finally, the scores of the lungs, LNs, and the remaining organs were summed to obtain the total macroscopic score.

For microscopic evaluation, tissue sections measuring 1.5 × 1 cm were cut at 3 µm and stained with haematoxylin–eosin using standard procedures. Histopathological TB-compatible lesions were evaluated in lungs, as well as head, pulmonary and abdominal LNs, the most frequent sites of lesion occurrence. Microscopic tuberculous granulomas were distinguished as a compact lesion composed of organised aggregates of immune cells, with a central core of mature macrophages surrounded by T and B cells, as well as fibroblasts. As previously defined (Wangoo et al. 2005), TB-granulomas were classified into four groups based on their size and composition: Stage I (initial), Stage II (solid), Stage III (minimal necrosis) and Stage IV (necrosis and mineralization). Granulomas were scored as; 0, no granulomas, 1, 1-10 granulomas; 2, 11-20 granulomas; 3, more than 21 granulomas. Additionally, the presence of epithelioid infiltrate was evaluated on a scale of severity: 0, absence (0% of tissue sample affected); 1, mild infiltrate (<30%); 2, moderate infiltrate (>30-70%); and 3, severe infiltrate (>70%). The final score was obtained by summing the scores of the lesions previously detailed.

2.5. Bacteriology

A pool of lung lobes as well as head and pulmonary LNs from each animal ($n = 48$), was stored at −80 °C and subsequently used for bacteriological culture in Löwenstein-Jensen with sodium pyruvate medium (Difco, Spain), as described elsewhere (Roy et al. 2020). The isolates observed were identified using DVR-spoligotyping (Kamerbeek et al. 1997).

2.6. Statistical analysis

Wilson's 95% confidence intervals (95% CI) were calculated for the percentage of positive reactors to the

different tests using WinPepi version 11.6. (Abramson 2004). Quantitative values, such as the increase in the skin fold thickness (SFT) (expressed in mm), IFN-γ levels (expressed as OD), E% and the score of TB-compatible lesions in animals from the different groups (naïve animals and pre-infected animals) and subgroups (immunised animals *vs* controls) at a specific sampling time were compared using the Kruskal-Wallis test followed by pairwise tests for multiple comparisons of mean rank sums after adjusting the *p*-value using the Bonferroni correction and Mann–Whitney U tests. Quantitative differences in the increase in SFT, OD and E% between different times of sampling were analysed using the Friedman test. The comparison of the proportions of reactors to different techniques used in the present study among the different groups and subgroups at a specific sampling point was carried out using chi-square test. The comparison of the proportions of test reactors within a given group between different sampling times was performed by using McNemar's test. Spearman's rank correlation coefficient (rs) was used to assess the relationship between SFT, OD and E% in serum samples and the score of post-mortem analysis obtained. Data analyses were performed using SPSS Statistics 25 (IBM, New York, NY, USA). Figures that representative the results of different diagnostic tools and gross and histopathological findings in lesions of different groups were performed in R version 3.6.2 (R Core Team 2019) using ggplot2 package (Wickham 2016).

3. Results

3.1. Cell-based diagnostic tests

3.1.1. SIT/SCIT tests

The number and percentage of positive reactors to the different diagnostic tests are summarised in Table 1. No significant differences ($p > 0.05$) were observed in the skin fold thickness increase (mm) between the immunised and control subgroups of naïve and pre-infected groups in any of the skin tests performed during the study. There was a significant increase in the number of reactors to SIT test in both the immunised and control subgroups of naïve animals in T7 compared to those observed at T2 (Table 1). Equally, the number of reactors to SCIT test at T7 was significantly higher compared to T2 in the immunised ($p = 0.031$) and control ($p = 0.004$) animals of this group. However, the differences in the number of reactors to SIT and SCIT tests in the immunised and control animals from the pre-infected group were not significant ($p > 0.05$), regardless of the technique used (SIT/SCIT), at T7 compared to T2 (Table 1).

3.1.2. IGRA. No significant differences ($p > 0.05$) on the OD of IGRA were observed between immunised and control subgroups of naïve and TB pre-infected groups at the different time points regardless of the antigen used (PPD-B and PPD-A, P22PI or ESAT-6/CFP-10). A progressive increase in the number of reactors to IGRA (regardless of the antigen used) was observed in the control and immunised animals in both groups (Table 1). There was a significant increase in the number of reactors to IGRA using PPDs ($p < 0.001$), P22PI ($p < 0.001$) or ESAT-6 ($p = 0.004$) in the immunised animals of the naïve group, and only using PPDs ($p = 0.031$) in the immunised animals of the pre-infected group.

3.2. Humoral-based diagnostic tests

There were no significant differences in the levels of E% between immunised and control animals in the naïve group at the T-1 ($p = 0.101$) and at T0 ($p = 0.551$). However, significant differences ($p < 0.05$) were observed between immunised and control animals in subsequent sampling periods (Figure 2A). All animals from the immunised subgroup were positive to P22 ELISA after the first (T0) and second (T1) immunisation (Table 1). These animals remained positive to P22 ELISA in all subsequent sampling periods, and there were no significant differences in the number of reactors within this subgroup between T2 and T7 ($p = 1$). A progressive increase in the number of reactors to P22 ELISA was observed in the control subgroup since these animals were exposed to the donor goats (Table 1, Figure 2A). The number of reactors to P22 ELISA was significantly ($p = 0.039$) higher at the T7 compared to the T2 (Figure 2A).

With regard to the pre-infected group, the differences observed in the levels of E% were not significant between immunised and control animals at T0 ($p = 0.114$) and T1 ($p = 0.551$). However, these differences were significant ($p < 0.05$) in all subsequent samplings (Figure 2B). As previously described in the naïve group, all animals from the immunised subgroup were positive to P22 ELISA in the samplings performed immediately after first and second immunisation in this group, and during the rest of the experiment (Table 1). Therefore, in these animals the differences in the number of reactors to P22 ELISA were not significant between T2 and T7. In the control subgroup, there was a progressive increase in the number of reactors from the initial exposure to the donor animals ($n = 4$) up to the last sampling ($n = 9$). However, differences in the number of reactors were not statistically significant ($p = 0.063$) between T7 and T2 (Figure 2B).

Regarding the correlation between the results of the ELISA P22 and the cell-based diagnostic tests, a significant increase ($p = 0.003$) in skin fold thickness was observed among the naïve animals testing positive for the P22 ELISA at T7 compared to those testing negative (Figure 3A). In contrast, in the TB pre-infected goats, there were no significant differences ($p = 0.122$) in the skin fold thickness increase in immunised and controls animals at that time (Figure 3B). With regard to IGRA, the OD levels were significantly higher in animals which were positive to P22 ELISA in the last testing before the end of the study

Table 1. Number and percentage of positive reactors in each experimental group using different diagnostic tests and antigens.

Test	Antigen	Group $n=24$	Subgroup $n=12$	T-2 % (CI 95%)[f]	T-1 % (CI 95%)	T0[g] % (CI 95%)	T1 % (CI 95%)	T2 % (CI 95%)	T3 % (CI 95%)	T5 % (CI 95%)	T7 % (CI 95%)
IGRA[a]	PPD-B[b]	Naïve animals	Immunised	NA	0 (0.0-0.24)	0 (0.0-0.24)	0 (0.0-0.24)	0 (0.0-0.24)	0 (0.0-0.24)	41.6 (19.3-68.1)	91.6 (64.6-98.5)
			Control	NA	0 (0.0-0.24)	0 (0.0-0.24)	0 (0.0-0.24)	0 (0.0-0.24)	8.33 (1.4-35.4)	50 (25.3-74.6)	50 (25.3-74.6)
		Pre-infected animals	Immunised	0 (0.0-0.24)	83.3 (55.2-95.3)	41.6 (19.3-68.1)	41.6 (19.3-68.1)	8.33 (1.4-35.4)	8.33 (1.4-35.4)	25 (8.8-53.2)	58.3 (31.9-80.0)
			Control	8.33 (1.4-35.4)	83.3 (55.2-95.3)	16.6 (4.7-44.8)	16.6 (4.7-44.8)	16.6 (4.7-44.8)	8.33 (1.4-35.4)	41.6 (19.3-68.1)	25 (8.8-53.2)
	P22[b]	Naïve animals	Immunised	NA	NA	0 (0.0-0.24)	0 (0.0-0.24)	8.33 (1.4-35.4)	8.33 (1.4-35.4)	41.6 (19.3-68.1)	100 (75.75-100)
			Control	NA	NA	8.33 (1.4-35.4)	0 (0.0-0.24)	0 (0.0-0.24)	8.33 (1.4-35.4)	58.3 (31.9-80.0)	41.6 (19.3-68.1)
		Pre-infected animals	Immunised	NA	NA	41.6 (19.3-68.1)	25 (8.8-53.2)	8.33 (1.4-35.4)	8.33 (1.4-35.4)	33.3 (13.8-60.9)	41.6 (19.3-68.1)
			Control	NA	NA	8.33 (1.4-35.4)	16.6 (4.7-44.8)	16.6 (4.7-44.8)	16.6 (4.7-44.8)	50 (25.3-74.6)	41.6 (19.3-68.1)
	E/C[b]	Naïve animals	Immunised	NA	NA	0 (0.0-0.24)	0 (0.0-0.24)	0 (0.0-0.24)	0 (0.0-0.24)	41.6 (19.3-68.1)	83.3 (55.2-95.3)
			Control	NA	NA	0 (0.0-0.24)	0 (0.0-0.24)	0 (0.0-0.24)	8.33 (1.4-35.4)	58.3 (31.9-80.0)	58.3 (31.9-80.0)
		Pre-infected animals	Immunised	NA	NA	25 (8.8-53.2)	16.6 (4.7-44.8)	0 (0.0-0.24)	16.6 (4.7-44.8)	25 (8.8-53.2)	50 (25.3-74.6)
			Control	NA	NA	8.33 (1.4-35.4)	8.33 (1.4-35.4)	8.33 (1.4-35.4)	0 (0.0-0.24)	41.6 (19.3-68.1)	33.3 (13.8-60.9)
SIT test[c]	PPD-B	Naïve animals	Immunised	NA	0 (0.0-0.24)	NA	NA	0 (0.0-0.24)	NA	75 (46.7-91.1)	91.6 (64.6-98.5)
			Control	NA	0 (0.0-0.24)	NA	NA	0 (0.0-0.24)	NA	50 (25.3-74.6)	83.3 (55.2-95.3)
		Pre-infected animals	Immunised	16.6 (4.7-44.8)	58.3 (31.9-80.0)	NA	NA	50 (25.3-74.6)	NA	100 (75.75-100)	83.3 (55.2-95.3)
			Control	8.33 (1.4-35.4)	33.3 (13.8-60.9)	NA	NA	50 (25.3-74.6)	NA	66.6 (39.0-86.1)	75 (46.7-91.1)
SCIT test[d]	PPD-B and PPD-A	Naïve animals	Immunised	NA	0 (0.0-0.24)	NA	NA	0 (0.0-0.24)	NA	58.3 (31.9-80.0)	50 (25.3-74.6)
			Control	NA	0 (0.0-0.24)	NA	NA	0 (0.0-0.24)	NA	33.3 (13.8-60.9)	75 (46.7-91.1)
		Pre-infected animals	Immunised	0 (0.0-0.24)	8.33 (1.4-35.4)	NA	NA	8.33 (1.4-35.4)	NA	41.6 (19.3-68.1)	50 (25.3-74.6)
			Control	0 (0.0-0.24)	0 (0.0-0.24)	NA	NA	0 (0.0-0.24)	NA	33.3 (13.8-60.9)	25 (8.8-53.2)

(*Continued*)

Table 1. Continued.

Test	Antigen	Group $n=24$	Subgroup $n=12$	T-2 % (CI 95%)[f]	T-1 % (CI 95%)	T0[g] % (CI 95%)	T1 % (CI 95%)	T2 % (CI 95%)	T3 % (CI 95%)	T5 % (CI 95%)	T7 % (CI 95%)
P22 ELISA[e]	P22	Naïve animals	Immunised	NA	0 (0.0-0.24)	16.6 (4.7-44.8)	100 (75.75-100)	100 (75.75-100)	91.6 (64.6-98.5)	100 (75.75-100)	100 (75.75-100)
			Control	NA	0 (0.0-0.24)	25 (8.8-53.2)	0 (0.0-0.24)	0 (0.0-0.24)	8.33 (1.4-35.4)	41.6 (19.3-68.1)	50 (25.3-74.6)
		Pre-infected animals	Immunised	58.3 (31.9-80.0)	50 (25.3-74.6)	91.6 (64.6-98.5)	100 (75.75-100)	91.6 (64.6-98.5)	100 (75.75-100)	100 (75.75-100)	100 (75.75-100)
			Control	8.33 (1.4-35.4)	66.6 (39.0-86.1)	66.6 (39.0-86.1)	33.3 (13.8-60.9)	33.3 (13.8-60.9)	41.6 (19.3-68.1)	50 (25.3-74.6)	75 (46.7-91.1)

[a]An animal was considered positive to the IGRA if the optical density (OD) of a sample stimulated with bovine PPD (PPD-B) minus the OD of PBS was greater 0.05 and greater than the OD of the sample stimulated with avian PPD (PPD-A).
[b]Cut-of 0.05.
[c]An animal was considered a positive reactor to the SIT test when there was an increase of ≥ 4 mm in the skin fold thickness and/or the presence of clinical signs was observed.
[d]An animal was considered a positive reactor to the SCIT test when the bovine reaction was greater than the avian reaction by more than 4 mm and/or there were clinical signs at the bovine PPD inoculation site.
[e]An animal was considered positive to P22 ELISA when the E% value was greater than 100.
[f]Wilson's 95% confidence intervals.
[g]Animals were immunized at T0 and T1, exposed to donor goats at T2 and euthanized after T7.
E/C: cocktail of ESAT-6/CFP-1; NA: not applicable.

in the naïve ($p<0.001$) (Figure 3C) and pre-infected ($p=0.022$) groups (Figure 3D).

3.2.1. *Pathology*

At necropsy, TB-compatible macroscopic and microscopic lesions were observed in 75.0% (9/12) of immunised animals and 58.3% (7/12) of the controls from the naïve group, primarily located in lung and pulmonary LNs (Figure 4), and sporadically in liver. Immunised animals exhibited a higher mean macroscopic (6.8) and microscopic (16.8) lesion score compared to the control group (6.0 and 11.6); however, no significant differences were reported between both subgroups (Figure 5A and C). Moreover, it is important to highlight the higher number of granulomas found in the different pulmonary lobes and LNs of immunised animals compared to those observed in the control animals, with a particular emphasis on necrotic granulomas (stage III and IV), which were predominant over solid granulomas (stage I and II) (Table 2). No granulomas were reported in the retropharyngeal, mesenteric or ileocecal LNs of the control animals (Table 2). The correlation (Spearman's rank correlation coefficient) between the total macroscopic TB-compatible lesions score and the levels of E% in serum samples was moderate (rs = 0.64). In contrast, the correlation between the total macroscopic TB-compatible lesions score and the skin fold thickness increase (mm) was moderate (rs = 0.40) and with the OD readings in plasma was weak (rs = 0.24). Regarding the microscopic TB-compatible lesions, the correlation between the total score and levels of E% in serum samples was strong (rs = 0.72). However, the correlation of the total score of microscopic lesions observed and the skin fold thickness (mm) (rs =0.35) and the OD levels in plasma samples (rs =0.34) was weak.

In the TB pre-infected group, 100% (12/12) of the immunised animals and 83.3% (10/12) of the controls showed TB-compatible macroscopic and microscopic lesions, with higher presence and severity in lung and pulmonary LNs (Figure 4), and occasionally extending to the spleen and liver. Although no significant differences were observed between the subgroups in macroscopic lesions (Figure 5A), statistically significant differences were found in microscopic lesions ($p=0.035$), with immunised animals showing a higher mean score (29.8) compared to controls (15.9) (Figure 5C). With regard to the reactivity to P22 ELISA, the score of TB-compatible macroscopic (Figure 5B) and microscopic lesions (Figure 5D) were significantly ($p<0.05$) higher in animals which were positive to P22 ELISA in the last testing before the end of the study in the naïve and pre-infected groups. The number of granulomas observed at the different pulmonary lobes were similar between immunised and control animals. However, goats in the immunised subgroup had three times more granulomas than control specimens, particularly evident in the mediastinal and mesenteric LNs (Table 2). These differences were statistically significant

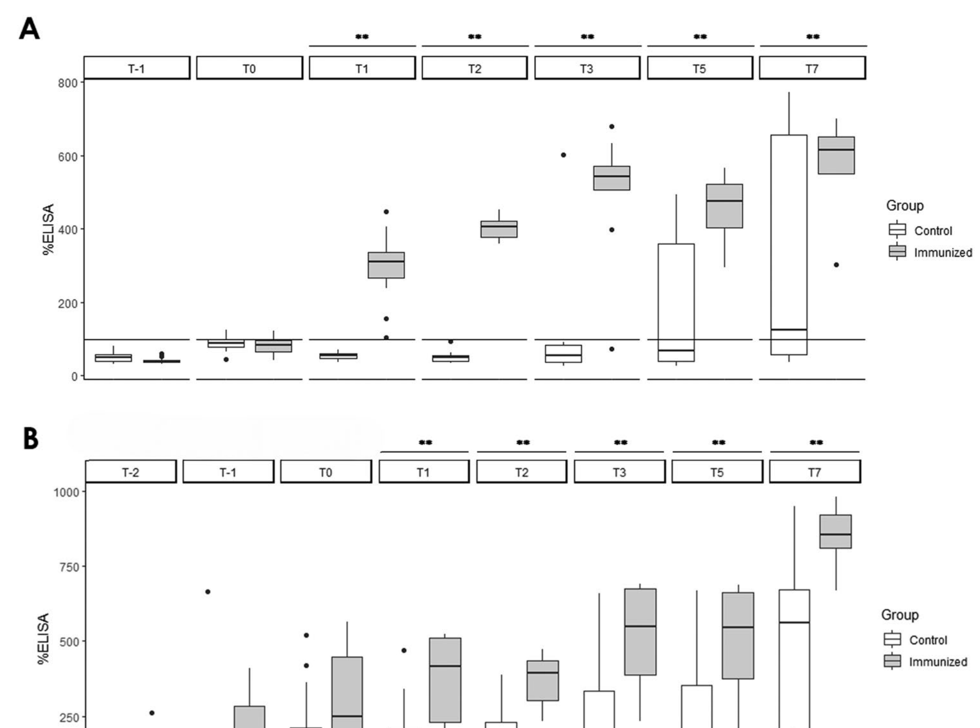

Figure 2. ELISA % in serum samples during different times of sampling in the naïve animal's group (A) and TB pre-infected goats (B) with a cut-off point of 100 E%. significant differences are described in the boxplot as follows: $^{**}p < 0.05$.

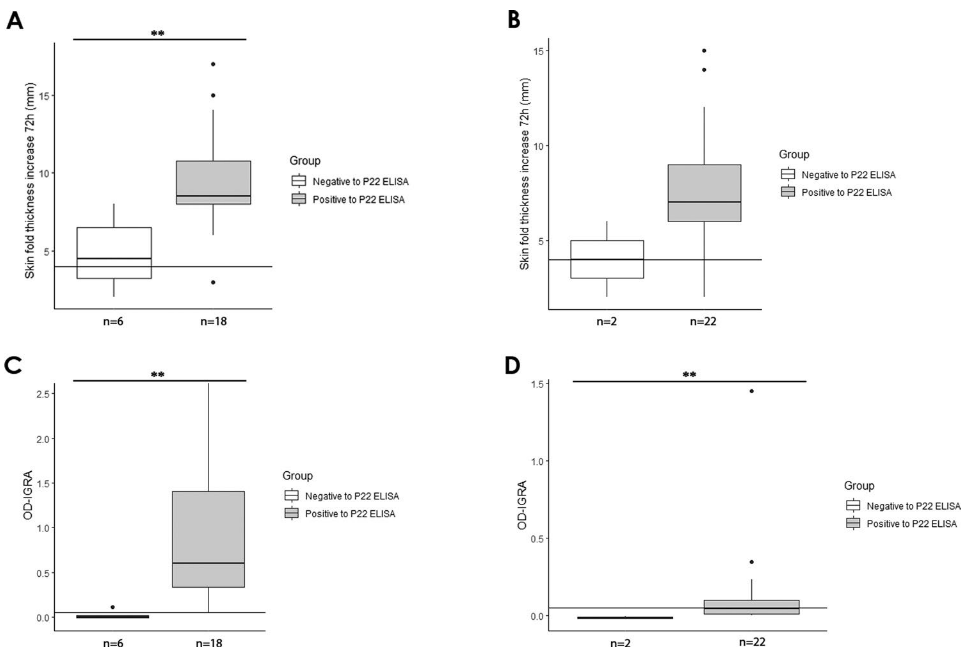

Figure 3. Summary of the differences in the median skinfold thickness increase (mm) after bovine PPD inoculation between positive and negative animals to P22 ELISA (cut-off point of 100 E%) in serum samples at the testing previous to the sacrifice in the naïve (A) and TB pre-infected (B) groups and differences in the interferon-gamma release assay expressed by optical density (OD) between positive and negative animals to P22 ELISA in serum samples at the testing previous to the sacrifice in the naïve (C) and TB pre-infected groups (D). Significant differences are described in the boxplot as follows: $^{**}p < 0.05$.

($p = 0.016$). As previously reported in the naïve animals, these lesions were predominantly characterised by stage III and IV granulomas. There were two animals of the control subgroup that showed no TB-compatible lesions in any organ during the *post-mortem* analysis. Interestingly, both animals were the only ones that tested negative to P22 ELISA at the end of the experiment (T7). The correlation between the total macroscopic TB-compatible lesions score and the levels of E% in serum samples was very weak (rs = 0.09) and weak (rs = 0.28) between the total macroscopic score and the skin fold thickness

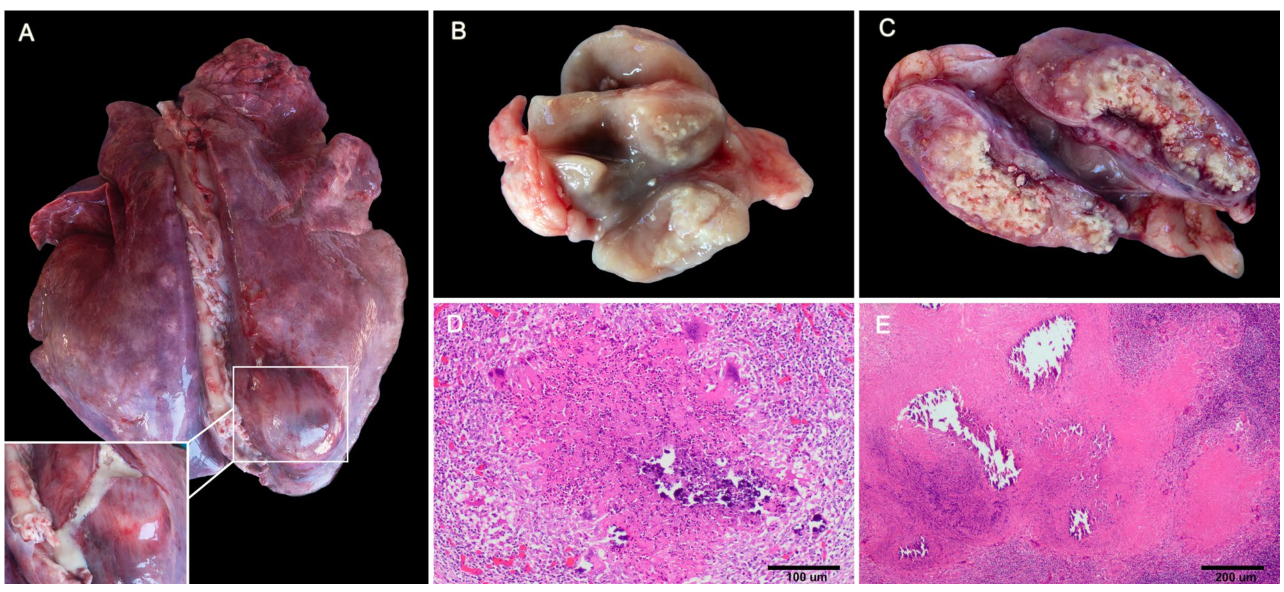

Figure 4. Representative images of gross and histopathological findings in lesions of immunised and control goats naturally-infected with *M. bovis* from naïve and TB pre-infected groups. Macroscopically, immunised and control animals exhibited visible TB-compatible lesions (TBL) in the lungs (a), with caseous and purulent (inset) consistency. In lymph nodes (LNs), control (B) and immunised (C) animals presented a yellowish appearance with caseous consistency, with immunised goats displaying higher severity. Microscopically, control animals displayed necrotic granulomas with some calcification foci surrounded by a diffuse mixture of inflammatory cells (D). Immunised animals exhibited multifocal necrotic granuloma in LNs with several calcification foci and extensive areas of necrosis (E).

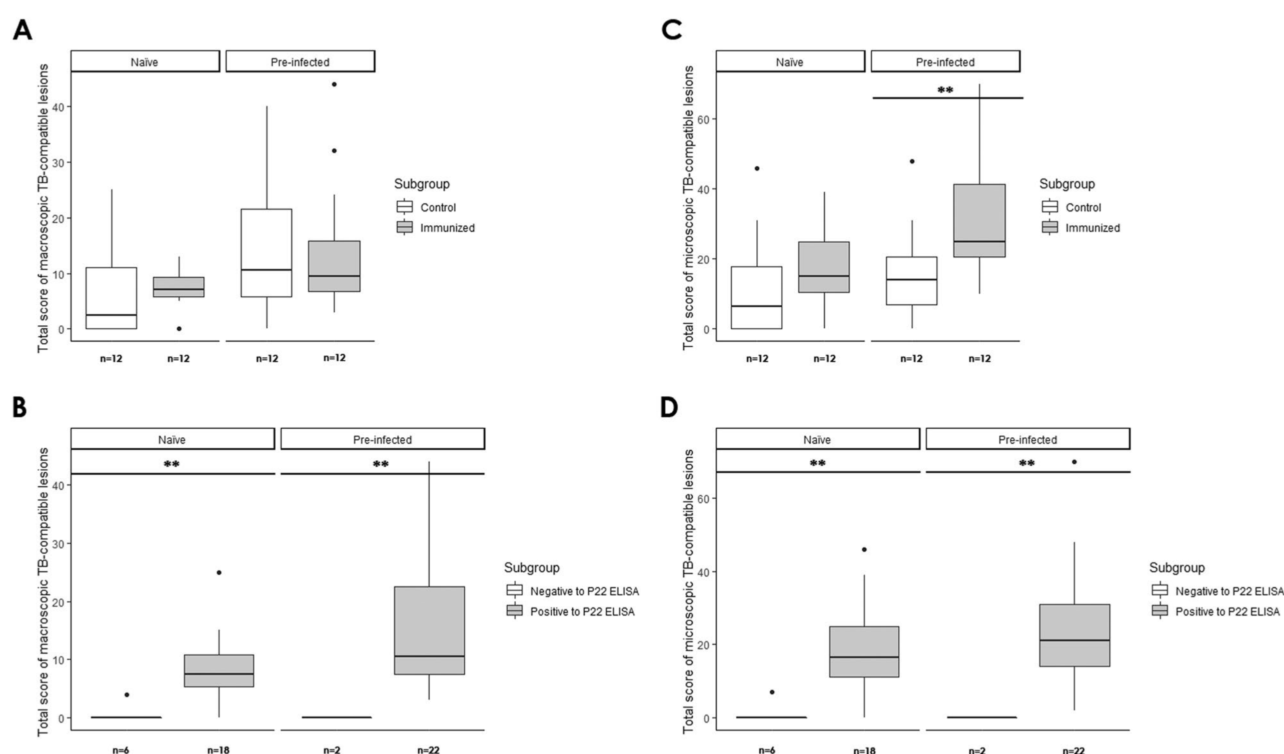

Figure 5. Differences in macroscopic (A) and microscopic (C) total scores between immunised and control subgroups in naïve and pre-infected animal groups and in macroscopic (B) and microscopic (D) total scores between positive and negative animals to P22 ELISA in naïve and pre-infected animal groups. Significant differences are described in the boxplot as follows: $^{**}p < 0.05$.

(mm). However, the correlation between the total score of macroscopic TB-compatible lesions and the quantitative results of IGRA in plasma samples was moderate (rs = 0.42). Regarding the microscopic TB-compatible lesions, the correlation between the total score and levels of E% in serum samples was strong (rs = 0.76). Nevertheless, the correlation of the total score of microscopic lesions observed and the skin fold thickness (mm) (rs =0.38) and the OD levels in plasma samples (rs =0.35) was weak.

Table 2. Characterization of TB-compatible histopathological lesions in lungs and lymph nodes (LNs) across study groups.

Lung								
Group	Subgroup	Left cranial S/N	Left caudal S/N	Right cranial S/N	Right middle S/N	Right accessory S/N	Right caudal S/N	Total lung granulomas S/N
Naïve animals	Immunised	1.3/0.0% (1/0)	10.7/20.6% (8/41)	40.0/12.1% (30/24)	5.3/18.1% (4/36)	8.0/8.5% (6/17)	34.7/40.7% (26/81)	100/100% (75/199)
	Control	4.0/9.4% (4/12)	6.9/13.4% (7/17)	33.7/26.7% (34/34)	11.9/15.0% (12/19)	1.0/2.4% (1/3)	42.5/33.1% (43/42)	100/100% (101/127)
Pre-infected animals	Immunised	4.6/7.6% (5/13)	49.1/28.2% (52/48)	15.1/13.0% (16/22)	5.7/1.2% (6/2)	8.5/10.6% (9/18)	17.0/39.4% (18/67)	100/100% (106/170)
	Control	0.0/0.6% (0/1)	6.8/25.8% (6/43)	17.1/28.1% (15/47)	0.0/0.0% (0/0)	6.8/13.8% (6/23)	69.3/31.7% (61/53)	100/100% (88/167)

Lymph nodes							
Group	Subgroup	Retropharyngeal S/N	Mediastinal S/N	Tracheobronchial S/N	Mesenteric S/N	Ileocecal S/N	Total LNs granulomas S/N
Naïve animals	Immunised	1.0/11.1% (1/29)	29.2/45.4% (28/119)	30.2/36.3% (29/95)	0.0/6.1% (0/16)	39.6/1.1% (38/3)	100/100% (96/262)
	Control	0.0/0.0% (0/0)	36.8/ (7/82)	63.2/ (12/74)	0.0/0.0% (0/0)	0.0/0.0% (0/0)	100/100% (19/156)
Pre-infected animals	Immunised	10.6/12.6% (28/43)	18.3/26.7% (48/91)	16.7/18.8% (44/64)	29.7/26.9% (78/92)	24.7/15.0% (65/51)	100/100% (263/341)
	Control	4.5/10.4% (3/13)	25.4/17.6% (17/22)	29.9/40.8% (20/51)	14.9/7.2% (10/9)	25.3/24.0% (17/30)	100/100% (67/125)

S: solid granulomas; N: necrotic granulomas.

3.3. MTC culture

Regarding the bacteriological culture results, *M. bovis* SB0121 was isolated from the respiratory pool of all (24/24) immunised animals and most of the control animals in both the naïve (6/12) and pre-infected (9/12) groups. Notably, all animals from which isolation was not achieved tested negative in the P22 ELISA.

4. Discussion

An immunisation based on the P22PI was developed and evaluated for the first time in naturally TB naïve and pre-infected goats, with a specific focus on the role of induced antibodies in preventing or reducing disease-induced pathology. The observed findings not only suggest that P22 immunisation, under the conditions of the present study, did not confer protection against TB in goats, but also indicate that high antibody levels could be associated with more severe TB-compatible lesions, increased mycobacterial culture isolation, and consequently, severe disease pathology and a higher risk of transmission within the farm.

According to the results obtained, the infection protocol (*via* contact with infected animals) implemented resulted in the infection of both immunised and control animals from the naïve group. This was evidenced by positive cultures, reactivity to the different diagnostic tests employed, and the presence of TB-compatible lesions observed in lungs, LNs and other analysed organs. This transmission pattern is consistent with recent goat studies investigating experimental vaccines, including SO2 (Bezos et al. 2017) and MTBVAC (Roy et al. 2019), where animals were subjected to a similar period of exposure. Although all animals were exposed to *M. bovis* for a period of 5 months, infection was not confirmed in all control goats from the naïve group. This could be attributed to the variability in infection rates, often encountered in natural transmission models, and inherent differences in susceptibility among animals (Smith et al. 2016). Additionally, the shorter exposure duration in our study, compared to other investigations where animals were in contact with donor animals for over 10 months (Ameni et al. 2018; Fromsa et al. 2024; Roy et al. 2018a), could have influenced the reported infection rates.

Nowadays, *M. bovis* BCG is the only available vaccine against TB in humans and it has been also evaluated in animals (Nemes et al. 2018). However, the effectiveness of BCG is highly variable in humans (Brewer 2000) and ruminants (Buddle et al. 2018; Mustafa et al. 2006). For this reason, several studies have been conducted in recent years with the aim of developing new vaccines or immunostimulants to complement or replace the BCG vaccine (Zhang et al. 2023). In ruminants, vaccine research efforts are primarily focused on the evaluation of potential protein antigens, including MPB70, MPB83, ESAT-6, and CFP10 (Maue et al. 2007; Vordermeier et al. 2002, 2000; Wedlock et al. 2003). These antigens are major components of the P22PIutilized in the present study (Infantes-Lorenzo et al. 2017).

Our study demonstrates the immunostimulatory potential of the P22PI, widely employed for TB humoral diagnosis in domestic and wild animals (Barral et al. 2022; Infantes-Lorenzo et al. 2017), due to its capacity to trigger a potent humoral and cellular immune response. The latter was evidenced by the positive result to cell-based tests conducted throughout the study, even before exposure to the donor animals. Different studies in cattle evaluated the immunostimulant potential of distinct components of the P22PI, including MPB70, MPB83, CFP10 and ESAT-6 (Maue et al. 2007; Vordermeier et al. 2000; Wedlock et al. 2003). Consistent with our

findings, Vordermeier and collaborators demonstrated that calves immunised with MPB83 or MPB70 showed enhanced cellular and humoral immune responses (Vordermeier et al. 2000). However, another study employing similar vaccine doses and an identical route of vaccination showed that a vaccination regimen based on MPB70 and MPB83 induced a minimal Th1 response as evidenced by the observed levels of IFN-γ and interleukin-2 (Wedlock et al. 2003). This discrepancy could potentially be attributed to variations in the genetic background of the cattle utilized (Wedlock et al. 2003). On the other hand, the administration of a DNA vaccine based on ESAT-6 and CFP10 resulted in increased frequencies of antigen-specific IFN-producing cells compared to control groups (Maue et al. 2007). These results suggest that immunisation with different antigens can lead to variable enhancements in both humoral and cellular immune responses, influenced by factors such as the specific antigen employed or the characteristics of the animal population selected. Therefore, the increased cellular and humoral responses observed in our study may be associated with the complex composition of the P22PI. Moreover, the multiple antigenic stimulation during the study period—including PTB vaccination, subsequent intradermal tests and P22PI immunisation—may not only have induced lymphocyte tolerance towards MTC members (Achkar et al. 2015; Boccasavia et al. 2021), affecting the protective outcomes of the experiment, but also could have interfered on the P22 ELISA results, as previously described (Roy et al. 2018b).

The OD levels of IGRA using PPDs, P22PI and ESAT-6 antigens were higher in immunised animals of naïve and pre-infected groups. However, no significant differences were reported between immunised and control goats from these groups. These results differed from published, where differences in PPD-B and P22PI-specific IFN-γ responses were significantly lower in vaccinated animals after exposure (Melgarejo et al. 2022). Additionally, Vordermeier and collaborators previously reported in cattle that the lack of ESAT-6-induced IFN-γ production *in vitro* within peripheral blood post-infection could serve as a robust indicator of the protective efficacy of BCG vaccination (Vordermeier et al. 2002). Similar findings have been observed in primate models of human TB, as previously reported (Langermans et al. 2001). In this context, optimal protection was achieved by administering an Ag85B, MPB64, and MPB83 DNA vaccine in combination with *M. bovis* BCG (Cai et al. 2006) or an ESAT-6: CFP10 DNA vaccine in conjunction with *M. bovis* BCG (Maue et al. 2007). Cai and collaborators demonstrated that a DNA vaccine prime followed by a BCG boost significantly reduced *M. bovis* levels in the lungs of calves infected with virulent *M. bovis* compared to calves immunised with the DNA vaccine alone or BCG alone (Cai et al. 2006). Similarly, Maue and collaborators observed that the combined administration of *M. bovis* BCG and ESAT-6: CFP10 + GMCSF + CD80/CD86 DNA vaccine provided the highest level of protection compared to administration of either the DNA vaccine alone or BCG alone (Maue et al. 2007). These findings suggest that integrating P22PI into similar combined vaccine regimens could potentially enhance the protective immune response against TB.

Regarding the post-mortem analysis, TB-compatible lesions were detected mainly in the lungs and local LNs of both groups, although lesions in extrathoracic LNs, liver and spleen were also reported. Our findings reveal that the immunisation protocol with P22PI used in the present study did not effectively prevent infection in TB-free animals and failed to reduce TB pathology in pre-infected goats despite the progressive increase in the antibody response after the P22PI administration. Indeed, far from protecting, immunised animals exhibited increased severity of TB-compatible lesions compared to controls in both naïve and TB pre-infected groups. These results were similar to those reported in a previous study in humans (Achkar et al. 2015) but also in cattle, where vaccination with MPB70 or MPB83 did not lead to a protective effect against TB infection (Wedlock et al. 2003). Indeed, the calves vaccinated with MPB70 showed a higher incidence of severe lung lesions compared to controls, as we reported. Microscopically, necrotic TB-granulomas were predominant over solid granulomas in naïve and TB pre-infected immunised animals, contrasting with findings reported by other authors in BCG-vaccinated cattle (Dean et al. 2015; Salguero et al. 2017). This predominance was also observed in control subgroups; however, immunised animals exhibited 1.7 and 2.7 times more necrotic granulomas than control animals in the naïve and pre-infected groups, respectively. These results underscore the lack of protection conferred by P22PI administration, as late-stage granulomas are associated with significant necrosis, extensive tissue damage, higher bacterial loads and elevated disease transmission rates (Palmer et al. 2022), factors closely linked to the detection of *M. bovis* reported in all immunised animals analysed using culture (Hai et al. 2019). Additionally, the widespread anatomical presence of tuberculous lesions in non-pulmonary LNs and the spleen in Immunised animals implies that the P22PI immunisation strategy employed may have negatively impacted the containment of mycobacteria locally, potentially favouring their lymphatic dissemination. Notably, these animals exhibited increased antibody levels against P22PI, which could be associated with accelerated disease progression and more severe lesions, as previously observed in humans (Bitencourt et al. 2021).

A key strength of this study lies in its utilisation of a natural transmission model, providing valuable insights into the potential effectiveness of P22PI immunisation under field conditions. Moreover, the inclusion of young animals, a practice standardised in previous studies (Pérez de Val et al. 2016; Roy et al. 2018a), is crucial in TB studies due to their susceptibility and role in disease transmission dynamics (Byrne et al. 2022). However, a limitation of this study is the assessment of a single concentration of P22PI

immunostimulant, which restricts our understanding of dose-dependent effects on protection or therapy efficacy. Additionally, the initial health status of the animals was unknown, and the possibility of pre-existing differences in lesion severity between subgroups at the beginning of the study cannot be excluded.

Therefore, under the particular conditions of the present study, the potential of the P22PI immunisation strategy to trigger a strong humoral response was demonstrated, which, far from being associated with protection, could have favoured disease progression. Future studies should investigate the underlying mechanisms driving this observed exacerbation of disease pathology, thus advancing our understanding of TB vaccine development.

Ethics approval

The animal experimental procedures were authorised by an institutional ethical committee and approved by the local authorities (PROEX 245.8/20, Comunidad de Madrid).

Author contributions

Concept formulation: JO, IAR, LD, MAR and JB. Methodology: JO, IAR, AR, IM, AGB, BR, EFC, LJ, MAR and JB. Data analysis: JO, IAR, LD, MD, MAR and JB. Writing the original draft: JO, IAR, MAR and JB. Critical revisions: all the authors. Funding acquisition: MD, MAR and JB.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The original contributions presented in the study are included in the article. Any materials utilized in this manuscript are publicly accessible in the referenced publications.

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