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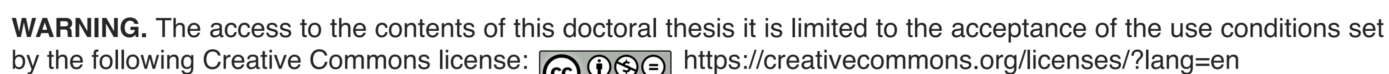
WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license:  https://creativecommons.org/licenses/?lang=en

Study of vaccines and new strategies for the control of goat tuberculosis

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PhD Thesis

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Study of vaccines and new strategies for the control of goat tuberculosis

Tesi doctoral presentada per **Claudia Cecilia Arrieta Villegas** per accedir al grau de Doctor en el marc del programa de Doctorat en Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció del Dr. **Bernat Pérez de Val** i tutoria del Dr. **Mariano Domingo Álvarez**.

Bellaterra, 23 de Setembre 2021

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Certifiquen:

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Doctorand

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A mi familia y a Salva

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LIST OF ABBREVIATIONS

APC	Antigen presenting cells
aPPD	Avian purified protein derivative
BCG	*Mycobacterium bovis* Bacille Calmette-Guérin
BCG LONFIELDT	Study I: Long-term efficacy of BCG vaccination in goat herds with a high prevalence of tuberculosis
BCG REVAC	Study II: Immunogenicity and protection against *M. caprae* challenge in goats vaccinated with BCG and revaccinated one year after
bPPD	Bovine purified protein derivative
CFP-10	Culture filtrate protein 10
CMI	Cell-mediated immune response
DC	Dendritic cells
DIVA	Differentiating infected from vaccinated animals
DTH	Delayed-type hypersensitivity
EC	Antigen cocktail based on ESAT-6 and CFP-10 proteins
ELISA	Enzyme-linked immunosorbent assay
EM	Environmental mycobacteria
ESAT-6	Early secreted antigen target 6 kDa
HIMB	Heat-inactivated *Mycobacterium bovis*
HIMB VAC	Study III: Efficacy of parenteral vaccination against

	tuberculosis with HIMB in experimentally challenged goats
IFN-γ	Interferon gamma
IGRA	Interferon gamma release assay
IL	Interleukin
LN	Lymph nodes
LTBI	Latent tuberculosis infection
MAC	*Mycobacterium avium* complex
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
MHC	Major histocompatibility complex
MTBC	*Mycobacterium tuberculosis* complex
NK	Natural killer cells
NTM	Non-tuberculous mycobacteria
OIE	Office International des Epizooties – World Organization for Animal Health
OTF	Officially bovine tuberculosis free country
p.c.	Post challenge
p.v.	Post vaccination
P22 BCGDIAG	Study IV: Evaluation of P22 antigenic complex for the immune-diagnosis of tuberculosis in BCG vaccinated and unvaccinated goats
PBS	Phosphate buffer saline

RD	Region of difference
SCIT	Single intradermal comparative test
Se	Sensitivity of diagnostic tests
SFT	Skinfold thickness
SIT	Single intradermal tuberculosis test
Sp	Specificity of diagnostic tests
ST	Skin tests
TB	Tuberculosis
TFP	Tetrafusion protein
TNF-α	Tumor Necrosis factor alpha
WHO	World Health Organization
wpc	Weeks post challenge

ABSTRACT

Tuberculosis (TB) is an infectious disease caused by different members of the *Mycobacterium tuberculosis* complex (MTBC) which can affect a wide range of hosts, including different species of domestic and wild animals and humans. TB is still an important animal health issue with a high economic impact worldwide. In domestic animals, such as goats, TB control programs based on test-and-slaughter of positives are not always feasible. Therefore, the interest in the research on TB vaccination and vaccine-associated diagnostic tests for the disease control in animals has been renewed. In this regard, the present thesis provided insights into the efficacy of different vaccination strategies against caprine TB and vaccine-associated diagnostic tests in goats.

The first study evaluated the efficacy in the field of a long-term vaccination of goats with the live-attenuated Bacilli Calmette-Guérin (BCG) vaccine (Danish 1331 strain) in five goat farms with different characteristics and management. The systematic vaccination of replacement goat kids with BCG contributed to a progressive reduction of TB incidence and prevalence in goat herds. The second study addressed the duration of immune responses elicited by BCG in goats and the efficacy of BCG vaccination and revaccination after one year. The lifespan of immune responses evoked by BCG vaccination was lower than one year and did not confer significant protection after *M. caprae* experimental challenge. On the contrary, BCG revaccination after one year of first vaccination provided better protection against *M. caprae* challenge. The third study evaluated the efficacy of the Heat-Inactivated *Mycobacterium bovis* (HIMB) vaccine candidate in experimentally *M. caprae* infected goats. Parenteral vaccination with HIMB showed similar protection to parenteral BCG vaccination, paving the way for its evaluation under field conditions. The fourth study assessed the performance of the antigen complex P22 (obtained by immunopurification of *M.*

bovis tuberculin) for cell-mediated and serological immunodiagnosis of TB in goats under different vaccination contexts: BCG and *Mycobacterium avium* subesp. *paratuberculosis* (MAP) vaccination and MAP vaccination alone. In BCG and MAP vaccinated animals, combined interpretation of serological diagnostic using P22 and skin tests was a performant TB diagnostic strategy in terms of specificity and sensitivity. In all studies of the present thesis, the antigen cocktail based on ESAT-6 and CFP-10 proteins, applied for the Interferon-gamma release assay, was used for Differentiating infected from vaccinated animals (DIVA). Any diagnostic interference reaction was detected neither in BCG nor in BCG revaccinated, nor in HIMB vaccinated goats, confirming the suitability of ESAT-6 and CFP-10 antigen cocktail as a DIVA diagnostic test.

Overall findings of the present thesis encourage the use of vaccines against TB and the vaccine-associated diagnostic tests as a useful strategy for control of TB in goats.

RESUMEN

La tuberculosis (TB) es una enfermedad infecciosa causada por diferentes miembros del complejo *Mycobacterium tuberculosis* (MTBC), la cual puede afectar a una amplia variedad de huéspedes, incluidos los seres humanos y diferentes especies de animales domésticos y salvajes. La TB sigue siendo un importante problema de salud animal con un alto impacto económico en todo el mundo. En los animales domésticos, como por ejemplo las cabras, los programas de control de la TB basados en la prueba y el sacrificio de los animales positivos no siempre se pueden llevar a cabo. Por este motivo, durante las últimas décadas se ha renovado el interés por la investigación sobre la vacunación antituberculosa y las pruebas de diagnóstico asociadas a la vacuna para el control de la TB animal. En este sentido, la presente tesis aporta información sobre la eficacia de diferentes estrategias de vacunación contra la tuberculosis en cabras y las pruebas de diagnóstico asociadas a la vacuna en esta especie.

El primer estudio evaluó la eficacia en el campo de una vacunación a largo plazo de cabras con la vacuna viva atenuada de *Mycobacterium bovis* Bacilo Calmette-Guérin (BCG) (cepa danesa 1331) en cinco granjas de cabras con diferentes características y manejo. La vacunación sistemática de cabritos de la reposición con BCG contribuyó a una reducción progresiva de la incidencia y de la prevalencia de la TB en los rebaños de cabras. El segundo estudio abordó la duración de las respuestas inmunes provocadas por la BCG en cabras, así como la eficacia de la vacunación y revacunación con BCG después de un año. La duración de la respuesta inmunitaria inducida por la vacunación con BCG fue inferior a un año y no confirió una protección significativa frente a la infección experimental con *M. caprae*. Por el contrario, la revacunación con BCG después de un año de la primera vacunación proporcionó una mejor protección contra el

desafío con *M. caprae*. En el tercer estudio se evaluó la eficacia de un candidato vacunal basado en *Mycobacterium bovis* inactivado por calor (HIMB) en cabras desafiadas experimentalmente con *M. caprae*. La vacunación parenteral con HIMB mostró una protección similar a la conferida por la vacuna BCG parenteral. Estos hallazgos abren el camino para su evaluación en condiciones de campo. El cuarto estudio evaluó el desempeño del complejo antigénico P22 (obtenido por inmunopurificación de la tuberculina de *M. bovis*) para el inmunodiagnóstico (serológico y mediado por células) de la TB en cabras. Este estudio se llevó a cabo en diferentes contextos de vacunación: vacuna BCG y vacuna contra la paratuberculosis (*Mycobacterium avium* subesp. *paratubercuclosis* - MAP) y vacunación contra MAP sola. En animales vacunados con BCG y MAP, la interpretación combinada del diagnóstico serológico utilizando P22 y pruebas cutáneas fue una estrategia de diagnóstico eficaz en términos de especificidad y sensibilidad. En todos los estudios de la presente tesis se utilizó el cóctel de antígenos basado en las proteínas ESAT-6 y CFP-10, aplicado para el ensayo de liberación de interferón-gamma, para la diferenciación de animales infectados de vacunados (DIVA). No se detectó ninguna reacción de interferencia diagnóstica ni en cabras vacunadas ni revacunadas con BCG, así como tampoco en cabras vacunadas con HIMB, lo que confirma la idoneidad del cóctel antigénico ESAT-6 y CFP-10 como prueba de diagnóstico DIVA.

Los hallazgos generales de la presente tesis apoyan el uso de vacunas contra la tuberculosis y de las pruebas de diagnóstico asociadas a la vacunación como una estrategia útil para el control de la TB en cabras.

RESUM

La tuberculosi (TB) és una malaltia infecciosa causada per diferents membres del complex *Mycobacterium tuberculosis* (MTBC), que pot afectar un ampli ventall d'hostes, inclosos els éssers humans i diferents espècies d'animals domèstics i salvatges. La TB continua sent un problema important de salut animal amb un elevat impacte econòmic a tot el món. En animals domèstics, com ara les cabres, els programes de control de la tuberculosi basats en la prova i el sacrifici dels animals positius no sempre són factibles. Per tant, en les últimes dècades s'ha renovat l'interès en la investigació sobre la vacunació contra la tuberculosi i les proves diagnòstiques associades a la vacuna per al control de la malaltia en els animals. En aquest sentit, la present tesi aporta informació sobre l'eficàcia de diferents estratègies de vacunació contra la TB caprina i les proves diagnòstiques associades a la vacuna en cabres.

En el primer estudi es va avaluar l'eficàcia en el camp de la vacunació a llarg termini de cabres amb la vacuna atenuada viva *Mycobacterium bovis* Bacil Calmette-Guérin (BCG) (soca danesa 1331) en cinc granges caprines amb diferents característiques i maneig. La vacunació sistemàtica de cabrits per a la reposició amb BCG va contribuir a una reducció progressiva de la incidència i de la prevalença de TB en els ramats de cabres. El segon estudi va tractar la durada de les respostes immunes induïdes per la BCG en cabres i l'eficàcia de la vacunació i revacunació amb BCG al cap d'un any. La durada de les respostes immunes induïdes per la vacunació amb BCG va ser inferior a un any i no va conferir una protecció significativa després del desafiament experimental amb *M. Caprae*, ans el contrari, la revacunació amb BCG després d'un any de la primera vacunació va proporcionar una millor protecció contra la infecció amb *M. caprae*. En el tercer estudi es va avaluar l'eficàcia d'un candidat vacunal basat en *Mycobacterium bovis* inactivat per calor (HIMB) en cabres desafiades

experimentalment amb *M. caprae*. La vacunació parenteral amb HIMB va conferir una protecció similar a la vacunació parenteral amb BCG, obrint el camí per a la seva avaluació en condicions de camp. En el quart estudi es va avaluar el complex antigènic P22 (obtingut per immunopurificació de la tuberculina del *M. bovis*) com a eina per al diagnòstic immunològic (humoral i mediat per cèl·lules) de la TB en cabres. L'estudi es va dur a terme en diferents contextos de vacunació: vacunació amb BCG i contra la paratuberculosi (*Mycobacterium avium* subesp. *paratuberculosis* - MAP) i vacuna MAP sola. En animals vacunats amb BCG i MAP, la interpretació combinada del diagnòstic serològic mitjançant P22 i proves cutànies va resultar en una bona estratègia de diagnòstic de la TB en termes d'especificitat i sensibilitat. En tots els estudis de la present tesi, el còctel d'antigen basat en les proteïnes ESAT-6 i CFP-10, aplicat per a l'assaig d'alliberament d'interferó-gamma, es va utilitzar per diferenciar els animals infectats dels vacunats (DIVA). No es va detectar cap reacció d'interferència diagnòstica en els animals vacunats i revacunats amb BCG ,ni en cabres vacunades amb HIMB, fet que confirma la idoneïtat del còctel antigènic ESAT-6 i CFP-10 com a prova diagnòstica DIVA.

En síntesi, les troballes de la present tesi encoratgen a l'ús de vacunes contra la TB i de les proves diagnòstiques associades a la vacunació com a estratègia útil per al control de la TB en cabres.

PUBLICATIONS

The results presented in this Thesis have been published in international scientific peer-reviewed journals:

- Arrieta-Villegas, C., Vidal, E., Verdés, J., Moll, X., Espada, Y., Singh, M., Villareal-Ramos, B., Domingo, M., and Pérez de Val, B. Immunogenicity and protection against *Mycobacterium caprae* challenge in goats vaccinated with BCG and revaccinated one year after. *Vaccines* 2020; 8, 751, pp. 1-12. DOI: 10.1038/s41598-020-77334-1.

- Arrieta-Villegas, C., Allepuz, A., Grasa, M., Martín, M., Cervera, Z., Mercader, I., López-Soria, S., Domingo, M., and Pérez de Val, B. Long-term efficacy of BCG vaccination in goat herds with a high prevalence of tuberculosis. *Scientific Reports*, 2020; 10, 2039, pp. 1-16. DOI: 10.3390/vaccines8040751.

- Arrieta-Villegas, C., Infantes-Lorenzo, JA., Bezos, J., Grasa, M., Vidal, E., Mercader., I., Singh, M., Domingo, M., de Juan, L., and Pérez de Val. Evaluation of P22 antigenic complex for the immuno-diagnosis of tuberculosis in BCG vaccinated and unvaccinated goats. *Frontiers in Veterinary Science*, 2020, Vol 7, 347, pp. 1-9. DOI: 10.3389/fvets.2020.00374.

- Arrieta-Villegas, C., Perálvarez, T., Vidal, E., Puighibet, Z., Moll, X., Canturri, A., Sevilla, IA., Espada, Y., Juste, RA., and Pérez de Val, B. Efficacy of parenteral vaccination against tuberculosis with heat-inactivated *Mycobacterium bovis* in experimentally challenged goats. *Plos One*, 2018 Vol 13(5):e0196948, pp. 1-14. DOI: 10.1371/journal.pone.0196948.

CHAPTER 1

General Introduction

1.1. HISTORY OF TUBERCULOSIS

Tuberculosis (TB) is an infectious disease caused by bacteria from *Mycobacterium tuberculosis* complex (MTBC). Throughout history, TB disease has killed more persons than any other microbial pathogen. In ancient Greece, TB was called Phtisis, and Hipocrates (460-377 BCE) defined it as the "most serious disease, the most difficult to cure and the most lethal". The infectious nature of tuberculosis was first hypothesized during the Roman Empire and Galeno (131-201) continued to promulgate it. However, the transmissibility of TB was not demonstrated until 1865. Jean Antoine Villemin (1827-1892) in his study "Cause et nature de la tuberculose: son inoculation de l'homme au lapin", proved that TB lesions from cattle inoculated with human TB, can be transmitted to rabbits and guinea pigs, evidencing the zoonotic potential of TB and postulating that the disease was caused by a specific microorganism. (Villemin, 1865). Later, on the 24th of march of 1882, the microbiologist Robert Koch (1843-1910) discovered the bacterial aetiology of TB. He reproduced the TB infection in guinea pigs by inoculating them with infectious material from humans and then performing culture of lung tubercles and subsequent staining of the bacilli with methylene blue (Koch, 1982).

The origin of human TB date back to Neolithic period (Buzic and Giuffra, 2020). First evidence of lesions suggesting bony tuberculous lesions in humans was observed in Egyptian mummies dating back to 3000-2500 BCE (Nerlich and Lösch, 2009). In animals, the first attempt to identify animal tuberculosis was carried out using DNA from lesions suggestive of tuberculosis from an extinct long-horned bison dating from the Pleistocene (17000 BP), attributing an ancient origin to animal TB caused by *Mycobacterium bovis* (Rothschild et al., 2001). However, the hypothesis that human TB (caused by *M. tuberculosis)* had evolved from bovine tuberculosis (caused by *M. bovis)* was disproved thanks to

the whole genome sequencing of both organisms (Cole et al., 1998; Garnier et al., 2003). The genetic diversity among MTBC members is mainly given by extensive sequence polymorphisms, such as Regions of difference (RD) (Brosch et al., 1998) and given that the events generating the RD are mainly unidirectional, the phylogeny of MTBC members could be inferred (Brosch et al., 2002). The common ancestor of MTBC species is closely related to the "modern" *M. tuberculosis* and *M. bovis* is the most distant species (Rodriguez-Campos et al., 2014). Thus, the RD of *M. bovis* with respect to *M. tuberculosis* are deletions rather than insertions in the *M. tuberculosis* genome (Gordon et al., 2001, 1999). Under this perspective, it is possible that selective pressure may induce host shifts, entailing changes in the ancestral *M. tuberculosis*, triggering the adaptation to different animal hosts and originating the different "ecotypes" of the MTBC (Figure 1.1). Thus, the evolution of the *M. tuberculosis* ancestor in domestic animals could be linked to the domestication process. Given that the maintenance hosts of *M. origys, M. bovis* and *M. caprae* (*Bos indicus, Bos taurus* and goats, respectively) are domestic species, it is possible that the *M. tuberculosis* common ancestor was transmitted from human populations to cattle and other livestock species, and the proximity between hosts along with high contact rates could determine the host range and specialization (Brites et al., 2018).

1.2. EPIDEMIOLOGY OF TUBERCULOSIS

TB can be transmitted from animals to humans, which is known as a zoonosis, and conversely, humans can transmit TB to animals, which is known as anthropozoonosis. The interdisciplinary approach in the prevention and control of the global epidemic of TB, implicating both veterinary and public health authorities, also called One Health, has been largely supported by international

organizations such as the World Health Organization (WHO), the world organization for animal health (Office International des Epizooties -OIE) and the Food and Agriculture Organization (FAO) from the United Nations.

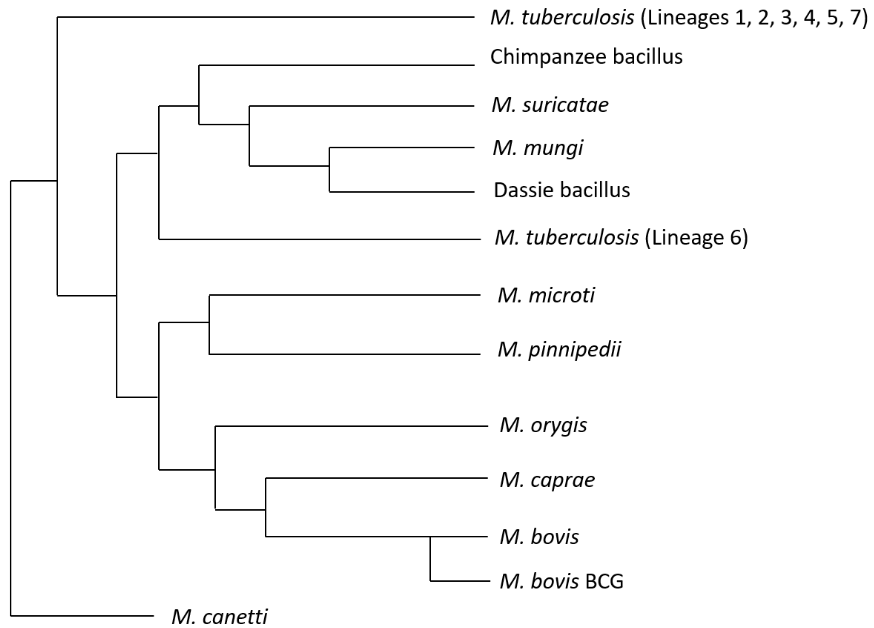


Figure 1.1. Phylogenetic tree of animal adapted *Mycobacterium tuberculosis* complex members. Adapted from (Brites et al., 2018).

1.2.1. Human tuberculosis

In 2019, approximately 10.0 million of people became ill with *M. tuberculosis* worldwide (WHO, 2020) and it has been estimated that one fourth of the world's population has latent TB infection (LTBI) (Cohen et al., 2019). However, it has been accepted that 5-10% of individuals with LTBI will develop active TB at some point of their lives (CDC, 2011). Moreover, recent evidence points that some infected people will clear the mycobacteria early after infection (Koeken et al., 2019; Verrall et al., 2020).

The incidence of TB in 2019 was estimated at 10 million new cases, from which 8.2% were HIV-positive people. The majority of new TB cases were located (Figure 1.2) in the WHO region of South-Est Asia (44%), Africa (25%) and the Western Pacific (18%). Two-thirds of the global total new TB cases were accumulated by eight countries: India (26%), Indonesia (8.5%), China (8.4%), the Philippines (6%), Pakistan (5.7%), Nigeria (4.4%), Bangladesh (3.6%) and South Africa (3.6%) (WHO, 2020).

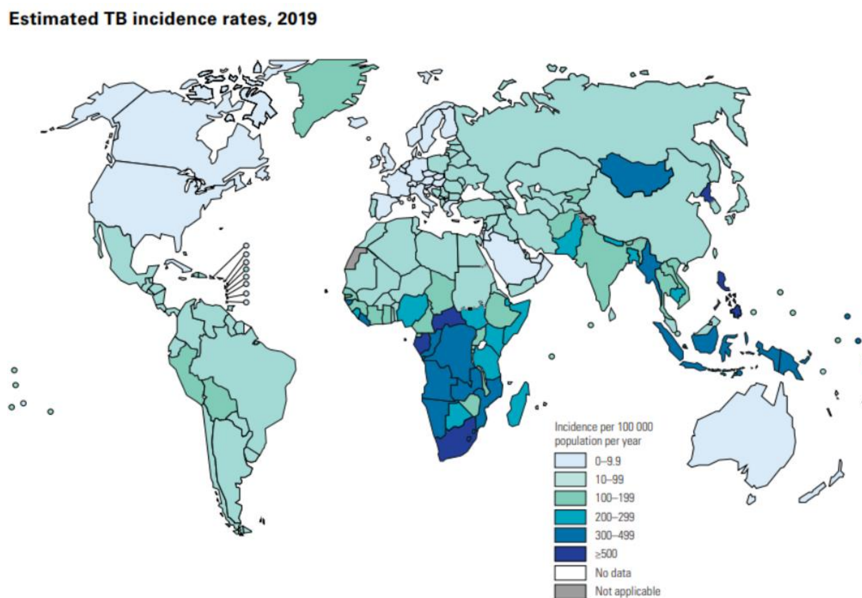


Figure 1.2. Estimated TB incidence rates in humans worldwide. (Source: Global tuberculosis report 2020. WHO, 2020. Available at: https://www.who.int/teams/global-tuberculosis-programme/tb-reports).

Moreover, antimicrobial TB resistance is still a major pitfall for public health worldwide. In 2019, around half a million people developed rifampicin-resistant TB (RR-TB), of which 78% had multidrug-resistant TB (MDR-TB, resistant to at least rifampicin and isoniazid) (WHO, 2020). India, China and the Russian federation were the countries with the largest number of drug-resistant TB cases. In 2016, it was estimated that 6.2% of people with MDR-TB have extensively drug-resistant TB (XDR-TB, resistant to isoniazid, rifampicin, at least one fluoroquinolone and one second line drug, such as amikacin, kanamycin, or capreomycin) (WHO, 2017a).

Current estimates suggest that, in 2019, 1.2 million HIV-negative people died from TB and 208,000 deaths were estimated among HIV-positive people. Compared to data from the year 2000, there was a reduction in the number of deaths, 0.5 million among HIV-negative and 462,000 from HIV positive people. However, due to the COVID-19 pandemic, an increase of 0.2-0.4 million deaths in 2020 is expected due to disruption in health services, affecting the detection and treatment of TB (WHO, 2020). On the other hand, predictions of the economic impact of pandemics suggest that the loss of incomes or unemployment will increase the number of people with TB, which is foreseen to one million of excess per year in the period 2020-2025 (WHO, 2020). Thus, for the next few years, efforts for control and eradication of human TB need to be intensified.

On the other hand, the real burden of Zoonotic TB remains underrated. In 2016, 147,000 new cases of zoonotic TB were estimated worldwide, and 125,000 people died because of the disease. The majority of cases were reported in the African region, followed by the South-East Asian region. (WHO, 2017b). In Europe, Zoonotic TB due to *M. bovis* or *M. caprae* accounted for 147 confirmed

human cases, and 69.4% of them were from not officially bovine tuberculosis free (non-OTF) countries (EFSA, 2021).

1.2.2. Animal tuberculosis

From a holistic point of view, animal tuberculosis is the infection of mammals caused by bacteria belonging to the MTBC. Despite all members of MTBC cause the disease in a broad range of domestic and wild animals, *M. bovis* is considered the main causative pathogen of animal TB and zoonotic TB (OIE, 2021a). The term "bovine tuberculosis" is often used to refer to the infection not only in bovines but in other animal hosts. Consequently, the majority of efforts for the control and eradication of animal TB are focused on cattle. In fact, despite the OIE the Terrestrial Animal Health Code from 2019 (OIE, 2021b) includes diseases caused by MTBC members in different species, such as goats, cervids and new world camelids, “bovine tuberculosis” remains the denomination used in the list of OIE notifiable diseases (OIE, 2021a). In any case, animal TB is still an important public health issue, particularly in developing countries, where it is estimated to be responsible for 10% of human tuberculosis cases (OIE, 2021a).

Animal TB has a wide distribution but the presence of the disease is heterogeneous among territories. Over 30 years (1986-2016) of reported data to the OIE, an overall reduction of more than 30% of notified TB cases evidenced a significant improvement on global control of the disease (Awada et al., 2018) and between January 2017 and June 2018, 82 out of 188 countries and territories reported bovine tuberculosis (Figure 1.3) (OIE, 2019). Nevertheless, the capacity of countries to monitor the evolution of TB infection differs, and not all countries provide detailed information to the OIE. During 2005-2015, 89% of total reported cases were from the regions of the Americas and Europe, showing

a significant decrease in TB cases over the period. Similarly, important reductions in TB cases were reported in Oceania. On the contrary, in the regions of Africa and Asia, a significant increase in the number of bovine TB cases was recorded (Awada et al., 2018). The strong reductions in TB cases reported in the Americas, Europe and Oceania are probably due to adequate eradication policies, allowing a more efficient control of the disease.

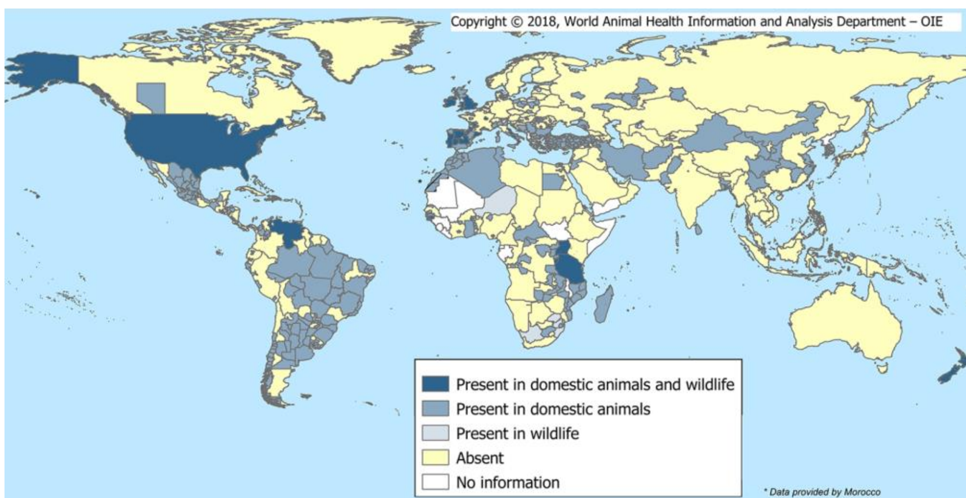


Figure 1.3. Global distribution of bovine tuberculosis between 2017 and first semester of 2018. (Source: OIE bulletin Panorama 2019-1: Controlling bovine tuberculosis: a One Health challenge. Dossier: Bovine tuberculosis: global distribution and implementation of prevention measures according to WAHIS data. Available at: https://oiebulletin.com/?page_id=115).

In developed countries, the strategy for TB eradication is based on *in vivo* diagnostic tests, such as the Single intradermal tuberculin test (SIT) or the Single intradermal cervical comparative tuberculin tests (SCIT), followed by the slaughter of positives. In recent years, despite efforts to reduce bovine TB by the strategy of test and slaughter, reductions in cases have stagnated in developed countries, such as Europe or New Zealand (EFSA, 2021; Nugent et al., 2018a),

probably due to the presence of other reservoir species, such as goats, sheep or wildlife animals, which may maintain the mycobacteria in the environment. For instance, authors argue that the presence of wildlife reservoirs in territories is a serious challenge due to potential recirculation of TB between cattle and wild mammal hosts, compromising the achievement of TB eradication (Gortazar et al., 2015; Nugent et al., 2018a). Therefore, the limited availability of epidemiological information from reservoir species is an important concern that might be urgently addressed.

In this context, animal tuberculosis continues to be a relevant issue for animal health entailing huge socio-economic costs for livestock industries and authorities. For farmers, the reduction in milk production of chronically TB infected cattle may entail important losses (Boland et al., 2010). The slaughter and replacement of positive animals, as well as the limitation of animal movements from herds, may increase the costs of TB disease for farmers. The overall resulting costs of bovine TB are highly related to the context. For instance, in developed countries, which usually have low TB prevalences, the main costs are related to trade barriers to live animals and animal products, and to the implementation of eradication programs based on TB testing by veterinarians (OIE, 2019). Moreover, the presence of wildlife reservoirs, such as wild boars, badgers, white-tailed deer or brushtail possums, hinder and increase the costs of eradication programs (Azami and Zinsstag, 2018). On the other hand, in the majority of developing countries, there is still a lot of work to be made on the awareness of the animal TB, and its burden remain to be estimated (Azami and Zinsstag, 2018). For instance, one estimation of costs of bovine TB in Ethiopia determined that an eradication program based on test and slaughter of positives was economically and logistically unachievable and thus, other

control strategies such as milk pasteurization, meat condemnation and control of animal movements must be considered (Tschopp and Aseffa, 2016).

In 2019, 17 member states of the European Union were officially bovine tuberculosis free (OTF) (Austria, Belgium, Czechia, Denmark, Estonia, Finland, France, Germany, Hungary, Latvia, Lithuania, Luxembourg, Netherlands, Poland, Slovakia, Slovenia and Sweden) and 11 were non-OTF (Bulgaria, Croatia, Cyprus, Grece, Ireland, Italy, Malta, Portugal, Romania, Spain and the United Kingdom) (Figure 1.4). It is important to consider that, four out eleven non-OTF countries have some OTF regions, which are Italy (8 regions and 14 provinces), Portugal (the Algarve region), Spain (the province of Pontevedra and the Canary Islands), and the United Kingdom (Scotland and the Isle of Man). The overall proportion of cattle herds infected with TB in the UE was very low (0.8%). In the 11 non-OTF member states 1.8% of cattle herds were reported as positive for bovine TB. In recent years, in the non-OTF regions, the annual prevalence of bovine tuberculosis reported in herds was heterogeneous, with a prevalence above 10% for Wales, England and Northern Ireland, and a low prevalence (between 2-5%) for Greece, Ireland and Spain, and a very low prevalence (<1%) for Italy and Portugal (EFSA, 2021).

In Spain, the programme for accelerated eradication of TB presented in 1987 (UE directives 77/931/CEE and 78/52/CEE), based on test and slaughter of positive cattle, allowed to a significant improvement of TB status throughout years, reducing the herd prevalence from 11% to a <2% in 2004, which was maintained until 2014. In 2015, a relapse in herd TB prevalence and animal incidence was observed but in 2019, the trend returned to < 2%. This phenomenon is probably due to the efforts that have been made to increase diagnostic sensitivity which may emerge residual infections in herds (MAPA, 2021).

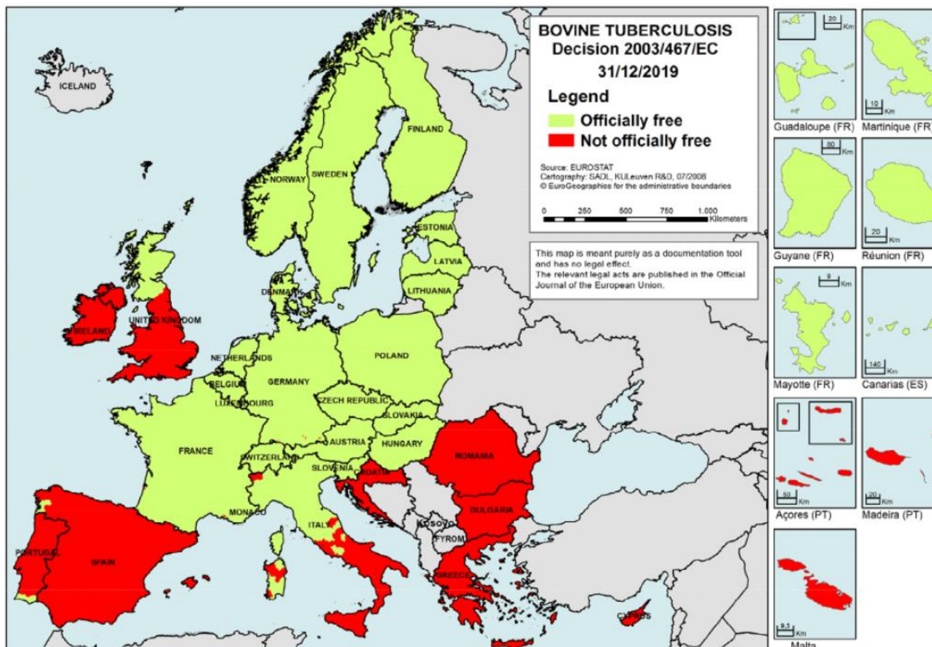


Figure 1.4. Status of countries on bovine tuberculosis EU/EA, 2019. (Source: EU One Health Zoonoses report 2019. EFSA Journal 2021; 19(2):6406. Available at: https://www.efsa.europa.eu/fr/efsajournal/pub/6406).

It is important to mention that differences in incidence among geographical areas in Spain are significant. The northern autonomous communities (Basque Country, Galicia and Asturias) have low rates of TB incidence in cattle (Figure 1.5), whereas in the South and Centre of the country (Andalusia, Castilla-La Mancha, Extremadura and Madrid), the incidence is higher (MAPA, 2021). These differences are likely due to the extensive production system, particularly in beef cattle in the south west and centre of Spain, and the presence of wildlife reservoirs. In extensive management systems, beef cattle share pasture and water sources with animals from different farms, such as goats, and with wild animals such as wild boar and red deer (Vicente et al., 2007).

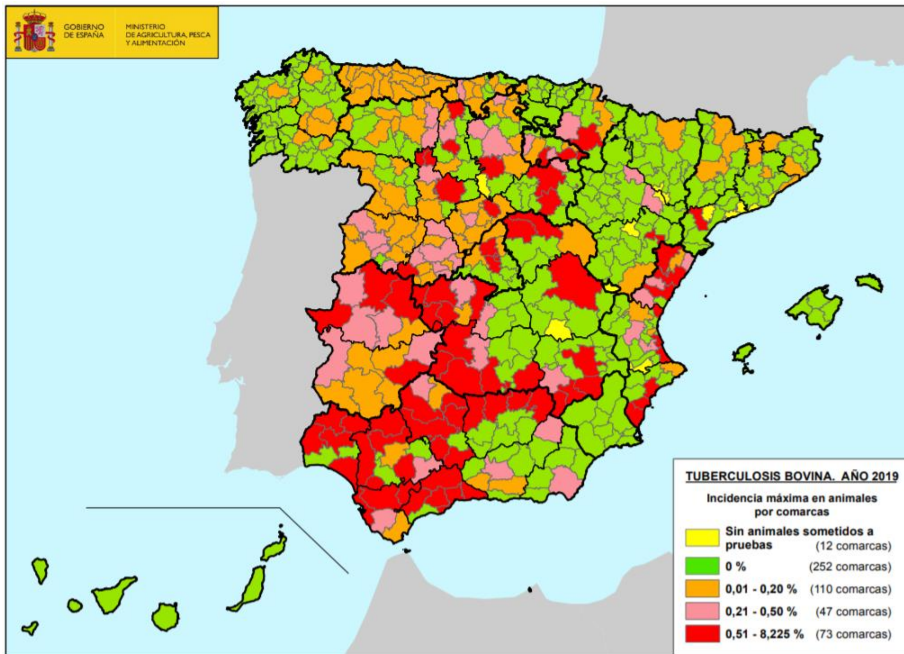


Figure 1.5. Incidence of bovine tuberculosis in cattle in Spanish districts in 2019. (Source: Programa nacional de erradicación de tuberculosis bovina 2021 (Infección por el complejo *Mycobacterium tuberculosis)*. Versión abril 2021. (MAPA, 2021).

1.2.3. Tuberculosis in goats

Caprine TB was long time believed to be a rare disease and related to the spread of TB in cattle (O'Reilly and Daborn, 1995). However, in the last decades, several cases of caprine TB have been reported worldwide (Cadmus et al., 2009; Higino et al., 2011; Naima et al., 2011). Considering that TB in goats has been reported in underdeveloped countries, and the vast majority of caprine census around the world is located in those countries (mainly in Asia and Africa) (FAOSTAT 2021, accessed on 17/08/21), where TB is highly endemic in humans and cattle, TB in goat flocks may pose a serious socio-economic burden for farmers and public health.

In Europe, caprine TB has been reported in Ireland (Shanahan et al., 2011), UK (Daniel et al., 2009) and particularly in Mediterranean countries and Portugal (Duarte et al., 2008; Napp et al., 2013; Quintas et al., 2010). In Spain, the main causative agent of caprine TB is *M. caprae* (Aranaz et al., 2003), representing 7.4% of isolates of MTBC from domestic and wild animals (Rodríguez et al., 2011). Besides, *M. caprae* has also been isolated in goats from other Mediterranean countries, such as Italy (Amato et al., 2018; Boniotti et al., 2009), and Greece (Ikonomopoulos et al., 2006).

In Europe, goat TB is of special concern for countries with high census and significant goat livestock industry, such as Greece and Spain (FAOSTAT 2021, accessed on 17/08/21). In intensive dairy goat production, a high density of animals housed all year is common, promoting a rapid transmission of TB within herds and high mortality rates (Michel, 2018). Thus, TB disease in goat herds may result in significant economic losses due to reduced milk production (Cvetnic et al., 2007), trade restrictions (Daniel et al., 2009), and may be a source of TB for humans (Prodinger et al., 2014) and other domestic and wild animals (Cano-Terriza et al., 2018; Ciaravino et al., 2020; Napp et al., 2013), hampering the efforts that have been made for eradication of the disease in cattle.

In Spain, caprine TB is considered endemic and studies suggest that prevalence in goat herds is high (Bezos et al., 2014b; Rodríguez et al., 2011). However, there is no unified official data of TB prevalence in goats, and only scarce information from local studies is available. This is likely due to the fact that caprine TB is not an OIE notifiable disease and goats are not subjected to an official eradication program in the UE, unless for goat flocks epidemiologically linked with cattle (MAPA, 2021). Still, several Spanish Autonomous Communities have currently implemented local programs for control of goat TB

(Murcia, Castilla y León, Andalucía, Extremadura, Valencia, Catalonia, Castilla-La Mancha). Nonetheless, the epidemiological situation of caprine and ovine TB in Spain deserves further study, in order to improve the decision making relative to the eradication of the disease in the country.

1.3. ETIOLOGY

1.3.1. General characteristics

The Mycobacteria belongs to the Phylum *Actinobacteria*, class *Actinobacteria*, order *Actinomycetales*, suborder *Corynebacterineae*, family *Mycobacteriaceae* and genus *Mycobacterium*. The genus *Mycobacterium* counts 194 species (List of procaryotic names with standing nomenclature. Source: lpsn.dmz.de/genus/mycobacterium Accessed on 17/08/21) which are aerobic, immobile, non-spore-forming and are characterized by acid-alcohol fastness, weak gram positivity, curved-rod shape, and high DNA G+C content. For practical purposes, the members of the genus can be classified depending on their fast or slow-growth in culture. Another classification used is *M. tuberculosis* complex (MTBC), and non-tuberculous mycobacteria (NTM) (Forbes et al., 2018).

The slow-growing mycobacteria include the MTBC and the *Mycobacterium avium* complex (MAC) (Gupta et al., 2018), which includes mycobacteria with important implications for veterinary and public health (Table 1.1). Concerning the MAC, one of the most important members for animal health is the *M. avium* subspecies *paratuberculosis* (MAP), which is the causative agent of paratuberculosis disease in ruminants (Barkema et al., 2010).

Table 1.1. Species of *Mycobacterium tuberculosis* complex and *Mycobacterium avium* complex.

Mycobacterium tuberculosis complex (MTBC)[a]	**Mycobacterium avium complex (MAC)[b]**
M. tuberculosis	*M. avium* subspecies *avium*
M. africanum West-African 1	*M. avium* sp *paratuberculosis*
M. africanum West-African 2	*M. avium* sp *hominisuis*
M. microti	*M. avium* sp *silvaticum*
M. mungi	*M. intracellulare*
M. suricatae	*M. colombiense*
M. pininipedi	*M. arosiense*
M. orygis	*M. vulneris*
M. caprae	*M. marseillense*
M. bovis	*M. timonense*
M. bovis BCG	*M. paraintracellulare*

a. Extracted from Rodriguez-Campos et al., 2014.
b. Extracted from Shin et al., 2020.

One of the special features of pathogenic mycobacteria is the complex structure of their cell envelope, particularly the cell wall and the capsule or outer membrane. The cell wall is composed of peptidoglycans, arabinogalactans and mycolic acids. The latter are long-chain fatty acids unique to the genera *Mycobacterium* and *Corynebacterium*. The particular cell envelope composition and organization confers hydrophobicity and plays a key role in driving the host-pathogen interactions and susceptibility to antibiotics (Dulberger et al., 2020; Jackson, 2014).

The species belonging to the MTBC cause TB in humans, and in a wide range of animal domestic and wild hosts. In animals, TB is mainly caused by *M. bovis*, *M. caprae* and *M. tuberculosis*, depending on geographical localizations.

1.3.2. MTBC members of interest for public and animal health

M. tuberculosis: Is the most important etiological agent of human TB, infecting about a quarter of the global population (WHO, 2020) and has also been isolated in domestic animals, such as cattle (Ameni et al., 2011), goats (Cadmus et al., 2009; Kassa et al., 2012), sheep (Arunmozhivarman et al., 2018), dogs (Martinho et al., 2013; Mentula et al., 2020), and in wild animals, including animals from zoos (Akhtar et al., 2019).

M. africanum: Is restricted to West Africa being responsible of the 50% of cases of human TB (de Jong et al., 2010). The progression of the infection seems to be slower (de Jong et al., 2008) and transmission rates are lower compared to *M. tuberculosis* in urban zones (Asare et al., 2018). In animals, *M. africanum* has been sporadically isolated in monkeys from central and West Africa (Thorel, 1980) and from cows (Rahim et al., 2007).

M. bovis: Is the main etiological agent of bovine TB but it can frequently affect other domestic species such as goats (Crawshaw et al., 2008; Daniel et al., 2009), sheep (Muñoz Mendoza et al., 2012) and other bovids such as bison (Wobeser, 2009), buffalos (Michel et al., 2007) and antelopes (Malama et al., 2014). Moreover, *M. bovis* has been isolated in humans (Nebreda-Mayoral et al., 2019), cats (Eroksuz et al., 2019), dogs (Rocha et al., 2017), and in a wide range of wildlife species such as wild boar and red deer (Madeira et al., 2017), badgers (Schroeder et al., 2020), and brushtail possums (Nugent et al., 2015) among others.

***M. caprae*:** It has been mainly reported in continental Europe (Prodinger et al., 2005). *M. caprae* is the main etiological agent of caprine TB in Spain (Aranaz et al., 2003; Rodríguez et al., 2011) and is responsible for 7.4% and 4% of bovine TB breakdowns in Spain (Rodríguez et al., 2011) and Portugal (Cunha et al., 2012), respectively. Moreover, *M. caprae* has been isolated in humans and cattle (Cvetnic et al., 2007; Prodinger et al., 2014, 2005; Rodríguez et al., 2009), sheep (Muñoz Mendoza et al., 2012; Reis et al., 2020), wild boar (Ciaravino et al., 2020; Reis et al., 2020) and other wild animals, such as foxes, bison and red deer (Chiari et al., 2014; Krajewska et al., 2015; Rodríguez et al., 2011). In recent years, there is growing evidence on the role of *M. caprae* in TB transmission cycle (Chiari et al., 2014; Ciaravino et al., 2020; Magnani et al., 2020; Rodríguez et al., 2011), hampering efforts made in eradication in cattle.

***M. microti*:** It was initially described as a pathogen of small rodents such as field voles (*Microtus agrestis*), bank voles (*Myodes glareolus*), wood mice (*Apodemus sylvatucius*) and shrews (*Sorax araneus*) (Cavanagh et al., 2002). The voles usually develop systemic disease with frequent involvement of spleen and liver (Kipar et al., 2014). Moreover, it is usually isolated from domestic cats (Smith et al., 2009) and it has been reported in ferrets, badgers and llamas (Emmanuel et al., 2007), as well as in cattle (Michelet et al., 2020, 2017) and goats (Michelet et al., 2016). Moreover, *M. microti* has been reported as a pathogen in immunocompromised and immunocompetent humans (Foudraine et al., 1998; Niemann et al., 2000).

***M. orygis*:** It was characterized as a MTBC subspecies in 2012 (van Ingen et al., 2012) but to date, its host range remains unknown. It has been isolated from oryxes, gazelles (van Soolingen et al., 1994), deer, antelope and waterbucks (Smith et al., 2006), cattle and humans (Dawson et al., 2012).

M. pinnipedii: is also known as "seal bacillus" in view of its natural host, otariid species such as Australian sea lions (*Neophoca cinerea*), Australian fur seal (*Arctocephalus pusillus doriferus*), New Zealand fur seal (*Arctocephalus forsteri*), as well as wild south American sea lion (*Otaria flavescens*) and wild subantartic fur seals (*Arctocephalus tropicalis*) (Cousins et al., 2003; Martins Melo et al., 2019). Moreover, *M. pinnipedii* zoonotic potential was confirmed by transmission to seal trainers who worked with seal colonies in Netherlands (Kiers et al., 2008).

1.4. PATHOGENESIS

The manifestation of TB infection in goats, as well as in humans, cattle and other mammals, will depend on different factors, including the route of infection, the virulence of the mycobacterial strain and host immune responses (Neill et al., 2001). The aerosols generated by a cough from TB infected individuals is considered the most common route of infection in animals (Pollock and Neill, 2002). Moreover, in animals, the oral infection could also be important. The ingestion of contaminated milk or feed, especially when climatic conditions allow the survival of the mycobacteria in pastures (Goodchild and Clifton-Hadley, 2001), could lead to the formation of intestinal lesions (Serrano et al., 2018). However, the route of infection does not always determine the localization of lesions, as demonstrated in cattle orally challenged with *M. bovis,* in which lesions were commonly found in lungs, mediastinal and tracheobronchial lymph nodes (LN) (Palmer 2004)

Once the Mycobacteria has reached the mucous membranes or the alveolar space, the mycobacterial cell wall is recognized by the host immune system, activating inflammatory signaling pathways. Then, the mycobacteria are

phagocytized by macrophages and neutrophils are attracted to the site of infection. These cells interact with other cells involved in innate and acquired immune responses (Arentz and Hawn, 2007). In immunocompetent humans, this initial immune response leads to the elimination or control mycobacteria multiplication in 90% of cases, and only a small proportion of cases progress towards active TB infection (O'Garra et al., 2013). On the contrary, in cattle, studies suggest that TB infection by *M. bovis* generally progress towards acute infection with active mycobacterial replication and development of severe stages of the disease (Sabio y García et al., 2020). Similar observations were made in goats challenged with *M. bovis* and *M. caprae*, although a lower number of viable *M. caprae* was recovered from tissues (Bezos et al., 2015a).

This initial infection may lead to the development of the granuloma (Figure1.6 A-B), the hallmark of tuberculous disease. The infected macrophages start the development of the granuloma by the recruitment of different proinflammatory cells, such as other macrophages (dendritic cells, epithelioid macrophages, giant multinucleated cells and foamy macrophages), T lymphocytes and neutrophils (Ehlers and Schaible, 2013; Silva Miranda et al., 2012). Subsequently, the host immune response drives the reorganization of the granuloma with central TB infected macrophages surrounded by a rim of lymphocytes (Ehlers and Schaible, 2013). Then a more mature granuloma takes place by emergence of central necrotic areas with a full or partial fibrotic capsule surrounding the granuloma (Figure 1.6B) (Wangoo et al., 2005). Moreover, after initial infection, phagocytic cells migrate though lymphatic capillary vessels to regional lymph nodes, where a new focus of infection can be stablished. This dual infection is known as primary complex (Domingo et al., 2014).

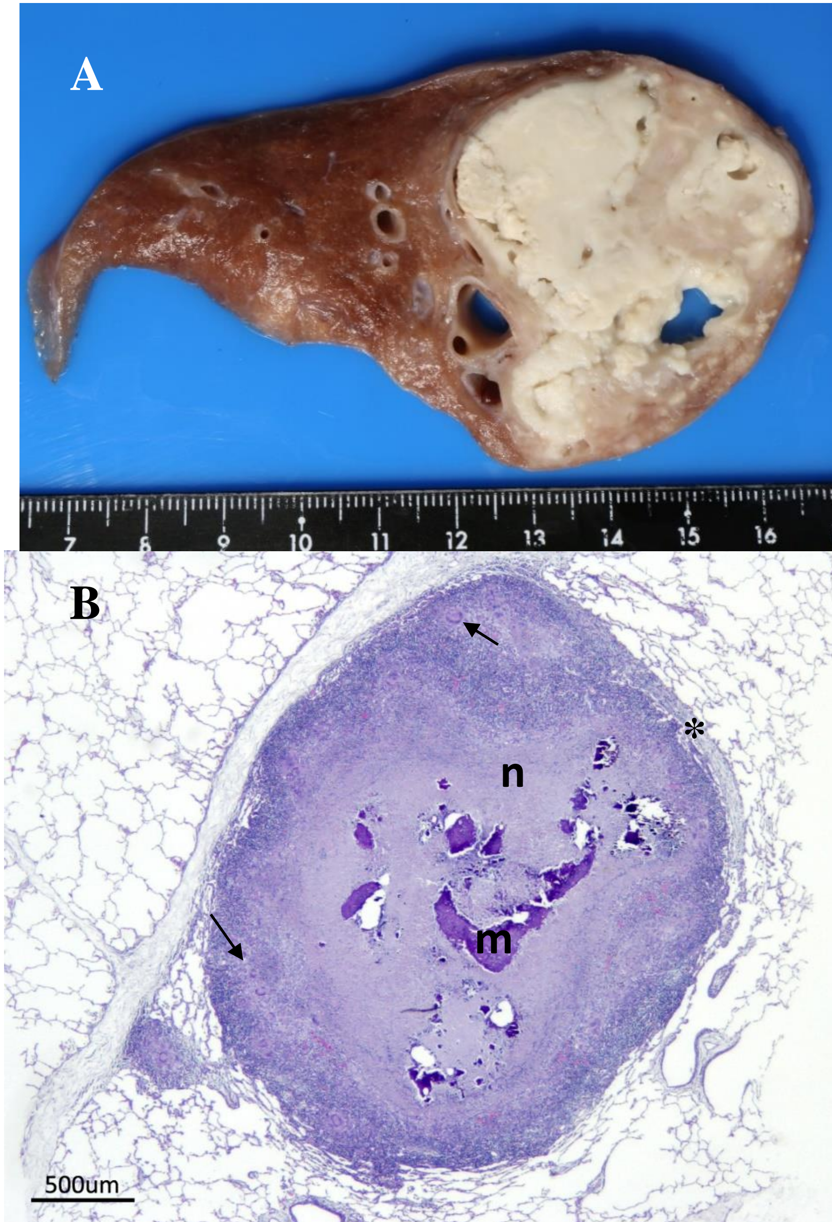


Figure 1.6. Tuberculous lesions in goats. A. Macroscopic lesion in the lung. B. Microscopic lesion in lungs with a necrotic center (n) with mineralization (m) and multinucleated cells (arrows), surrounded by a fibrotic capsule (asterisk). Hematoxylin – Eosin stain.

For a long time, it has been assumed that the structure of granuloma was organized, static and allowed the containment of the mycobacterial multiplication. However, recent research suggests that the complex structure and cell population of granulomas are dynamic, involving continuous loss of cells and replacement by cellular recruitment, as well as tissular and vascular remodeling (Ehlers and Schaible, 2013; Orme and Basaraba, 2014). This may explain the coexistence of granulomas in different developmental stages within the same individual and the regression or progression of lesions (Ehlers and Schaible, 2013). Moreover, it has been suggested that early innate immune responses in the early granuloma are not effective for controlling the *M. tuberculosis* infection and rather, promote its replication and spread in other tissues (Ehlers and Schaible, 2013; Orme and Basaraba, 2014). It has been demonstrated that when the bacteria in the infected macrophage reach the number of 20, the macrophage can break. The released mycobacteria are phagocytized again by alveolar macrophages or by Dendritic cells (DC) (Rossitto and Spagnolo, 2020). The DC leave the site of infection through lymphatic drainage, to the local lymph node (LN) (Wolf et al., 2007), or through blood leading to the development of secondary lesions or generalization of TB infection. In LN, the DC present Mycobacterial antigens to T naive cells, leading to their activation to CD4+, which induces the production of different cytokines (notably Interferon-gamma - IFN-γ), and drives the host immune responses (Rossitto and Spagnolo, 2020).

Macroscopically, in cattle (subjected to active surveillance of the infection), the initial TB lesion in the lungs is usually absent or too small to be detected at necropsy (Domingo et al., 2014). However, in animals non subjected to an eradication program, such as goats, which usually show a rapid course of TB, advanced stages of infection or chronic TB are frequently observed (Figure

1.6A) (Domingo et al., 2009). The initial lesion could progress becoming a circumscribed yellowish granulomatous nodule completely or partially surrounded by a fibrotic capsule, containing caseous necrotic mineralized material, and, eventually, cavitated (Domingo et al., 2014). It is important to note that cavitation is a typical feature of TB lesions in lungs but not in other localizations (i.e. lymph nodes). The precise immune mechanisms involved in the formation of cavities are not fully understood but it seems that cavitated granulomatous lesions may release the mycobacteria in the respiratory airways, probably promoting its transmission by the production of aerosols by cough (Urbanowski et al., 2020).

Other factors, the most important of which are the host immune response, age, breed and the production system, may influence the course of TB infection. In humans, it is well known that in young children (<5 years) TB disease has a rapid progression with high risk of developing disseminated infections (Basu Roy et al., 2019). Similarly, a more severe disease, in terms of development of lesions, was observed in naturally TB infected wild boar piglets than in adult individuals (Martín-Hernando et al., 2007). Moreover, studies on genetic variation in resistance to bovine TB in cattle suggests that selective breeding may help to reduce TB transmission (Tsairidou et al., 2018). However, the abovementioned aspects have not yet been investigated in goats.

On the other hand, intensive dairy management usually involves larger sizes of herds, which contributes to within-herd transmission of TB (Ramírez-Villaescusa et al., 2010), and greater stress due to high levels of milk production, which probably trigger the disease.

The infecting strain is also an important factor to take into account. It was shown that in goats, the challenge with *M. bovis* produced more severe lesions

than the challenge with *M. caprae*, and the challenge with *M. tuberculosis* produced a reduced number of lesions with low severity (Bezos et al., 2015a). Moreover, bacteria from MTBC have different virulence factors involved in the interaction with the host immune responses, which allows the mycobacteria to adapt to the macrophage limited nutritional conditions, counteract the host cell responses by arresting the formation of the phagosome, increase the resistance to toxic compounds, thanks to the cell wall and specific effectors, and avoid the development of some immune responses (Forrellad et al., 2013). The infective dose and the route of infection may also influence the distribution of lesions and the disease onset. Experimentally, the aerosol challenge with *M. bovis* (Gonzalez-Juarrero et al., 2013) and the endobronchial challenge with *M. caprae* (Pérez de Val et al., 2011) in goats at a similar dose ~ 1×10^3 CFU, sowed different pictures, being more severe the endobronchial challenge.

1.5. IMMUNE RESPONSES

The immune responses against mycobacterial infections are driven by interactions between innate and adaptative immunity. The first response against Mycobacteria is piloted by the initial innate immune response, although the exact mechanisms of adaptative immunity remain to be defined. The understanding of these mechanisms is of paramount importance for the development of tuberculosis vaccines in humans and animals (Hope and Werling, 2018). The immune responses against TB have been studied mainly in humans (Flynn, 2004). However, in large animals, studies in cattle have been undertaken (Waters et al., 2011a), mainly to understand immune responses underlying diagnostic tests and responses against TB vaccines, particularly the *M. bovis* Bacille Calmette-Guérin (BCG) vaccine. In goats, only one study intended to understand the cell lines and immune mechanisms involved in the

development of TB granulomatous lesion (Caro et al., 2001). Although scarce information exists on general immune responses in goats, it is considered that their immune responses are similar to immune responses in cattle, which is considered similar to that observed in humans, except for some physiological differences (Waters et al., 2011a).

1.5.1. Innate immune response

The innate immune response is the first to respond against pathogens and responsible for maintaining the integrity of tissues and repair them. Moreover, the innate immune resposnse participate in the activation and direction of the adaptative immune response. The main cells involved in the innate response are macrophages, dendritic cells (DC), gamma delta T cells (γδ T cells), neutrophils and Natural Killer (NK) cells (Carrisoza-Urbina et al., 2018).

The innate immune response identifies the mycobacteria though different immunity cells. This interaction leads to the production of pro-inflammatory cytokines such as tumor-necrosis factor alfa (TNF-α), type 1 Interferons (IFNs), Interleukins (IL) 1 beta, IL-18 and IL-12, as well as chemokines and antimicrobial proteins. As a result, different cells of the innate immune system are recruited at the site of infection, which subsequently will activate the adaptative immune response to contain the infection (Carrisoza-Urbina et al., 2018). However, it has been demonstrated that virulent mycobacteria have the capacity to evade immune responses and multiplicate inside macrophages (Forrellad et al., 2013), leading to the development of the disease.

During mycobacterial infection, macrophages and dendritic cells (DC), which are antigen-presenting cells (APC), recognize the Mycobacteria and initiate the immune response to control the spread of the bacilli (Hussain Bhat and

Mukhopadhyay, 2015). Macrophages are specialized phagocytic cells, considered the first line of defense against mycobacteria and are necessary for tissue homeostasis, development and repair of injured tissue (Carrisoza-Urbina et al., 2018). Classical activation of macrophages through phagocytosis of virulent *M. bovis* induces large levels of Nitric Oxide (NO) release, increasing the microbicidal activity in the macrophage (Esquivel-Solís et al., 2013). However, different virulence factors of *M. tuberculosis* and *M. bovis* confer resistance to oxidative stress and the capacity to impair trafficking of mycobacteria to phagolysosome (a process known as maturation of phagosome) within macrophages (Forrellad et al., 2019, 2013).

DC are specialized in antigen presentation. After phagocytosis, DC processed the antigens and upload them into the major histocompatibility complex (MHC) molecules for presentation in lymph nodes (LN) (Hope et al., 2004). DC present antigens to activate different immunity cells, such as NK, γδ T cells and naive T cells, thus being critical for the induction of immune responses (Siddiqui et al., 2012). However, in mycobacterial infections, DC may act as a double-edged sword. Interaction of DC with *M. bovis* induced expression of IL-10, an immunosuppressant cytokine, which inhibits the migration and maturation of DC (Hope et al., 2004), delaying the immune responses against mycobacteria. Moreover, internalized *M. bovis* is able to increase its replication in DC, carrying the mycobacteria to LN and to other localizations (Denis and Buddle, 2008). On the other hand, DC infection by *M. tuberculosis* or *M. bovis* BCG has been correlated with enhanced expression of proinflammatory cytokines (Hope et al., 2004). Particularly, IFN-γ probably released by NK cells and γδ T cells, enhances the secretion of IL-12 by DC (Siddiqui et al., 2012), which at its turn, in the adaptative immune response, will trigger the secretion of IFN-γ and TNF-

α from T cells, after which the microbicidal activity of macrophages and NK cells increased (Denis and Buddle, 2008; Hope et al., 2004).

The NK cells are granular lymphocytes with different functions in the immune system, notably cytotoxicity and cytokine secretion. In BCG vaccinated cattle, activated NK cells can induce death in targeted cells by degranulation of cytotoxic proteins such as perforins and granulysins (Siddiqui et al., 2012). It has been demonstrated that NK cells in contact with infected bovine macrophages and under stimulation of IL-12 can reduce *M. bovis* growth (Denis et al., 2007).

Recent research suggest that goats have a low proportion of γδ T cells in both young and adult animals (Baliu-Piqué et al., 2019), in contrast to other ruminants (10% of γδ T Cell in goats vs 60% in cattle) and its proportion do not decrease with age after 6 months (Yirsaw et al., 2021). In cattle, the γδ T Cell subset expressing workshop cluster antigen 1 (WC1) is particularly involved in mycobacterial infections due to their secretion of IFN-γ (Carrisoza-Urbina et al., 2018). However, in TB infection in goats, despite a wider diversity of WC1 subset, γδ T cells do not seem to have a significant role (Yirsaw and Baldwin, 2021). The γδ T cells are considered transient T cells of the immune response (Carrisoza-Urbina et al., 2018). In the innate immune response, γδ T cells are able to recognize pathogen-associated and damage-associated molecular patterns (Vantourout and Hayday, 2013). Besides, in adaptative immune response, it has been suggested that γδ T cells participate in an early drive towards a Th1 response (Price and Hope, 2009).

Recent studies suggests that innate immune system is able to increase specific responses to a secondary infection, against the same or different microorganisms (Netea et al., 2011). This type of immunity, known as “trained immunity”, is

driven by monocytes, macrophages and NK cells, independently of T and B cell responses (Kleinnijenhuis et al., 2014). In cattle, in vitro exposure of monocytes to BCG resulted in increased TNF-α and IL-6 production after agonist stimulation of the Toll-like Receptors from APC's. Similarly, aerosol BCG vaccination of calves amplified proinflammatory cytokine production, and trained immunity effects on PBMCs were still noticeable after 3 months of vaccination (Guerra-Maupome et al., 2019). Thus, in a secondary exposure, trained monocytes rapidly recognized the pathogen and enhanced cytokine production (Kleinnijenhuis et al., 2014).

Overall, it is important to note that the outcome of TB infections can be determined by virulence of MTBC strain but also by resistance to infection by host immune responses (Wilkinson et al., 2017).

1.5.2. Adaptative immune response

In the adaptative immune response against TB, both cell-mediated immune (CMI) and humoral responses participate. However, it is assumed that the CMI plays the most important role in dealing with intracellular pathogens, depending on the interplay between virulent factors of mycobacteria and the host immune responses. These responses will lead to protection against infection or to the progression and development of the TB disease. The CMI responses involve different T cell subsets, mainly the TCD4+, TCD8+, γδ T cells and memory T cells, as well as cytokines, such as IFN-γ. Adaptative immunity play a key role in the immune response against vaccines, vaccination strategies and constitute the basis for current diagnostic tests of tuberculosis (Skin tests and interferon gamma release assay).

Due to scarce information on immunological responses in goats, we will consider that responses against TB and TB vaccines are similar to that observed in bovines, which are at its turn considered similar to human responses, except for few differences (Waters et al., 2011a).

The adaptative immune response starts with the presentation of MTBC processed antigens by APC's (mainly DC's) to naive T cells in the regional lymph node. Studies in mice had shown that activation of naive T cells takes place around 9-11 days after TB infection. However, the development of the adaptative immune response can be delayed depending on MTBC dose (Reiley et al., 2008) and variable host factors (Urdahl et al., 2011). Naive T cells express in their surface T cell receptors (TCR) which interacts with the DC through the MHC to present MTBC antigens. DC presents antigens through MHC class II to CD4 T cells and through MHC class I to the CD8 T cells.

CD4 and CD8 T cell-specific responses are essential for effective control of MTBC infections. After activation, CD4 T cells replicate, produce cytokines such as IFN-γ and TNF-α, acquire effector functions and leave lymph nodes to be recruited at the site of infection, and to other organs around 15 days post-infection (Reiley et al., 2008). The secretion IFN-γ by innate immune effectors promotes secretion of IL-12 from infected DC which induces the differentiation of CD4 T cells in T helper 1 (Th1) cells (Siddiqui et al., 2012). The IFN-γ is a key cytokine for the maintenance of a Th1 biased CMI response which has been associated with a more controlled infection in cattle (Welsh et al., 2005). During MTBC infections CD4 T cells are the main source of IFN-γ (Ngai et al., 2007), although, in cattle, other cells (CD8 and γδ T cells and NK cells) contribute to its secretion. Moreover, vaccine induction of IFN-γ from CD4 T cell is fundamental for inducing protective immunity (Fletcher and Dockrell, 2016),

however, IFN-γ levels are not directly related to vaccine protection (Waters et al., 2012).

CD8 T cells are also induced by MTBC infections and are recruited at the site of infection. Activated CD8 T cells possess anti-mycobacterial activity by degranulation of granulysins and perforins (Endsley et al., 2007, 2004). It seems that CD8 T cells have an effector function in a CD4 T cell dependent fashion (Behar et al., 2007) and are involved in protective responses in vaccinated animals (Ryan et al., 2009). However, their role in human and animal TB remains to be clearly elucidated.

In the adaptative immune response, γδ T cells seem to play a role in driving the immune response towards Th1 (Price and Hope, 2009). After BCG vaccination or *M. bovis* infection, γδ T cells are strongly activated and expanded, and are involved in cytotoxic and regulatory activity (McGill et al., 2014). Moreover, γδ T cells are able to actively respond to *M. bovis*, trafficking to the site of infection and are important for early granuloma formation (McGill et al., 2014; Salguero et al., 2016). In goats, these roles of γδ T cells remain to be studied.

In TB infections, as well as in other inflammatory or autoimmune diseases, an excessive inflammatory response may induce extensive tissue damage. To modulate these responses, the lymphocyte subset Treg, characterized by CD3 and CD4, CD25, FoxP3^{+} markers, plays an important role (Cardona and Cardona, 2019). Treg responses involve the secretion of immunosuppressive cytokines such as IL-10 and TGF-β (Saraiva and O'Garra, 2010), which downregulate production of IFN-γ, required for controlling mycobacterial growth. Moreover, animal models showed that Treg depletion worsened the outcome of TB infections and higher Treg frequencies were related to less susceptible mice strains and improved pathology in non-human primates

(Cardona and Cardona, 2019). Thus, the role of Treg responses needs to be further elucidated and research in domestic animals and under vaccination contexts need to be made.

Effector memory and central memory T cell subsets are also important for adaptative immune responses as they are able to rapidly respond to posterior infections. When MTBC antigens are encountered, the number of specific T cells increase by clonal expansion and lead to the development of memory cells (Pollock et al., 2001). In cattle, T memory cells (Tm) have been identified by surface receptors such as CD45RO, CD62L and CCR7 (Blunt et al., 2015; Maggioli et al., 2015). The T central memory cells (CD45RO+ CCR7+ CD62L hi) in cattle are the first line of cells responding to *M. bovis* infection in IFN-γ ELISPOT long term cultures (Blunt et al., 2015; Maggioli et al., 2015). Although for vaccination, this type of responses is of paramount importance, the specific role in vaccine-induced protection of memory cells needs to be further studied.

1.5.3. Humoral responses

It has been a long time assumed that in TB infections, humoral responses were not relevant (Maglione and Chan, 2009), due to the intracellular nature of mycobacteria. However, B cells may contribute to the immune response against TB through antigen presentation and cytokine secretion. In fact, antibodies can regulate the APC function and antibody-dependent cytotoxicity may play a role in immune responses (Achkar et al., 2015). Moreover, it has been demonstrated that in early granulomas (stages I and II) in cattle, scattered B cells were observed, but in advanced stages of granulomas (III and IV) clusters of B cells were organized around the fibrous capsule (Salguero et al., 2016), and in

vaccinated animals, that displayed early stages of granulomas, the number of B cells was greater compared to unvaccinated animals.

Overall, the immune responses against TB are highly complex and are governed by different mechanisms and multiparametric factors that need to be balanced to achieve the control of the infection.

1.6. IMMUNOLOGICAL DIAGNOSIS

TB in goats, such as in cattle, is difficult to diagnose due to slow progress of infection and unspecific clinical signs, such as weight loss, reductions in milk production or cough. Moreover, in vivo detection of Mycobacteria in nasal secretions or other body excretions is not always possible because of intermittent excretion of the bacilli. On the other hand, diagnostics by detection of lesions at the slaughterhouse could be useful to confirm the infection in goat herds. However, small lesions may go unnoticed and awareness of active search of lesions should be promoted. Suspected lesions might be extracted from the carcass and send to the laboratory for histopathologic diagnostics (by Hematoxylin / Eosin and Ziehl Neelsen stain) and/or bacterial culture and spoligotyping.

In that light, ante mortem diagnostics based on the immunological responses could be highly useful for disease control. In TB infected cattle or goats, since *M. bovis* and *M. caprae* display high immunogenicity, activation of cell responses by antigen stimulation could be a good indicator of infection. Particularly, the measurement of the specific release of IFN-γ in the blood may the advantage of allowing the detection of the disease at the early stages of infection, even when the bacterial load is not high enough to be cultured (Vordermeier et al., 2004). Then, animals become reactive to skin test using

tuberculins and late, antibody responses are detected. Although, as explained in previous chapters, these timings can be shorter or larger depending on the host immune responses and the increase in bacterial load and pathology (Figure 1.7- (Vordermeier et al., 2004).

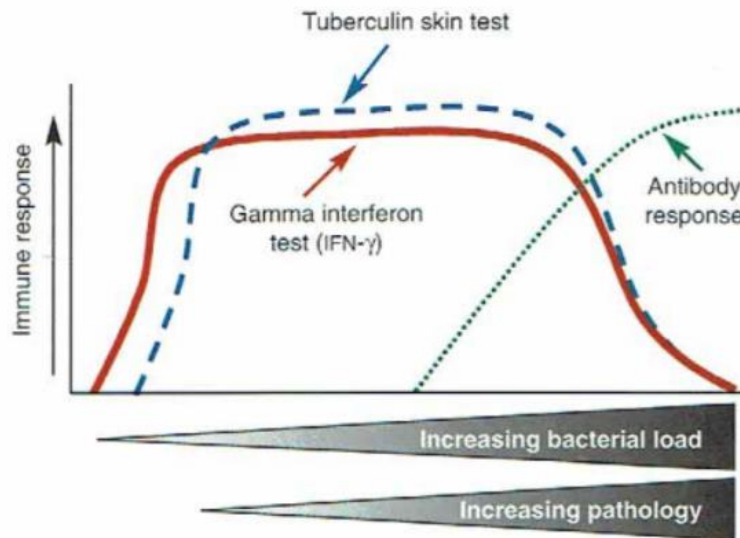


Figure 1.7. Schematic representation of time course of the pathology and immune responses in cattle infected with bovine tuberculosis. Red and blue dashed line represents Cell-mediated immune responses detectable by Interferon gamma secretion (IFN-γ) and skin induration after tuberculin injection (tuberculin skin test). Green dotted line indicates antibody responses against MTBC epitopes (Source: Vordermeier et al, 2004).

Early diagnostics of TB infected animals is crucial for the success of eradication or control programs, and it has been assumed that the diagnostic tests based on CMI (SIT, SCIT and IGRA) are the most useful tools. Indeed, eradication programs based on testing by SIT or SCIT and slaughter of positives allowed the eradication of TB in cattle from Australia (Cousins and Roberts, 2001), and highly reduced the presence of the disease in cattle herds from Canada, New Zealand and some European countries (Good and Duignan, 2011; Rivière et al.,

2014). However, complete eradication becomes difficult due to the presence of reservoirs.

On the other hand, in goats that are not subjected to an eradication program, it is common that the TB infection progress, leading to the development of extensive pathology and bacterial growth (Domingo et al., 2014). In this context, the demand for CMI is constantly high, which could result in an exhaustion of cellular responses (Khan, 2017) and consequent false negatives outcomes in diagnostic tests. This condition is called anergy. At this stage of the disease, the antibody response is still detectable, and thus serological diagnosis could be an interesting option to use, at least for screening in goat herds. Moreover, it has been demonstrated that performing in parallel different diagnostic tests based on CMI and antibody responses improved the sensitivity of TB diagnostics (Bezos et al., 2018; Casal et al., 2017). Although, the use of different tests in parallel is not always feasible due to logistic and economic issues.

1.6.1. Antigens for TB diagnostics

The members from the MTBC possesses highly preserved epitopes recognized by T cells and B cells. Some of these antigens are highly immunogenic, and are key compounds of the protein derivatives used for TB diagnostics.

- **ESAT-6 secretion system (ESX)**: EXS are bacterial secretion systems identified after discover the 6kDa early secretory antigen target (ESAT-6 or also known as EsxA) and are located at the RD1 of *M. tuberculosis* genome, which is absent in *M. bovis* BCG vaccine. Five EXS have been described in *M. tuberculosis* and three of them (ESX-1, ESX-3 and ESX-5) are necessary for virulence. Particularly, the ESX-1 is key for resistance and evasion of host immune responses, inducing, for instance,

phagosomal break with the consequent release of bacteria into the cytosol of phagocytes. ESX-1 secretes two immunodominant antigens, ESAT-6 and CFP-10, both forming a heterodimer that is involved in transport substrates through the inner membrane of the mycobacteria (Gröschel et al., 2016). ESAT-6 and CFP-10 are potent inductors of T cell responses and subsequent production of IFN-γ (Abebe et al., 2017), and have been used in different assays for diagnostic of TB in humans (Pai et al., 2008) and cattle (Vordermeier et al., 2016). Among the family of the ESAT-6 proteins, Rv3020c has been identified in macrophages from *M. bovis* infected cattle as a potent inductor of IFN-γ responses, but not in macrophages from BCG vaccinated animals, suggesting its use for differentiating vaccinated from infected animals (DIVA) diagnostic (Jones et al., 2010). Another immunodominant protein from the EXS systems is Rv3615c, also known as EspC. This protein is an Esx-1 substrate which secretion is dependent on ESAT-6, but are not encoded by the same region of the gene esx-1 (Gröschel et al., 2016). Thus, it is considered that this antigen is not secreted by BCG, becoming a candidate for DIVA diagnostic that has been tested in cattle (Jones et al., 2017; Middleton et al., 2021; Parlane et al., 2016) and goats (Bezos et al., 2015b; Pérez de Val et al., 2012a).

- **Acr (alpha crystallin) family**: The Acr protein (also known as HspX or Rv2031) is related to latent TB and stimulates T cell responses resulting in the production of IFN-γ, TNF-α and IL-10 (Rizvi et al., 2016). Another protein from the Acr family, the Acr2 (also known as HspX or RV0251c), has also been identified as an inductor of T cell IFN-γ release in early TB infection in cattle and humans (Wilkinson et al., 2005).

- **Cell wall antigens**: Two major antigens have been identified in the surface of the MTBC, MPB70 and MPB83 in *M. bovis*, or MPT70 and MPT83 for their orthologous in *M. tuberculosis*. Besides cell wall, these proteins have been found in culture filtrates of *M. bovis* grown in liquid culture, suggesting that are also secreted. MPB70 and MPB83 also induce T cell secretion of IFN-γ and TNF-α, although responses against MPB83 seem to be stronger. *M. bovis* express higher amounts of MPB83 and MPB70 than the orthologous in *M. tuberculosis* (Wiker, 2009). Particularly, antibodies against MPB83 appear early after experimental infections (5-107 days) and is a promising antigen for serological diagnostics that have been tested in cattle (Marassi et al., 2011), badgers (Lesellier et al., 2008), white-tailed deer (Waters et al., 2004b), and domestic and wild suids (Miller et al., 2019), among others.

The characterization of the purified protein derivative (PPD) of *M. bovis* also called bovine tuberculin (bPPD), used as a reagent for current diagnostic tests of TB in cattle and goats, revealed the most abundant antigens: ESAT-6, CFP10, MPB70, MPB83, Acr and other ESAT-6 like proteins, accounting for around 50% of the total of proteins identified (Infantes-Lorenzo et al., 2017). Moreover, a total of 146 proteins were identified as common between bovine and *Mycobacterium avium* subsp *avium* PPD, thus animals infected with other mycobacteria could cross-react in diagnostic tests based on bPPD. Under the basis of the most abundant proteins of the bPPD, a new antigenic complex called P22, has been developed (Infantes-Lorenzo et al., 2017) and has been tested for serological diagnostics in different species, showing promising results (Casal et al., 2017; Infantes-Lorenzo et al., 2019a; Thomas et al., 2019a). However, the usefulness of the P22 antigenic complex in diagnostics based on

CMI remains to be investigated, as well as other antigens that potentially could improve the current diagnostic of TB.

1.6.2. Immunological diagnosis based on Cell-Mediated Immune responses

1.6.2.1. Skin tests

The underlaying immunological mechanism of skin tests in TB is a delayed-type hypersensitivity (DTH) response, also known as type IV hypersensitivity reaction, which relies on CMI. This immunological response was described by Koch, by means of the inoculation of inactivated culture filtrates of MTBC in guinea pigs and humans. In infected animals or in animals with previous contact with a Mycobacteria, the subcutaneous injection of PPD may sensitize T cells, activating Th1 CD4 T cells and macrophages, inducing the secretion of cytokines such as IFN-γ and TNF-α. These cytokines attract and activate other inflammatory cells from blood towards the site of injection (Kobayashi et al., 2001), inducing skin induration, swelling and redness after 24-78 hours.

The specifications for the procedure (reagents, dose) and interpretation of the Single and Comparative Cervical Intradermal tuberculin test (SIT and SCIT) for cattle are included in the European directive 64/432/EEC and in the Spanish Royal Decree Number 047/2003.

Briefly, for the performance of the SCIT in cattle as well as in goats, before the inoculation of the bovine PPD (CZ Veterinaria, protein filtrate from *Mycobacterium bovis* strain AN-5 culture, 25000 UI/mL), the proximal zone of the left side of the neck is shaved and basal skinfold thickness (SFT) is measured by using a calliper. Then, 100uL of the bPPD is injected by using a

Dermojet (R) syringe. 72 h after inoculation, the skinfold thickness is measured again. The standard interpretation criteria are detailed in Table 1.2.

MTBC share epitopes with other members of the mycobacterium family. Thus, in cases where the infection of cattle and goats by other mycobacteria is suspected and in case of paratuberculosis vaccination (*M. avium* sp *paratuberculosis* - MAP), it is recommended to perform the SCIT, in order to discriminate other mycobacterial infections from tuberculosis. The procedure for the SCIT is similar to the SIT but the avian PPD (CZ Veterinaria, protein filtrate from *Mycobacterium avium* sbsp *avium* strain D4 ER culture, 25000 UI/mL) is inoculated at the right side of the neck. The standard interpretation criteria are detailed in Table1.2.

Skin tests are widely used for TB diagnostics in cattle and previous studies of its application in goat herds have shown variable outcomes in terms of specificity (Sp) and sensitivity (Se). For instance, both SIT and SCIT showed specificities ranging from 97.6-99.2% and 99.6-100%, respectively, in eight goat herds, using the standard interpretation (Bezos et al., 2012a). However, using a severe interpretation (positivity: SFT bPPD > 2mm or SFT bPPD - SFT aPPD > 2mm), which is recommended in herds to clear the TB infection or in herds with persistent TB infection (Buendía et al., 2013), the Sp obtained was lower for the SCIT (93-96%). These differences were probably due to the epidemiological context of farms (infection by *Corynebacterium pseudotuberculosis* and vaccination against paratuberculosis) and intrinsic factors (different breeds and wide range of age, varying from 12 months to 11 years). Moreover, a temporal analysis of the interference of MAP vaccination demonstrated a reduction of interferences in SIT and SCIT tests irrespectively of the criteria used from 3 to 9 months after vaccination, although 100% of Sp was recovered after 12 months (Roy et al., 2018a).

Table 1.2. Interpretation criterion of skin test.[1]

Result	SIT	SCIT
Negative	SFT bPPDB < 2mm or absence of clinical signs[2]	SFT bPPDB <2mm or SFT bPPD = SFT aPPD in absence of clinical signs
Doubtful	SFT bPPD >2mm and < 4mm in absence of clinical signs	SFT bPPD > 4mm or SFT bPPD >2mm and < 4mm and SFT bPPD > aPPD in 1 to 4 mm, in absence of clinical signs
Positive	SFT bPPD > 4mm or presence of clinical signs	SFT bPPD > aPPD in more than 4mm or presence of clinical signs

1. Interpretation according to Spanish Ministry of Agriculture (MAPA 2019). 2. Clinical signs: swelling, oedema, exudation or necrosis. SIT: Single cervical intradermal tuberculin test, SCIT: Single comparative cervical intradermal tuberculin test, SFT: Skinfold thickness, bPPD: bovine tuberculin, aPPD: avian tuberculin.

In terms of Se, different studies have been carried out with different results. Bezos and collaborators revised different studies in goats and found that for the SCIT using severe interpretation, results ranged from 53.2-88.8% and for the SCIT, ranged from 29.2-83.7 (Bezos et al., 2012b). Buendía and collaborators tested the SCIT in four goat herds with persistent infection with *Mycobacterium spp*, showing poor Se of 44.5% (Buendía et al., 2013). Moreover, the sensitivity of SIT and SCIT was evaluated in 7 goat flocks which had a high proportion of reactors at a first testing event (Bezos et al., 2014b). In four out of seven herds, paratuberculosis vaccination was carried out and significant differences in SFT depending on the age was detected. Overall, the results of Se among farms were highly variable, ranging from 19.4 to 85.5% for the SIT with severe interpretation, and Se for SCIT with severe interpretation ranged from 8.1 to 60.7%, and highly decreased using standard interpretation.

Globally, skin tests are diagnostic techniques that can be easily applied in field conditions at a limited cost. However, there is a need for improvement of its sensitivity in goats and it would be necessary to study how to adapt the diagnostic strategies to the herd context and study new reagents that allow discrimination between infected and paratuberculosis vaccinated animals.

1.6.2.2. Interferon gamma release assays (IGRA)

The IGRA is an official ancillary test to SIT or SCIT for TB diagnostics, and it is included in the European Directive 64/432. The protocol, reagents and interpretation cut-off points are the same used for bovines and are detailed in the Spanish Royal Decree 1047/2003 (MAGRAMA, 2015). The IFN-γ has been used in parallel to skin tests in eradication and control programs in New Zealand, Australia, EEUU and in the UE, in order to improve the sensitivity of TB diagnostics.

The IGRA test is performed in two phases. First, the whole blood collected in heparinized tubs is stimulated with bPPD, aPPD and Phosphate buffer saline (PBS for blank control), and incubated for 16-24h at 37ºC with CO_2. After incubation, the plasma is harvested and the IFN-γ levels from plasma samples are detected by an Enzyme-linked Immunosorbent assay (ELISA) from Bovigam® (Thermofisher Scientific, Switzeland) or IDscreen Ruminant IFN-G® (IDvet, Grabels, France). In TB infected animals, during the first phase of the technique, blood cells are stimulated by antigens from PPD, which are presented by APC's to effector T cells. This results in the secretion of different cytokines, such as IL-2 for cloning expansion of T effector cells, and production of IFN-γ.

In experimental infections in goats, CMI responses in the blood, in terms of IFN-γ levels, are detected between 2-4 weeks post-challenge (Bezos et al., 2010; Pérez de Val et al., 2011). Thus, despite a chronic onset of the disease, immunological responses can be detected at the early stages of infection. However, the intensity of IFN-γ responses is variable among individuals.

First studies on the use of IGRA in goats showed high Se, ranging from 83.7% (Gutiérrez et al., 1998) and 87.2% (Liébana et al., 1998). However, more recent studies showed lower sensitivities: 71% (Álvarez et al., 2008), 67.9% (Zanardi et al., 2013) and 50.8% (Bezos et al., 2018). These differences probably took into account diagnostic interferences derived from paratuberculosis coinfection or vaccination, which is a common practice in small ruminants. It has been demonstrated that these interferences also reduce the Sp of the IGRA test (Bezos et al., 2012a; Pérez de Val et al., 2012a; Roy et al., 2018a). However, this interference seems to decrease after one year of vaccination (Roy et al., 2018a).

1.6.2.3. Factors influencing diagnostic performance

Besides paratuberculosis infection or vaccination, other confounding factors may influence the results of tests based on CMI. For instance, whole blood might be stimulated within the first 8 hours after collection, otherwise, the Se of the IFN-γ is significantly reduced (Bezos et al., 2011b). Thus, important logistic resources might be employed to ensure correct diagnostics.

Severe pathology, characterized by cavitary lesions, or persistent infections with extensive pathology, are factors that have been associated with reduced Se of SCIT (Buendía et al., 2013; Gutiérrez et al., 1998). Although in *M. caprae* experimentally infected goats a positive correlation was observed between pathology and IFN-γ levels using bPPD (Bezos et al., 2010; Pérez de Val et al.,

2011), extensive pathology or persistent infections may also induce exhaustion of the CMI response, resulting in a reduction of IFN-γ secreted and a subsequently decreasing the Se of IGRA tests.

On the other hand, caseous lymphadenitis caused by *Corynebacterium pseudotuberculosis*, which is a common pathogen in Spanish goat herds, does not induce interferences in diagnostic tests using standard interpretations, however, it may induce positive results when using stringent criteria (Bezos et al., 2015c). Thus, depending on the TB, MAP and *C. pseudotuberculosis* status, and the objective of diagnostics (eradiation or control), the interpretation criterion must be adapted.

Another important concern for diagnostics based on CMI is parasitic infections, particularly helminths. In cattle, coinfections with *Fasciola hepatica* impaired the results of TB diagnostic tests (Claridge et al., 2012; Flynn et al., 2009, 2007). The reduction in Se of TB diagnostic tests seems to be associated with a drift towards Th2 response, which is driven by the parasite and characterized by the secretion of regulatory cytokines such as IL-10 and TGF-β (Nutman, 2015).

1.6.3. Immunological diagnosis based on humoral responses

In recent years, antibody-based diagnostics is gaining importance. It has been reconsidered as an option for TB diagnostics, particularly owing to ease collection of samples, storage and analysis. The drawback of antibody-based diagnosis has been its low Se. However, serological tests based on serodominant antigens of *M. bovis* (MPB83, MPB70) have been validated in cattle (Waters et al., 2011b). Its use has been approved by the OIE and the US Department of Agriculture only as an ancillary test to confirm MTBC infection and detect anergic cattle to skin tests.

In general, antibody tests have been recommended to be performed after the skin tests. Prior injection of bPPD will induce a boost on antibody responses, increasing the number of TB positive animals and thus, increasing the sensitivity of diagnostics, compared to the skin test alone (Casal et al., 2014). Although, the most suitable strategy would be the use of an antibody test able to detect infected animals without boosting responses after bPPD injection. In this regard, the P22 antigenic complex has been developed and tested in cattle, showing high sensitivity (87.6%) (Casal et al., 2017).

In goats, antibody tests, using the main antigenic epitopes of MTBC (MPB83, MPB70, ESAT-6 and CFP-10) had shown divergent results without previous boosting by skin tests (Table 1.3). These results are probably due to advanced stages of TB disease currently observed in goats (because of the lack of an eradication program for this species), and probably, to strain involved in the infection (*M. bovis* or *M. caprae*). Indeed, it has been experimentally demonstrated that infection by *M. bovis* in goats result in a more severe pathology (Bezos et al., 2015a). As well as in cattle, the use of the P22 antigenic complex showed promising results in terms of Se (Bezos et al., 2018; Casal et al., 2017). Similarly, its use in serum and milk samples allowed the detection of a high number of animals (43/52 and 40/52, respectively) in a persistently infected goat herd (Roy et al., 2020b). Thus, the P22 antigenic complex should be further tested in goat herds under different epidemiological situations to validate its use for diagnostics of TB in this species.

Table 1.3. Sensitivity of antibody detection tests by enzyme-linked immunosorbent assay (ELISA) without previous boosting with bovine tuberculin skin test.

ELISA Antigen	Sensitivity	MTBC strain	Reference
MPB70	93.4%	*M. bovis*	Acosta et al., 2000
MPB70	95.6%	*M. bovis*	Marassi et al., 2009
MPB70	66.3%	*M. caprae*	Buendía et al., 2013
Multiplex assay[a]	98.3%[b]	*M. bovis*	Shuralev et al., 2012
Multiplex assay[a]	85.7%	*M. caprae*	Shuralev et al., 2012
MPB83[c]	21.4% – 38.9%	*M. caprae*	Bezos et al., 2018
P22 antigenic complex	82.4% - 83.2%	*M. caprae*	Bezos et al., 2018

a. Enferplex (Enfer chemiluminescent ELISA, Enfer Scientific, Newhall, Ireland). Five antigens were tested in the multiplex assay: MPB70 peptide preparation, MPB70, MPB83, Rv3616c and CFP10.
b. SCIT was considered as the gold standard test.
c. Ingezim tuberculosis DR (INGENASA, Madrid, Spain).

1.7. PREVENTION AND CONTROL

The control of TB in animals remains a challenge for public and animal health worldwide. Despite the zoonotic potential of caprine TB and the inclusion of goats in the recommendations for infections with MTBC (OIE, 2021a), the disease is still covered by the bovine TB definition. Thus, there is still a gap to rule the notification of goat TB cases. In Spain, goat flocks with an epidemiological link with cattle herds are included in the bovine tuberculosis national eradication program (MAPA, 2021) and are subsequently tested for TB using skin tests or, accessorily, with IFN-γ. In these conditions, the vast majority

of goat herds are not subjected to TB control programs. Fortunately, different Spanish autonomous communities have implemented mandatory or voluntary eradication programs based on the test and slaughter of positives and test for control of animal movements (see epidemiology section). However, it is considered that goat TB is highly prevalent in Spain (Rodríguez et al., 2011) and these eradication programs are expensive for authorities and goat livestock industries.

In that light, other measures for the control of TB in goats should be undertaken:

- Segregation of goat kids: in infected goat herds, it would be interesting to apply systematic segregation of goat kids after birth, in order to avoid the ingestion of MTBC infected colostrum or contact with infected aerosols or secretions from adults.
- Pasteurization of colostrum: This procedure will maintain immunoglobulins indispensable for the development of goat kids and would contribute to the reduction of TB transmission to replacement animals.
- Biosecurity measures: controlled access to farms (to people, vehicles and wild animals), cleaning and disinfection planning. TB control for entry or exit of animals from the farm.
- Vaccination: vaccination against TB has been suggested as a long-term strategy for the reduction of TB prevalence. However, the major caveat for its implementation is the diagnostic interference with current TB diagnostic tests.

1.7.1. Vaccination

Over the past twenty years, a renewed interest in the use of vaccines for the control of animal TB has been raised and several studies in cattle and other animal species have been undertaken (reviewed in Buddle et al., 2018). The most used vaccine has been the live attenuated *M. bovis* Bacille Calmette-Guérin (BCG) which is the only licenced vaccine for humans, and recently for badgers in the UK (Chambers et al., 2014).

BCG vaccine was developed in 1921 by the microbiologist Albert Calmette and the veterinary Camille Guérin at the Institut Pasteur of Lille (France), after 231 passages in biliated potato medium, during 13 years (Locht, 2010). In 1924, the first results of vaccination with BCG were published (Calmette and Guérin, 1924) and in 1927 the first study of BCG efficacy in humans was published (Calmette, 1927). The vaccine was widespread around the world and different strains of BCG were created (Bottai and Brosch, 2016).

For the control of TB disease in animals, BCG has some advantages such as safety and inexpensive production (Buddle and de Lisle, 2014). However, the vaccine needs refrigeration for its conservation, which is disadvantageous for its use, particularly in developing countries. Moreover, efficacy studies of BCG in cattle and wildlife species showed variable outcomes (Buddle et al., 2018). The major caveats for the implementation of BCG vaccination in livestock species are incomplete protection and sensitization of animals to current diagnostic tests (Buddle et al., 2018). In the light of this data, other vaccine candidates and reagents allowing differentiation vaccinated from infected animals are being investigated and validated, and goats would be useful animal models for this purpose. Indeed, in goats, TB disease onset seems rapid with the development of large TB lesions (Domingo et al., 2009).

On the other hand, the induction of protective immunity and the duration of this immunity are characteristics of paramount importance for the development of TB vaccines and vaccination strategies. After vaccination, antigen-specific immune responses can be measured by induction of IFN-γ responses and skin test reactions. In goats, it has been demonstrated that BCG is a safe in goat kids and adult milking goats and induces tuberculin-specific IFN-γ responses until 24 weeks after vaccination (Pérez de Val et al., 2016). In cattle, it has been demonstrated that the immune responses elicited by BCG waned after one year (Thom et al., 2012), and revaccination after two years of first vaccinations conferred protection against *M. bovis* challenge (Parlane et al., 2014). In humans, BCG revaccinated individuals showed enhanced TB specific responses from NK cells, γδ, CD4 and CD8 T cells (Rakshit et al., 2019). Nevertheless, human BCG revaccination is a controversial issue. BCG revaccination of young children from Brazil did not provide substantial protection (Rodrigues et al., 2005) but in South African adolescents prevented sustained QuantiFERON Gold in tube assay conversion, which is indicative of sustained TB infection (Nemes et al., 2018). However, in goats, the duration of protective immunity and mechanisms involved in recalling responses elicited by revaccination with BCG, have not yet been studied.

1.7.1.1. Vaccine efficacy studies

The first efficacy studies of vaccination in goats (Table 1.4) used an endobronchial experimental infection model with *M. caprae* (Pérez de Val et al., 2011), and compared the protection conferred by BCG alone with goats primed with BCG and boosted with adenoviral-vectored vaccines, namely AdAg85A (AdHu5 expressing Ag85A) (Pérez de Val et al., 2012b) and AdATBF (which expresses Ag85A, TB 10.4, TB9.8 and Acr2 antigens) (Pérez de Val et al.,

2013). Protection against TB infection, in terms of reduction of bacterial load in pulmonary lymph nodes and absence extrapulmonary TB lesions, was observed using all three vaccines. In addition, animals vaccinated either with BCG (1331 Danish strain) or with prime-boost regime showed a reduction of lung lesions measured by computed tomography. However, animals vaccinated with a prime-boosted regime showed improved protection compared to animals that received only BCG.

Another live attenuated vaccine that has been tested in goats is the SO2 vaccine, which is a human vaccine candidate based on the deletion of *phoP* from *Mycobacterium tuberculosis* strain MT103 (Bezos et al., 2017). In this study, under a natural infection model, both BCG and SO2 vaccines conferred partial protection against TB infection, in terms of reduction of culture scores and reduction in the number and severity of TB lesions. However, only SO2 vaccinated animals showed significant reductions in culture scores of mediastinal lymph nodes and mycobacterial culture compared to unvaccinated animals. Similar results were observed in another study with the same characteristics to test the MTBVAC vaccine (Roy et al., 2019), which is a human vaccine candidate currently in phase II of clinical trials in neonates and adolescents. MTBVAC is a *M. tuberculosis* strain MT103 attenuated by a double independent deletion of *phoP* and *fadD26* genes, developed by the University of Zaragoza and produced by Biofabri SL (Porriño, Spain). Although both BCG and MTBVAC vaccinated animals showed lower lesion scores, differences were only significant when comparing the total lesion score in lungs from BCG with the respect to the unvaccinated animals.

Table 1.4 Experimental studies of TB vaccines in goats.

Species	Vaccine	Route[a]	Dose	Challenge[b]	Results
Pérez de Val et al., 2012	BCG (Pasteur)	SC	5×10^5 CFU	EB, 1.5×10^3 CFU of *M. caprae*	Significant reduction in lung TBL and mycobacterial burden compared to unvaccinated group. No extrapulmonary TBL.
	BCG (Pasteur) + AdAg85A	SC IM	5×10^5 CFU 10^9 PFU		Significant reduction in lung TBL compared to control and BCG groups. Significant reduction in mycobacterial burden compared to unvaccinated group. No extrapulmonary TBL.
Pérez de Val et al., 2013	BCG (Danish)	SC	5×10^5 CFU	EB, 1.5×10^3 CFU of *M. caprae*	Significant body weight gains and reduction of gross TBL and mycobacterial burden compared to unvaccinated group.
	BCG (Danish) + AdTBF	SC IM	5×10^5 CFU 10^9 IU		Significant body weight gains and reduction of gross TBL and mycobacterial burden compared to unvaccinated group. Significant reduction of TBL in lungs and pulmonary LN compared to BCG group.

a. Route of vaccination: O: oral, IM: intramuscular, SC: Subcutaneous
b. Challenge: EB: Endobronchial, N: Natural

CFU: Colony forming units, PFU: Plaque-forming units, IU: Infectious units, TBL: Tuberculous lesions, LN: lymph nodes

Table 1.4 Experimental studies of TB vaccines in goats (continuation).

Species	Vaccine	Route[a]	Dose	Challenge[b]	Results
Bezos et al., 2017	BCG (Danish)	SC	$1\text{-}4 \times 10^5$ CFU	Natural. 9 - 10 months in contact (*M. bovis* and *M. caprae*)	Vaccinated groups showed significant reduction in mycobacterial culture scores and TBL scores compared to unvaccinated group.
	SO_2 (attenuated M. tuberculosis) [c]	SC	1×10^5 CFU		
Roy et al., 2018b	HIMB (heat-inactivated *M. bovis*)	IM	2 doses of 10^7 CFU	Natural. 10 months in contact *M. caprae*	Reduction of 44% in lesion score (reduction not statistically significant).
Roy et al., 2019	BCG (Danish)	SC	$2\text{-}8 \times 10^5$ CFU	Natural. 9 months in contact *M. caprae*	Vaccinated groups showed significant reduction in TBL scores in lungs and pulmonary LN compared to unvaccinated group. Lower number of animals with extrapulmonary TBL compared to unvaccinated group.
	MTBVAC (attenuated *M. tuberculosis*)[d]	SC	5×10^5 CFU		

a. Route of vaccination: O: oral, IM: intramuscular, SC: Subcutaneous
b. Challenge: EB: Endobronchial, N: Natural
c. SO2 vaccine (Biofabri SL, Porriño, Spain): based in *M. tuberculosis* strain MT103 attenuated by deletion of *phoP* gene.
d. MTBVAC (Biofabri SL, Porriño, Spain): based in *M. tuberculosis* strain MT103 attenuated by independent deletions of *phoP* and *fadD26* genes.

CFU: Colony forming units, PFU: Plaque-forming units, IU: Infectious units, TBL: Tuberculous lesions, LN: lymph nodes

On the other hand, a heat-inactivated *Mycobacterium bovis* (HIMB) vaccine has been tested in goats under a natural infection model (Roy et al., 2018b). This type of vaccine is more stable at ambient temperatures overcoming the necessity of refrigeration for its conservation. In vaccinated animals, lesions were localized and there was a 44% of reduction in the presence TB lesions, although differences were not significant compared to the control group.

1.7.1.2. Field studies

Human clinical trials of vaccination with BCG have shown heterogeneous results, varying from substantial to absence of protection (Mangtani et al., 2014). In cattle, a field trials of vaccination with BCG have been conducted in the last decade (Ameni et al., 2018, 2010; Bayissa et al., 2021; Lopez-Valencia et al., 2010; Nugent et al., 2018b, 2017; Retamal et al., 2021), showing that although animals were not fully protected, vaccination reduced the transmission of mycobacteria (Buddle et al., 2018), by decreasing the mycobacterial shedding (Lopez-Valencia et al., 2010), and TB pathology (Ameni et al., 2018). Moreover, efficacy studies in cattle have shown a high proportion of protected animals, in terms of reduction of TB lesions and bacterial load, ranging from 67.4% (Nugent et al., 2017) to 77.9% (Nugent et al., 2018b) and, a vaccine efficacy of 66.5%, in terms of reduction of incidence after 18 months of vaccination, as well as an increased quality and milk production (Retamal et al., 2021). Besides, the unique study that has been performed in goats, showed a reduction in the number of animals with TB lesions compared to the unvaccinated animals (35% and 77%, respectively) (Vidal et al., 2017) after 16 months of exposure in a herd with high prevalence (70% of TB reactors).

In the light of all these data, long-term field trials are necessary to evaluate the contribution of BCG vaccination strategy for the control of TB spread within goat herds.

1.7.2. Differentiating infected from vaccinated animals (DIVA) diagnostics

Despite vaccination confers a certain degree of protection and can be a cost-effective tool for reducing TB prevalence in goat herds with high prevalence, the main drawback for implementation of vaccination programs in animals is its interference with current TB diagnostic tests based on tuberculins. In goats, previous studies using BCG, HIMB and MTBVAC have shown diagnostic interferences with the SIT, SICCT and the standard IFN-γ using tuberculins (Pérez de Val et al., 2016; Roy et al., 2019, 2018b). To overcome these diagnostic interferences, specific antigens to differentiate infected from vaccinated animals (DIVA) have been developed during the past two decades (Chandran et al., 2019; Srinivasan et al., 2019; Vordermeier et al., 2014, 2016).

The design of DIVA reagents for diagnostics using BCG is based on the selection of antigens encoded in the RD1 region, which is absent in the BCG genome, or antigens secreted by virulent MTBC, such as Rv3615c, but not by BCG. ESAT-6 and CFP-10 are antigens encoded by the RD1 region and have been used for stimulation of whole blood in IGRA's in cattle in field conditions (Bezos et al., 2014a; Vordermeier et al., 2001; Waters et al., 2004a). Moreover, a DIVA reagent containing ESAT-6, CFP-10, MPB70, MPB83, and alternatively Rv3615c, did not induce skin test reactions in BCG vaccinated cattle (Whelan et al., 2010). Similarly, a peptide cocktail containing ESAT-6, CFP-10 and Rv3615c, detected more TB infected animals than bPPD in a herd with high mycobacterial burden and presenting high sensitization to

environmental mycobacteria, and interferences in vaccinated animals were not detected (Srinivasan et al., 2019). Another study testing two peptide cocktails PC1 (ESAT-6, CFP-10 and Rv3615c) and PC2 (ESAT-6, CFP-10, Rv3615c and Rv3020c) in BCG, formalin-inactivated BCG and HIMB vaccinated cattle (van der Heijden et al., 2017), detected slightly increased skin test reactions in the BCG and BCG inactivated groups (but without statistically significant differences compared to unvaccinated animals). However, in the HIMB group, diagnostic interferences were observed irrespectively of the cocktail used.

In goats, experimental and field studies of BCG vaccination have shown a 100% of specificity of a cocktail of ESAT-6 and CFP-10 recombinant proteins (EC) used for stimulating whole blood for IFN-γ assay (Pérez de Val et al., 2016, 2012b). Moreover, the sensitivity of this peptide cocktail in field studies ranged from 50% to 80%, being similar or slightly lower to the Se obtained with the IFN-γ using tuberculins (Bezos et al., 2011a). For DIVA skin testing, a reagent including ESAT-6, CFP-10, Rv3615c and Rv3020 proteins, has been tested in BCG vaccinated goats showing promising results because no diagnostic interference was observed (Bezos et al., 2015c).

Overall, further investigation is needed to improve the diagnosis of TB in vaccinated goats with mycobacterial-based vaccines. The use of ESAT-6/CFP10 antigens for IGRA tests seems to be a useful alternative for diagnostics under vaccination programs and DIVA skin testing need to be further explored. However, the cost-effectiveness of this strategy for goat livestock industries must be further evaluated.

CHAPTER 2

Hypothesis and Objectives

Our hypothesis was that vaccination coupled with vaccine-adapted diagnostic tests could be an effective approach to control TB in goat herds. Under this rationale, the aim of the present PhD thesis was to evaluate the efficacy of different strategies of vaccination, as well as the suitability of new reagents to improve the diagnosis of caprine TB in different control and eradication scenarios.

To achieve this general goal, the following specific objectives were set:

- To assess the efficacy of a long-term systematic BCG vaccination strategy in TB naturally infected goat herds with different farm characteristics and management conditions (*Study I*).
- To study the duration of the immunity of BCG and to characterize the immunity induced by revaccination after one year in goats (*Study II*).
- To assess the efficacy of BCG revaccination one year after of first vaccination in *M. caprae* experimentally challenged goats (*Study II*).
- To evaluate the efficacy of a vaccine candidate based on a Heat-Inactivated *Mycobacterium bovis* (HIMB) in *M. caprae* experimentally challenged goats (*Study III*).
- To evaluate the performance of the antigenic complex P22 for the immune-diagnosis of TB in goats under three different contexts: TB infection, BCG and paratuberculosis vaccination, and paratuberculosis vaccination alone (*Study IV*).

CHAPTER 3

Study I

Long-term efficacy of BCG vaccination in goat herds with a high prevalence of tuberculosis

(BCG LONGFIELDT)

3.1. INTRODUCTION

TB infected goats participate in the epidemiology of animal TB in multi-host settings (Aranaz et al., 2004; Cano-Terriza et al., 2018; Ciaravino et al., 2020; Napp et al., 2013; Vidal et al., 2018) and pose a risk of zoonotic TB (Prodinger et al., 2014).

Moreover, goat TB in endemic herds implies large economic losses due to the reduction of milk production in TB infected goats (Cvetnic et al., 2007), and to trade restrictions and depopulation of positive herds (Daniel et al., 2009; Seva et al., 2002). In underdeveloped countries or high TB prevalent settings (such as Spain), eradication programs based only on test and slaughter are not always the best cost-effective strategy. Vaccination of goats against TB has been promoted as an ancillary tool for reducing TB prevalence (Conlan et al., 2015).

The *M. bovis* bacillus Calmette-Guérin (BCG), the only TB licensed vaccine in humans, and badgers in the United Kingdom, has been demonstrated to be safe and relatively inexpensive to produce (Buddle and de Lisle, 2014). The efficacy of BCG against TB has been evaluated in some livestock and wildlife species, yielding variable results (Buddle et al., 2018). A few field trials have been conducted in cattle (Ameni et al., 2018, 2010; Lopez-Valencia et al., 2010; Nugent et al., 2018b, 2017), goats (Vidal et al., 2017), and wildlife (Gormley et al., 2017; Gormley and Corner, 2009). Although protection was incomplete, encouraging results were observed in reducing the extent of lesions and the transmission of TB.

One of the major challenges for BCG vaccination in livestock is its interference with TB current diagnostic tests, namely the single intradermal test (SIT) or the

single intradermal cervical comparative tuberculin test (SICCT) and the Interferon-gamma (IFN-γ) release assay (IGRA). Antigens to differentiate infected from vaccinated animals (DIVA) have been developed in the two last decades to overcome these interferences (Vordermeier et al., 2016) and were evaluated in goats in both infection and vaccination settings (Arrieta-Villegas et al., 2020b; Bezos et al., 2011a; Pérez de Val et al., 2016).

Finally, identifying factors that increase or reduce the persistence of TB in infected herds become crucial for eradicating and controlling strategies. Herd size, housing type, or feeding practices have been identified as risk factors for TB persistence in cattle herds (Skuce et al., 2012; Veloso et al., 2016). Even though previous experimental studies demonstrated that BCG vaccination of goats confers protection against TB (Arrieta-Villegas et al., 2018; Pérez de Val et al., 2013), the factors influencing TB persistence should be disclosed to assess the efficacy of vaccination strategies at a herd level.

3.2. MATERIAL AND METHODS

3.2.1. Study design and animals

The present research was carried out in the framework of preliminary studies to establish an action plan for the control and eradication of caprine TB of the Department of Agriculture, Livestock, Fisheries, and Food of the Catalan Government (DARP). It was a field trial including five goat farms from Catalonia (Spain) with confirmed TB infection by culture (*M. caprae*) from tuberculous lesions in necropsied goats. Farms were selected due to the high proportion of positive animals detected in an initial screening carried out by the single-intradermal cervical comparative skin test (SICCT) (above 30% of

positivity). The *M. caprae* strains (www.Mbovis.org) from each farm are detailed in Table 3.1. Farms had different sizes, production, and management characteristics.

Table 3.1. Characteristics of goat farms.

Farm	Breed	No. of animals	Production	Management	Replacement Origin	*M. caprae* spoligotype
1	Blanca de Rasquera	180	Meat	Extensive	Own	SB0415
2	Malagueña	557	Milk	Intensive	Own	SB0157
3	Murciana-granadina	458	Milk	Intensive	Mixed	SB0415 SB0157
4	Murciana-granadina / Alpine /mixed	434	Milk	Intensive[1]	Mixed	SB0415
5	Mixed breeds	174	Milk	Extensive	Own	SB0415

[1] Ecological farm (daily grazing for 8h, no use of antibiotics).

The trial was conducted for 36 months. At the initial test sampling (Month 0), all adult and young (> 6 months) goats were tested with SICCT and IGRA, using *M. bovis* and *M. avium* tuberculins (CZV vaccines, Porriño, Spain) and a reagent for differentiating infected from vaccinated (DIVA) based on ESAT-6 and CFP-10 MTBC antigens (Lionex, Braunschweig, Germany). Animals positive at least to one test were classified as positive, and animals negative to all tests were vaccinated subcutaneously with BCG Danish 1331 strain *(ATCC, Ref. 35733)* behind the axillar region. All replacement goat kids added to the herd during the trial were systematically vaccinated at 2-3 months of age, and batches (B) were named depending on the month of first entry on sampling

routine (B-06, B-12, B-24, and B36, respectively). Replacement animals were managed in the herd following the productive routine of each farm. All goats with more than six months of age were tested at months (M) 6, 12, 24, and 36 after the initial vaccination (at Month 0) by the IGRA with tuberculins and DIVA reagents. BCG vaccinated animals were considered as positive depending only on the results of the DIVA test.

3.2.2. Ethics approval

All procedures were approved by the Research Ethics Commission of the Generalitat de Catalunya (procedure number 8697). All animals were managed by the personnel of the farm following conventional procedures and qualified veterinarians, authorized by the Departament d'Agricultura, Ramaderia, Pesca i Alimentació de la Generalitat de Catalunya (DARP), performed testing and sampling according to European (86/609/CEE) and Spanish (RD 53/2013) legislation.

3.2.3. Vaccine

The *M. bovis* BCG Danish 1331 strain (ATCC, Ref. 35733) was prepared as described previously (Pérez de Val et al., 2013). Briefly, an aliquot of BCG was subcultured in the Middelbrook 7H9 medium (BD diagnostics, sparks MD USA) and incubated for 28 days at 37 ºC. Then, an aliquot of the growth culture was 10-fold diluted on phosphate-buffered saline (PBS) with 0.05% Tween 80, and plated on 7H11 solid media (BD diagnostics) and incubated for 28 days at 37 ºC, then the remaining growth culture divided in 1ml aliquots that were stored at -80ºC. After incubation, the bacterial count was performed. For vaccine preparation, a suspension of one aliquot of growth culture was thawed and

diluted in PBS to reach approximately10^6 CFU/ml. An aliquot of 0.5ml of this suspension was subcutaneously injected in goats, and an aliquot of 70µl of the suspension was 10-fold diluted to verify the titer of the vaccine (2-8 $\times 10^5$ CFU/ml).

3.2.4. Single intradermal cervical comparative skin test (SICCT)

Before intradermal inoculation, the area was shaved, and skinfold thickness (SFT) was measured. The SICCT was performed by intradermal inoculation of 0.1 ml of purified protein derivative from *M. bovis* (PPD-B) and *M. avium* (PPD-A) at 25000 IU/ml each one, at the left-hand side and the right-hand side of the neck, respectively, by using a Dermojet syringe (Akra Dermojet, Pau, France). The increase of SFT was measured 72 h after inoculation. SICCT was interpreted as recommended by the Spanish bovine TB eradication program (MAPA, 2019). A goat was considered a positive reactor if SFT PPD-B > 2 mm and SFT PPD-B - SFT PPD-A > 1 mm, or if the presence of clinical signs in the PPD-B inoculation site.

3.2.5. Whole blood interferon-gamma release assay (IGRA)

Blood samples from the jugular vein were collected in heparinized tubes and were processed under 8 hours of bleeding, as described previously (Pérez de Val et al., 2016). Briefly, blood samples were stimulated with PPD-A, PPD-B, and the DIVA reagent (ESAT-6/CFP10 cocktail) at a final concentration of 20 µg/ml each. PBS was used for the unstimulated control. Samples were then incubated overnight at 37±1 ºC with 0.5% CO_2. Plasma supernatants were collected by centrifugation and analysed by ELISA (BOVIGAM, Thermo Fisher Scientific, Waltham, MA, USA) and read at 450 nm using a spectrophotometer (Biotek

Power Wave XS). The tuberculin IGRA based test was interpreted as recommended by the manufacturer: an animal was considered positive if PPD-B OD – PBS OD ≥ 0.05 and PPD-B OD > PPD-A OD, and for the DIVA reagent, an animal was considered positive if ESAT-6/CFP-10 OD – PBS OD ≥ 0.05.

3.2.6. Parameters considered for the analysis

In order to assess the effects of BCG vaccination, different parameters were calculated:

- The prevalence (P), estimated as the percentage of TB cases (new and old) at each "i" sampling time.
- Prevalence reduction between each follow-up period: estimated on the basis of the attributable fraction (AF) between periods as follows:

$$\mathbf{AF(i+1) = \frac{P(i) - P(i+1)}{P(i)}}$$

- The incidence risk (IR) in BCG vaccinated animals at each "i" sampling time. In order to account for the animals that entered (new) or leaved (lost) the group of vaccinated animals (N) throughout each follow-up period, we followed this approximation:

$$\mathbf{IR(i+1) = \frac{New\ cases(i+1)}{N(i) + Replacement\ goat\ kids\ (i+1) - \frac{1}{2} * Lost(i+1)}}$$

3.2.7. Statistical analysis

Differences between the number of TB positive animals at the beginning of the study (Month 0) and the end of the study (Month 36) for each studied farm,

were calculated using a chi-square test. A P-value < 0.05 was considered as significant.

In addition, differences in TB incidence risk among BCG vaccinated batches was evaluated taking into account the time in contact with infected animals of the herd of each vaccinated goat (i.e., less or more than one year, more than one year to two years, and more than two years to three years of being introduced in the TB positive farm) and the batch of animals. The time in contact was calculated based on the time that each animal remained negative on the farm. The interaction between both variables was included in the analysis. Besides, two qualitative variables (i.e., intensive or extensive management, high or low ventilated facilities) and five quantitative variables (i.e., number of unvaccinated animals in herds at the end of the study, initial prevalence, initial census, vaccinated positive adult and goat kids at the end of the study) were also considered. At a first step, we fit a generalized mixed-effect model with a Poisson error structure (as the number of cases is a count variable) separately for each variable. Variables with significant influence ($P < 0.05$) or with $P < 0.3$, were selected. In a second step, a multilevel model, also with a Poisson error structure, was fit, which included the farm as a random effect to take into account the lack of independence of the observations. Model selection was based on the Akaike information criterion (AIC), starting with a complex model including all variables with significant influence. Then, the complexity of the model was reduced by removing one by one of the variables without significant influence (based on P- values > 0.05). The AIC's of models generated were compared. Finally, the model with the lowest AIC was selected, and Tukey comparisons were performed in order to compare the incidence risk among batches depending on the time of exposure. All the analyses were performed

using RStudio Team (2019). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL: http://www.rstudio.com/.

3.3. RESULTS

3.3.1. Vaccine efficacy at a herd level

The evolution of TB prevalence and the prevalence reduction estimated on the basis of the attributable fraction (AF) were variable among farms throughout the vaccine trial (Figure 3.1). At the end of the trial (month 36), a favourable outcome was observed in four out of five farms. Overall AF (from Month 0 to Month 36) of 93.5%, 28.5% and 23.2% were obtained in farms 1, 2, and 3, respectively (Figure 3.1a-c). In farm 5, there was a dramatic decrease in the census in the six first months of the trial (from 174 to 135 animals), and the prevalence raised from 46.0% at month 0 (M0) to 60.7% at month 6 (M6). The overall AF in farm 5 was 14,32% (Figure 3.1d). In farm 4, the percentage of positive animals remained stable until M12 (~33%) but increased to 46.5% from M12 to M36. Thus, no prevalence reduction was obtained in farm 4 (Figure 3.1e). The overall results of the five farms are represented in Figure 3.1f. Altogether, the overall initial prevalence (M0 = 41%) started to drop at M6 (37.1%), remained stable at M12 (38.3%) and M24 (38.1%), and continued to drop at M36 (33.6%). The overall AF between M0 and M36 in the five farms was 18.1%.

Farms 1, 2, 3, and 5 showed a significant overall reduction of TB positive animals ($P < 0.001$, chi-square test), passing from 546 TB positives out of 1255 goats at M0 (i.e. 43.5% of TB positive animals) to 451 out of 1506 at M36 (142 TB positive goats vaccinated and 148 TB positive unvaccinated goats, i.e. 30%

of TB positive animals), with an overall percentage of prevalence reduction (AF) of 31% at the end of the study. The overall prevalence (% of TB positive animals) and AF of these four farms that showed a favourable vaccination outcome are summarized in Table 3.2. The overall proportion of TB-infected animals significantly decreased at each sampling time point while the AF increased. The evolution of the goat census in terms of TB status in farms is shown in Figure 3.2. In farms with a positive outcome, at M36, the unvaccinated animals that remained in the herds represented 32.8% of the total of TB positive animals. The vast majority of goats (90.2%) from these farms were vaccinated, and 77.7% of them remained TB negative.

Table 3.2. Overall evolution of TB status of farms 1, 2, 3 and 5 throughout 36 months of vaccination trial

	Month 0	**Month 6**	**Month 12**	**Month 24**	**Month 36**
Total Census	1255	1387	1335	1345	1506
% TB+ animals	44% (33-63)	39% (23-61)*	39% (5-65)*	35% (1-57)**	30% (2-48)**
Attributable fraction[a]	-	11% (0-32)	11% (0-85)	19% (0-97)	31% (14-94)

[a]Attributable Fraction: Percentage of prevalence reduction between prevalence at M0 and the prevalence at subsequent samplings. Ranges are shown in parenthesis. * $P < 0,05$. ** $P < 0,001$. Chi square test. Significant differences respect to the initial proportion of positive animals at M0.

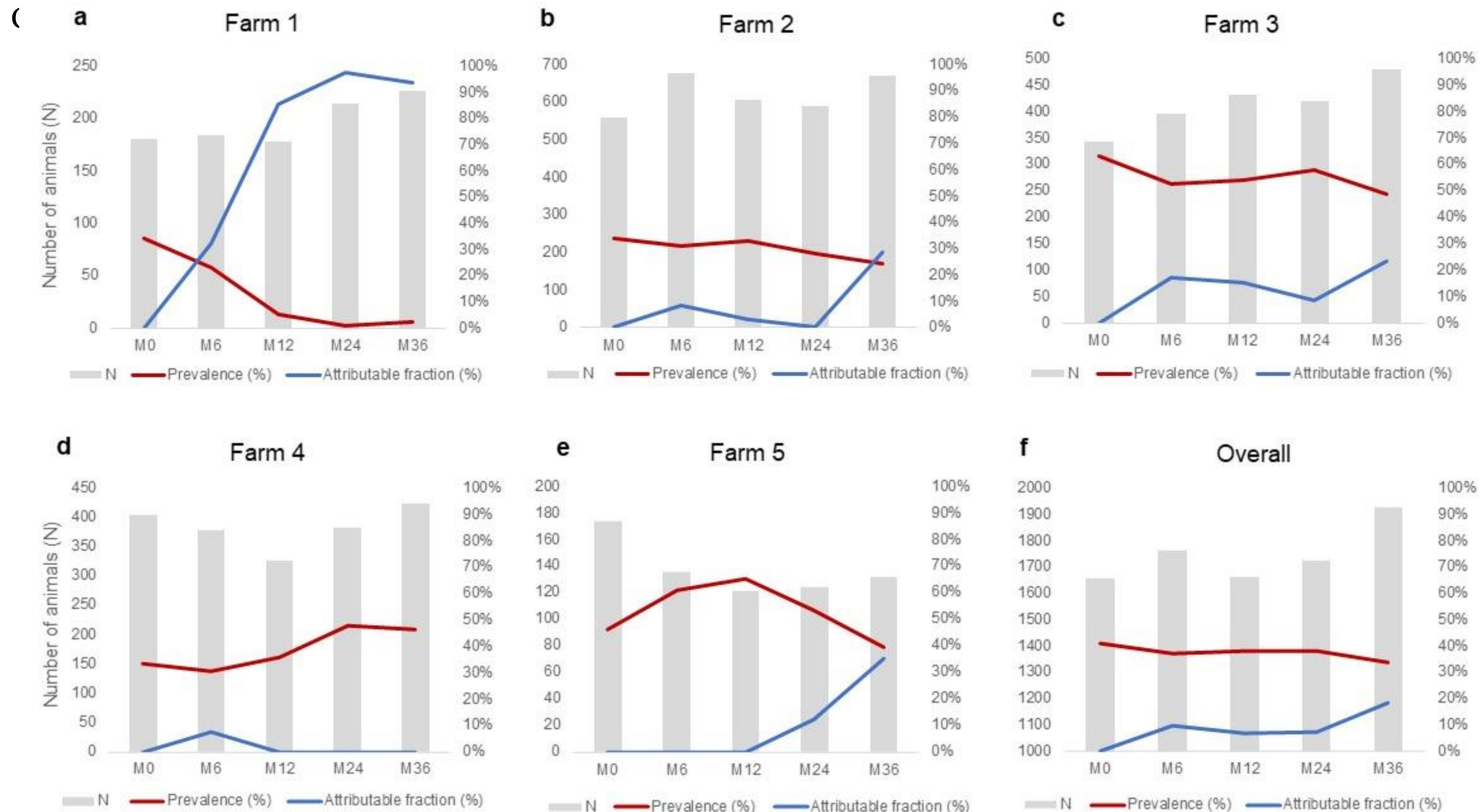


Figure 3.1. Evolution of prevalence throughout the BCG vaccination trial. Evolution of census (grey bars), prevalence (red lines) and percentage of prevalence reduction (blue lines) in farms 1 (a), 2 (b), 3 (c), 4 (d) and 5 (e), and overall reduction of five farms (f). The prevalence was measured as the percentage (%) of TB positive animals = number of TB positive goats / total census. The reduction of prevalence was measured as attributable fraction (AF) = (% of TB positive at M0 - % of TB positive at M6 or M12 or M24 or M36) / % of TB positive at M0.

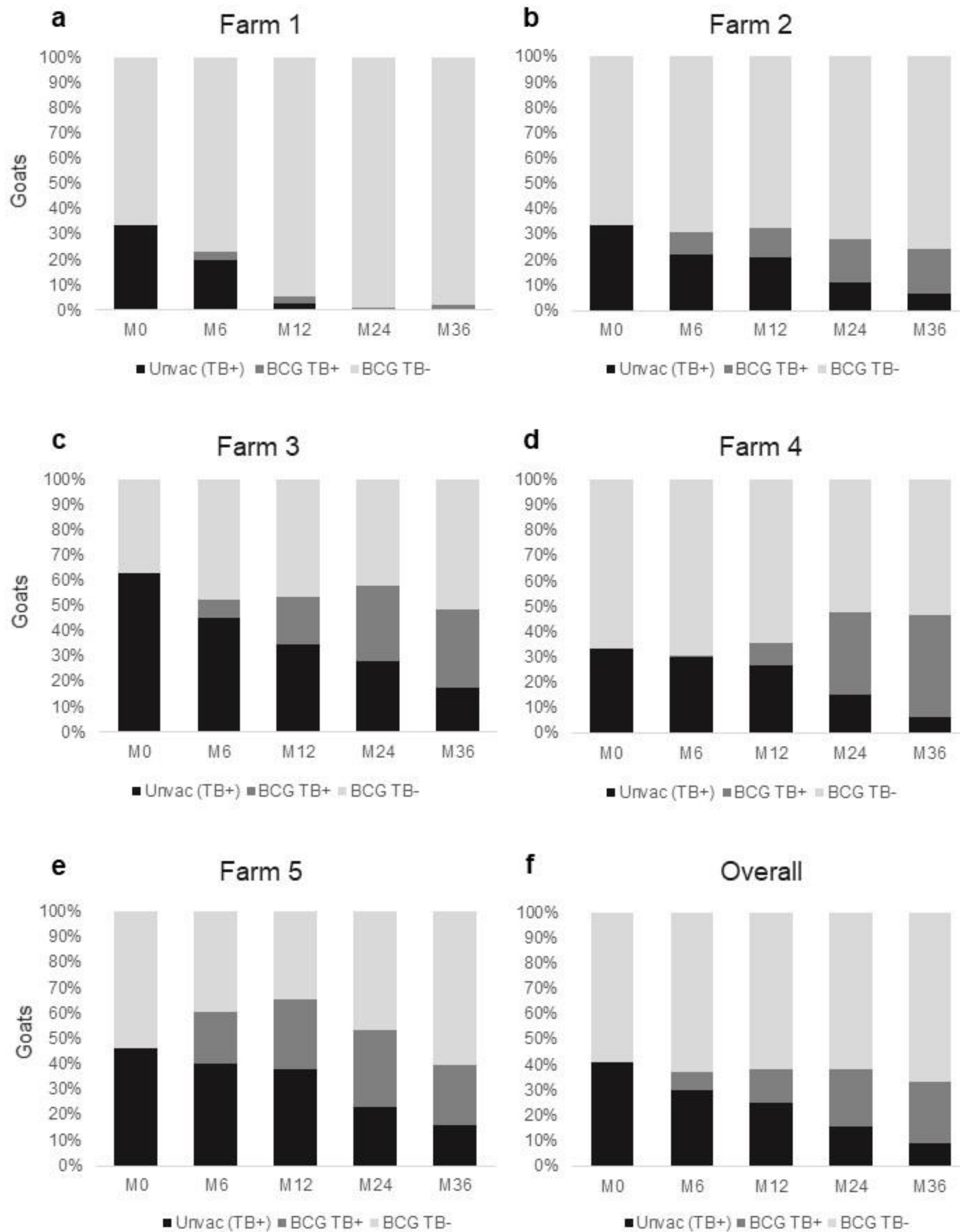


Figure 3.2. Proportion of TB positive and BCG vaccinated goats from farms 1 (a), 2 (b), 3 (c), 4 (d), 5(e) and all farms (f) throughout the trial. Evolution of population of vaccinated goats TB negative (light grey), vaccinated TB positive goats (dark grey) and unvaccinated animals (black) during the 36 months (M) of trial. Number of animals of each population indicated at each sampling time point.

The incidence (Ia) of TB in BCG vaccinated goats from farms was also calculated (Table 3.3). The overall data from all five farms did not show reductions in the incidence between samplings. In farms with a favourable outcome (farms 1, 2, 3, and 5), in general, there was a progressive reduction of incidence between sampling time points, until the end of the trial, and a 50% of overall incidence reduction was observed from M6 to M36.

Table 3.3. Evolution of TB incidence risk (IR) in BCG vaccinated goats from farms throughout the study.

	M0-M6			M6-M12			M12-M24			M24-M36		
Farm	**TB+**	**Total[a]**	**IR**	**TB+**	**Total**	**IR**	**TB+**	**Total**	**IR**	**TB+**	**Total**	**IR**
1	6	109	**6%**	5	131	**4%**	2	106	**2%**	4	120	**3%**
2	55	360	**15%**	21	454	**5%**	42	369	**11%**	49	576	**9%**
3	23	121	**19%**	53	183	**29%**	32	116	**28%**	27	247	**11%**
4	2	255	**1%**	24	244	**10%**	73	188	**39%**	87	330	**26%**
5	28	90	**31%**	8	53	**15%**	8	38	**21%**	6	88	**7%**

[a]Total of susceptible animals was calculated by the addition of the new vaccinated goat kids minus the half of the number of vaccinated animals that died or were slaughtered between sampling time points (we assumed that they were present a half of the period).

3.3.2. Efficacy of BCG in the different vaccinated batches

At the end of the trial, in farms 1, 2, 3, and 5, adult goats vaccinated at the beginning of the study showed a higher proportion of TB positive animals compared to the goats vaccinated at 2-3 months of age (Table 3.4).

Table 3.4. TB prevalence at month 36 of trial by age of vaccination of goats from farms 1, 2, 3 and 5.

	TB+	TB-	Total	% TB+ at M36
Adult[a]	142	179	321	44%
Goat kids[b]	161	876	1037	16%

[a]Adult goats vaccinated with BCG at M0. [b] Goat kids vaccinated at 2-3 months of age throughout the trial.

The evolution of TB status throughout the trial in BCG vaccinated batches is shown in Figure 3.3. Among farms, adult goats vaccinated at M0 (Batch B-0) showed the highest TB incidence during the first year in contact with infected animals of the herd regardless of the farm (Figure 3.3, B-0 showed the highest decrease in the proportion of TB negative animals during this period). In farms 1, 2, and 5 all batches of vaccinated goat kids (B-06, B-12, B-24, and B-36) showed a low risk of infection irrespectively of the time of contact, with incidences between periods ranging from 0% to 3.5 %, 0% to 10% and 6.25% to 9.23%%, for farms 1, 2 and 5, respectively (Figure 3.3 a, b and e). In farm 3, the proportion of TB negative adult vaccinated goats at M0 (Batch B-0), and goat kids vaccinated at M0 (Batch B-06) decreased dramatically after the first year of contact, with only 54% and 69% of goats remaining TB negative, respectively (Figure 3.3c). After three years of contact, only 35% and 38% of individuals of these two batches remained TB negative, respectively. In addition, in batch B-12 (goat kids vaccinated during the first year of the trial), the proportion of TB negatives dropped to 57% after three years of contact. In contrast, the proportion of TB negative animals in batches B-24 and B-36 (goat kids vaccinated in the

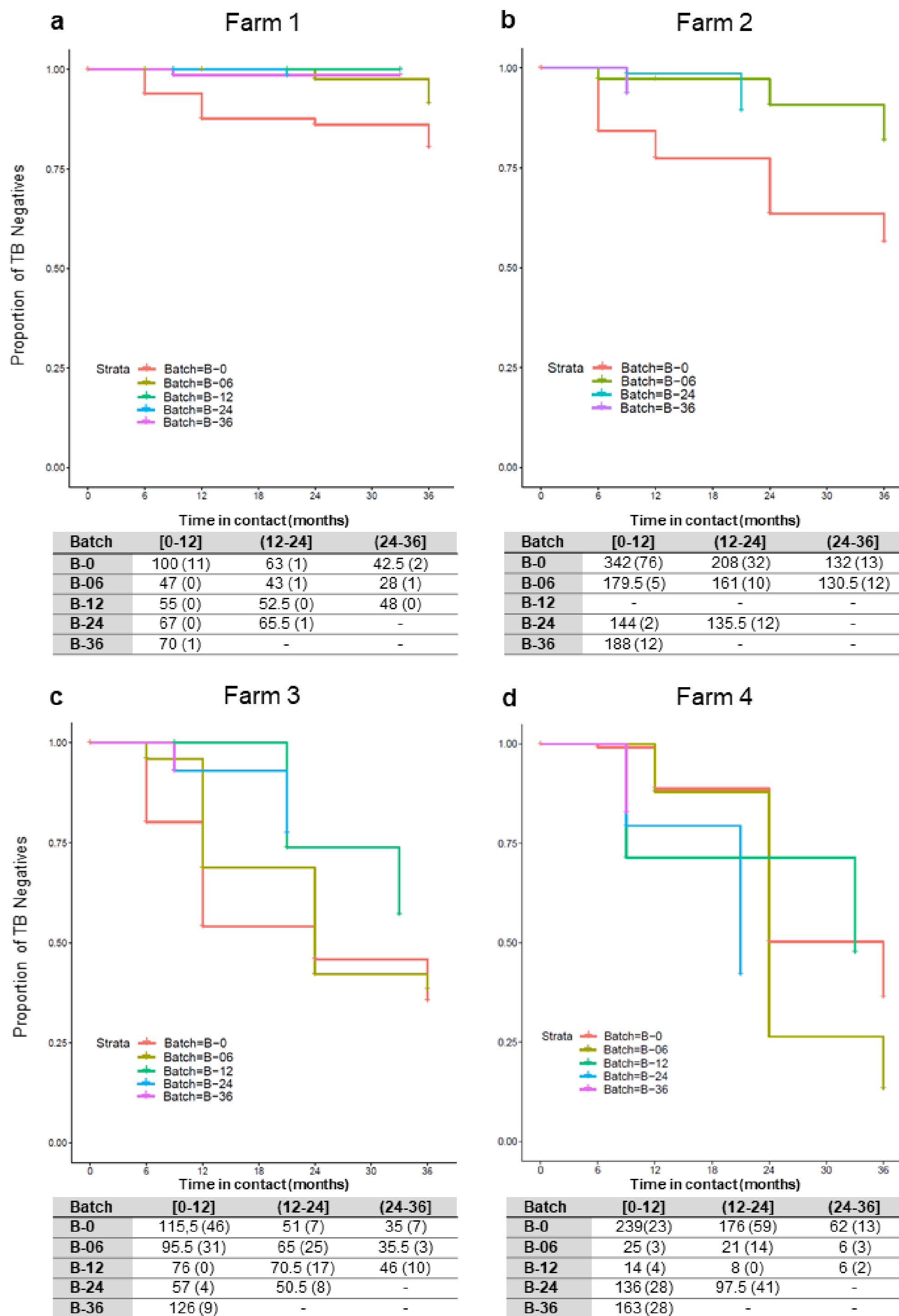
second and third year of the trial), that were in contact for two and one years of study, respectively, were 77.5% and 93%, respectively. In farm 4 (Figure 3d), after three years of contact, the proportion of TB negatives within adult vaccinated goats (B-0) decreased to reach a 36% and in animals vaccinated when goat kids, B-06, B-12, decreased to 13% and 47%, respectively. In batches B-24 and B-36, after two and one years of exposure, the proportion of TB negatives was 42% and 83%, respectively.

The TB incidence risk was evaluated using the multilevel model with the lowest AIC. Results of the paired comparisons between batches of the four farms during the first year in contact with the TB positive herd are described in Table 3.5. The risk of TB in batch B-0 (adult goats vaccinated at M0) during the first year of contact was significantly higher compared to three out of the four batches of vaccinated goat kids (B-06, B-24 and B-36). No differences were observed among batches vaccinated when goat kids, neither among batches after two or three years of contact with TB positive herds regardless of the age of vaccination.

Table 3.5. Risk of TB infection per batch during the first year in contact with the TB positive herd.

Comparisons between batches	Risk Estimate[1]
B-0 vs B-06	0.927 (± 0.186)[a]
B-0 vs B-12	19.78 (± 323.87)
B-0 vs B-24	1.89 (± 0.299)[b]
B-0 vs B-36	1.61 (± 0.219)[b]

[1]Risk estimate coefficient of TB infection (±SE) between batches in farms 1, 2, 3 and 5, determined by a generalized mixed-effect model with *post hoc* Tukey comparisons between batches. [a] $P = 0.0001$, [b]$P < 0.0001$.



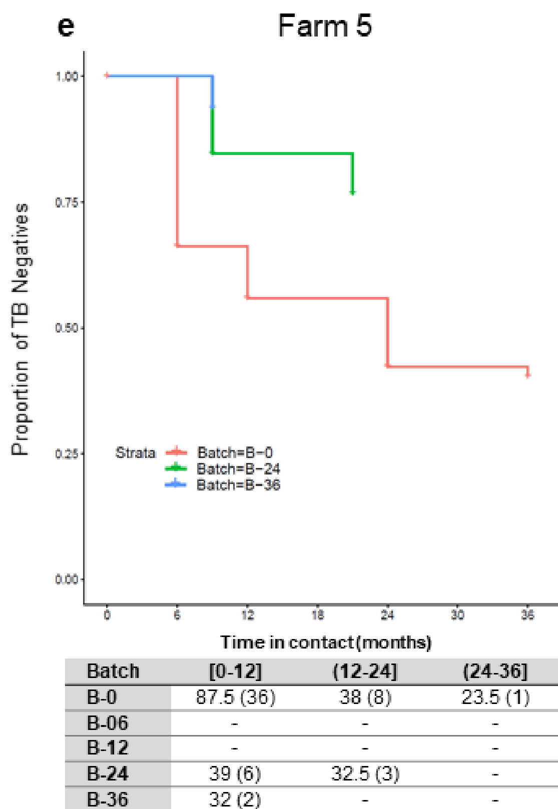
Batch	[0-12]	(12-24]	(24-36]
B-0	100 (11)	63 (1)	42.5 (2)
B-06	47 (0)	43 (1)	28 (1)
B-12	55 (0)	52.5 (0)	48 (0)
B-24	67 (0)	65.5 (1)	-
B-36	70 (1)	-	-

Batch	[0-12]	(12-24]	(24-36]
B-0	342 (76)	208 (32)	132 (13)
B-06	179.5 (5)	161 (10)	130.5 (12)
B-12	-	-	-
B-24	144 (2)	135.5 (12)	-
B-36	188 (12)	-	-

Batch	[0-12]	(12-24]	(24-36]
B-0	115,5 (46)	51 (7)	35 (7)
B-06	95.5 (31)	65 (25)	35.5 (3)
B-12	76 (0)	70.5 (17)	46 (10)
B-24	57 (4)	50.5 (8)	-
B-36	126 (9)	-	-

Batch	[0-12]	(12-24]	(24-36]
B-0	239(23)	176 (59)	62 (13)
B-06	25 (3)	21 (14)	6 (3)
B-12	14 (4)	8 (0)	6 (2)
B-24	136 (28)	97.5 (41)	-
B-36	163 (28)	-	-

Figure 3.3. TB Incidence in BCG vaccinated batches from farms 1 (a), 2 (b), 3 (c), 4 (d) and 5 (e) throughout the trial. Proportion of TB negative animals (y axis) throughout the time in contact



Batch	[0-12]	(12-24]	(24-36]
B-0	87.5 (36)	38 (8)	23.5 (1)
B-06	-	-	-
B-12	-	-	-
B-24	39 (6)	32.5 (3)	-
B-36	32 (2)	-	-

Figure 3.3. (Continued). (x axis) with the TB positive herd in months. The table represents the incidence of TB in each batch (B) depending on the time in contact, between 0 and 12 months ([0-12]), more than twelve to twenty-four months ((12-24]) and more than 24 to 36 months ((24-36]). The animals at risk, which were the TB negative animals between sampling time points (some animals were slaughtered or died between periods. Those animals were considered present during the half of the period and its number was divided by two). In parenthesis are represented the number of TB new positive goats during a given period.

3.3.3. Factors influencing the incidence of TB in vaccinated animals

The generalized mixed-effect model of each risk factor showed that the qualitative variable of facilities ventilation (high or low), and four quantitative variables (the initial prevalence, the number of positive unvaccinated animals remaining in herds, the number of adult BCG vaccinated TB positive goats at the end of the study and, the number of BCG vaccinated TB positive goat kids at the end of the study) potentially influenced the incidence in vaccinated animals.

Thus, these six variables were included in the multilevel model. The initial census and management (intensive or extensive) did not show any relevant effect (*P* > 0.3) at the individual analysis and thus, were discarded for the multilevel model. Based on the multilevel model with the lowest AIC, the number of positive unvaccinated animals remaining in herds and the number of positive adult vaccinated goats (B-0) were significant risk factors (*P* < 0.05) for TB infection in BCG vaccinated animals. The initial herd prevalence was still not a significant TB risk factor *(P* < 0.1). The other parameters included in the model were not detected as significant risk factors for TB infection in BCG vaccinated goat herds.

3.4. DISCUSSION

In this field study, the efficacy of long-term vaccination with BCG of adult and replacement goat kids was evaluated. The vaccine efficacy was determined as the reduction of prevalence, AF, and incidence of each herd during three years, using IGRA responses against DIVA antigens. The results of vaccine efficacy were heterogeneous among farms, similar to that observed in previous human studies (Mangtani et al., 2014), varying from substantial to absence of protection. Taking into account the data from all farms, the moderate vaccine efficacy observed in the present study was similar to that observed in a systematic review of BCG vaccination trials in children (Roy et al., 2014). Protection of goat kids against TB with BCG has been previously reported in a preliminary study conducted in field conditions, as a reduction in the number of animals with TB lesions in the vaccinated group compared to the unvaccinated one (35% and 77%, respectively), after 18 months of exposure to natural infection in the herd (Vidal et al., 2017). In the light of these results, the present study was aimed to assess the long-term efficacy of a blanket vaccination

program in a real field situation with productive goats in which all susceptible animals (TB negative adults and replacement goat kids) were vaccinated. Four out of five goat farms (farm1, 2, 3, and 5) showed a favourable outcome in terms of vaccine efficacy (AF, prevalence and incidence reductions), with a majority of vaccinated animals remaining negative. Other field trials to assess BCG efficacy in cattle showed an even higher proportion of protected animals, although it was determined using different parameters. Thus, protections of 77.9% and 67.4%, measured as TB lesions and bacterial load reduction, were observed in subcutaneously (BCG 3×10^5 CFU) and orally (BCG 1×10^8 CFU) vaccinated cattle, respectively (Nugent et al., 2018b, 2017).

Comparisons of BCG efficacy between field trials should be addressed with caution since the studies were conducted under different conditions, animals were exposed to different TB infection rates, and efficacy was measured using different parameters. Here, the initial TB prevalence of farms ranged between 33-63%, whereas in cattle studies, the herds had a proportion of TB positive reactors under 6%. These differences in herd prevalence may explain the higher BCG efficacy rates described in cattle than in the present study in goats. Another explanatory factor is the apparent higher intra-herd transmission of TB within the goat herds of the present study. Most of the herds (four out of five) had highly intensive management which implies that animals are grouped, often in high densities, and usually remaining in buildings with or without access to the outdoors, in contrast to extensive management where densities are low and animals usually have permanent access to outdoors for grazing. Thus, this intensive management in farms may have entailed closer contact among animals, with the exception of farm 1, in which the vaccine showed the highest protection rate and the management was extensive.

Even though BCG vaccination does not provide full protection against TB, it reduces the transmission of MTBC (Buddle et al., 2018). Previous field and experimental studies in cattle and goats showed that BCG decreased either mycobacterial shedding (Lopez-Valencia et al., 2010) or TB pathology (Ameni et al., 2018; Arrieta-Villegas et al., 2018; Vidal et al., 2017). Different practices may affect the evolution of incidence and prevalence among goat farms. All of them discarded (more or less intensively) the old unvaccinated TB positive animals (results of tests were made available to farmers) for the slaughterhouse, which is one of the most common control measures. In this sense, Ameni and collaborators suggested that gradual removal of positive animals and their replacement by BCG vaccinated cattle, can be a valuable approach for TB control when intensive test and cull of positive animals is not affordable (Ameni et al., 2010). Accordingly, the transmission of *M. caprae* in the present field trial, measured as TB incidence in vaccinated animals, was progressively reduced in four out five farms, attaining an overall reduction of 50% (Table 3-3).

Intriguingly, in farm 5, even with a positive outcome at the end of the trial, the initial prevalence increased six months after vaccination. In this case, a reduction of the census and a raise of incidence among vaccinated goats were observed. This farm had the lowest census among farms from the study and these fluctuations in the number of animals had a high impact on the prevalence outcome. Some deaths might be explained by productive reasons (8 goats from B-0 were discarded) and by the cold weather, which can worsen the symptoms of respiratory diseases (Matthews, 2016). These weather conditions might force the farmer to maintain animals enclosed. In this particular case, the buildings had poor airflow, and the proximity between animals probably promoted TB transmission and other respiratory diseases/pathogens. To our disappointment,

the vaccine failed to confer protection on farm 4. A single factor cannot explain this outcome. However, the results lead us to speculate that the most likely cause of vaccine failure might be that some batches of goat kids could be infected before vaccination. Those animals were vaccinated after two months of age, most of them were in contact with adult goats after birth, and were potentially fed with unpasteurized colostrum. Nevertheless, overall findings suggest that the BCG vaccine is effective in reducing TB prevalence in goat farms. However, its effects are highly influenced by intrinsic factors of the farm (i.e., management practices, facilities, etc.).

The direct effect of vaccination among batches was also evaluated. For that purpose, incidence and time to IFN-γ conversion (DIVA test) of each animal were measured. TB incidence in adult vaccinated goats (batch B-0) was higher compared to vaccinated goat kids (batches B-06, B-24, and B36), mainly during the first year in contact with the TB positive herd. Similarly, vaccination of yearling buffaloes with BCG failed to protect them against *M. bovis* challenge (de Klerk et al., 2010). The lack of efficacy of BCG in adult buffaloes was probably due to contact with environmental mycobacteria (EM). Indeed, adult goats had more chances to be sensitized by the exposure to EM prior to vaccination than goat kids. Contact with EM before BCG vaccination was associated with reduced vaccine efficacy in cattle (Buddle et al., 2002) and humans (Black et al., 2002). In addition, goats from five farms were usually infected by helminths. Pre-existing infections with helminths may reduce the efficacy of vaccines against TB (Elias et al., 2008; Hatherill et al., 2009). Indeed, BCG vaccinated cattle co-infected with *Fasciola hepatica* and *M. bovis* showed lower IFN-γ responses against PPD-B and reduced the number of reactors to SICCT (Flynn et al., 2009). Helminthic infections usually drift the immune response towards a type T-helper 2 response and downregulate the T-

helper 1 (Brown et al., 1994). The latter immune response is necessary for the production of IFN-γ and effective responses against *M. bovis* (Welsh et al., 2005). These responses are triggered after BCG vaccination, but it seems that pre-existing infections with helminths do not lead to the development of those responses[43]. Another relevant factor that cannot be ruled out is that some animals vaccinated at the beginning of the study (B-0) after showing a negative result to the IFN-γ DIVA test could be false negatives. In fact, chronic TB infections inducing anergy have been observed early in life in goats (Domingo et al., 2009) and in young calves (Houlihan et al., 2008) probably due to the exhaustion of antigen-specific cell-mediated immune responses against the mycobacteria after a long period of infection (Khan, 2017).

The multilevel model detected a higher risk of TB infection in adult vaccinated goats (B-0) during the first year in contact with the TB positive herd. The risk was significantly higher compared to B-6, B-24, and B-36. Differences between B-0 and B12 were not significant, probably because only two farms had a batch B-12, and the number of goats in this batch was low (see Figure 3-3). These findings also reinforce the hypothesis that vaccination of adult goats could be less effective. In addition, the incidence and the risk of TB infection in replacement goats detected by the multilevel model were similar regardless of the time of contact and the batch of vaccinated goat kids. Nonetheless, after one year of contact, the risk of TB infection was similar among batches irrespectively of the age of vaccination. Thus, the protective immunity of BCG vaccination decayed after one year. In the same way, in BCG vaccinated cattle, no significant protection by the reduction in gross pathology was observed after challenge with *M. bovis* after 24 months of BCG vaccination (Thom et al., 2012). However, significant protection was still detected when the challenge was carried out at 12 months post-vaccination (Thom et al., 2012). Here, despite

a decrease in BCG vaccine efficacy after one year of vaccination regardless of the batch, some degree of individual protection may remain, and systematic vaccination of replacement goat kids, progressively reduced the TB prevalence, as observed in oral BCG vaccinated badgers (Gormley et al., 2017). It suggests that high vaccination coverage may increase the protection against TB infection within the herd and improve the vaccine efficacy. Even so, other strategies to boost the protective immunity against TB, such as BCG revaccination, that showed improved protection than the single-dose regime in cattle (Parlane et al., 2014), might be further explored in large scale field trials.

On the other hand, as mentioned before, other control measures and intrinsic factors from farms may contribute to the control of TB and may favour the success or failure of the vaccine program. In this context, the multilevel model was also used to evaluate different factors influencing the TB incidence among farms. The TB positive unvaccinated animals remaining in the herds and the adult vaccinated goats were identified as the two factors that increased the risk of TB cases in herds. As expected, initial TB positive animals were a source of infection for vaccinated animals. Indeed, some adult animals, probably chronically infected, may act as super shedders (Santos et al., 2015). Regarding the risk of adult vaccinated goats, as previously observed in humans, vaccination of adults was not protective (Mangtani et al., 2014). Moreover, as discussed above, it is possible that some vaccinated animals were anergic before BCG vaccination, which is a phenomenon that can appear relatively early in goats (at 2-3 years of age) (Domingo et al., 2009). Thus, despite vaccination, in our conditions, adult vaccinated goats may act as the unvaccinated goats, contributing to the spread of the TB infection in herds. On the other hand, the initial prevalence was not detected as a significant risk factor for TB infection in the herds, however, a high initial prevalence may increase the pressure of

infection, hampering the positive effects of vaccination. Interestingly, BCG vaccinated goat kids that converted positively to IGRA did not appear to represent a risk for TB infection for other vaccinated animals. Probably these goats were less likely to infect other animals because they were partially protected and did not excrete or had reduced the mycobacterial shedding (Lopez-Valencia et al., 2010). Also, previous experimental infections with *M. caprae* showed slower progression of the TB infection, and a lower degree of lung lesions in BCG vaccinated goats compared to unvaccinated controls (Arrieta-Villegas et al., 2018; Pérez de Val et al., 2013, 2012b; Roy et al., 2019). This reduction of pathology does not favour the mycobacterial excretion by coughing (Urbanowski et al., 2020). Thus, both the progressive slaughter of TB positive unvaccinated animals and the systematic BCG vaccination of replacement goat kids may contribute to the reduction of TB incidence and TB prevalence in goat farms. Furthermore, previous studies have identified a high census of cattle (Skuce et al., 2012) and intensive management (Griffin et al., 1993) as risk factors for TB disease. However, in our conditions, these factors did not show a significant impact on the risk of TB infection. It is important to remark that risk assessments should be taken with caution because sample size, the measures used, the variables analysed, and the size of herds may differ among studies (Skuce et al., 2012).

Finally, the SICCT and the IGRA with tuberculins and DIVA reagents were used as indicators of TB infection in order to maximize the detection of infected animals prior to vaccination. However, as BCG vaccination causes interferences with the SICCT and the IGRA standard, at least after one year of vaccination (Thom et al., 2012), the only available diagnostic tool for the diagnosis of TB in vaccinated goats was the IGRA using DIVA reagents. The main caveat of this DIVA test in goats is that, the observed Sensitivity in previous studies in TB

infected goat herds was around 70% (Arrieta-Villegas et al., 2020b; Bezos et al., 2011a), despite being highly specific (100%) (Arrieta-Villegas et al., 2020b). On the other hand, as responses developed against BCG vaccine contribute to the containment of the infection (Pérez de Val et al., 2013), it is possible that some vaccinated-infected animals do not develop a detectable response by the IGRA DIVA. Thus, up to 30% of false negatives among vaccinated animals had to be assumed in the present study and this could attenuate the intervallic incidence risk. However, the same criterion for positivity was used in all time-points, enabling to assess the vaccine efficacy by the evolution of TB prevalence and AF.

In conclusion, our results showed that systematic BCG vaccination of replacement goat kids could contribute to reducing the transmission of *M. caprae*, steady reducing the overall TB prevalence within the herds, even when individual vaccine efficacy decreased one year after vaccination. Moreover, removing positive unvaccinated goats from the herds reduced the risk of new TB cases, favouring the success of the vaccination program. Therefore, besides vaccination, additional measures, such as test and cull of positive animals, are strongly recommended. The present study provides relevant data to evaluate the cost-benefit of a long-term vaccination strategy to the control of caprine TB.

CHAPTER 4

Study II

Immunogenicity and protection against *Mycobacterium caprae* challenge in goats vaccinated with BCG and revaccinated after one year

(BCG REVAC)

4.1. INTRODUCTION

The induction of protective immunity and its duration are key points for vaccine development. Antigen-specific immune responses to mycobacteria are generally measured through the evaluation of the in vitro secretion of Interferon-gamma (IFN-γ) by lymphocytes cultured with MTBC antigens. A requisite for a successful vaccine is the induction and differentiation of T-cells into IFN-γ secreting antigen-specific memory cells that may rapidly reach the site of the infection (Kipnis et al., 2005; Orme and Henao-Tamayo, 2018). In addition, the durability of vaccine-induced immunity is thought to be critical for the development of effective vaccination strategies against TB, enabling the host to respond more quickly and effectively after re-exposure to MTBC antigens. It has been shown that protection conferred by BCG in cattle wanes with time (Thom et al., 2012); and that BCG revaccination prolonged the duration of protection conferred against *M. bovis* infection by the primary vaccination (Parlane et al., 2014).

Moreover, the major constraint for the use of BCG either in cattle or goats is the interference of vaccination with the tuberculin skin test and IFN-γ release assay (IGRA), which are the approved tests for the diagnosis of TB in livestock. In this sense, the suitability of other MTBC-specific antigens as potential diagnostic test targets for differentiating infected from vaccinated animals (DIVA tests) is another focus of interest on animal TB research (Vordermeier et al., 2016).

In the present study, we evaluated: (1) the efficacy of BCG revaccination against *M. caprae* challenge in goats; (2) the duration of the immunity induced by BCG vaccination in goats and characterize the immunity induced after revaccination; and (3) the long-term BCG-induced interferences on TB immunodiagnosis.

4.2. MATERIAL AND METHODS

4.2.1. Animals, vaccination schedule and experimental infection

Thirty Murciano-granadina female goat kids, aged 6 to 7 weeks, were sourced from the experimental farm of the *Universitat Autònoma de Barcelona* (Catalonia, Spain). Experimental animals were negative to the IGRA as performed using a commercial kit (ID screen® Ruminant IFN-γ, ID.vet, Grabels, France). Goats were randomly assigned to three experimental groups of 10 animals each: Unvaccinated control, BCG, and BCG-BCG. Animals from BCG and BCG-BCG groups were subcutaneously inoculated (right axilla) with 0.5 mL (5×10^5 Colony Forming Units/animal) of *M. bovis* BCG Danish 1331 strain (ATCC, Ref. 35733TM) at week 0. BCG-BCG animals were re-vaccinated 56 weeks later. The BCG vaccine stock was generated as described previously (Balseiro et al., 2017) and diluted in sterile phosphate buffered saline (PBS) to reach a suspension of 1×10^6 Colony Forming Units (CFU)/mL before use, and 0.5 mL were inoculated into each animal. Blood samples from all animals were taken every 8 weeks from week 0 to week 56, and at week 59 (3 weeks after re-vaccination).

At week 63, all animals from the three experimental groups (unvaccinated control, BCG, and BCG-BCG) were transferred from the experimental farm to the Biosafety Level 3 (BLS3) facilities of IRTA-CReSA. Animals were housed in groups of 10 with three or four animals from each group. One week later, goats were sedated intramuscularly (with Equipromacina® at 0.05 mg/kg and Torbugesic® at 0.2 mg/kg). Subsequently, goats were anaesthetized intraveonusly (with Propofol Lipuro® at 4–5 mg/kg), and then endobronchially challenged with *M. caprae* (~3×10^3 CFU), as previously described (Arrieta-Villegas et al., 2018). The inoculum stock of *M. caprae* strain SB0416

(www.Mbovis.org) was prepared as described previously (Arrieta-Villegas et al., 2018). After the challenge, animals were monitored daily for clinical signs of TB, weighed and bled every two weeks, and rectal temperature was measured every week. Animals were euthanized at week 72 (9 weeks after challenge).

Animals were fed with hay and food supplements as necessary and maintained with water ad libitum throughout the experiment.

4.2.2. Ethics statement

All experimental animal procedures were approved by the Animal Research Ethics Commission of the Generalitat de Catalunya (procedure number 8697/2015), according to current European legislation for the protection of experimental animals (86/609/EEC, 91/628/EEC, 92/65/EEC and 90/425/EEC).

4.2.3. In vitro IFN-γ release assay (IGRA)

Whole Blood samples from all experimental goats were collected from the jugular vein in 10 mL heparinized tubes at weeks 0 (prior to vaccination), 8, 16, 24, 32, 40, 48, 56 (before revaccination), 59, 64 (prior to challenge), 66, 68, 70 and 72 of the experiment. Whole blood samples were stimulated in 96-deep well cell culture plates (Eppendorf Ibérica, Madrid, Spain) with *M. bovis* (PPD-B) and with *M. avium* (PPD-A) tuberculins (CZ Veterinaria, Porriño, Galicia, Spain), and a cocktail of the recombinant proteins ESAT-6 and CFP-10 (EC) (Pérez de Val et al., 2016) at a final concentration for all three reagents of 20 µg/mL. PBS was used as a negative control for the stimulation of blood samples from each animal. Samples were incubated at 37 °C in a 5% CO_2, 95% humidity atmosphere. After 16–20 h of incubation, plasma supernatants were harvested by centrifugation at 18× *g* for 10 min and analyzed immediately by sandwich

ELISA for bovine IFN-γ (ID.vet) or stored at −20 °C for subsequent analysis. The IGRA was performed following the manufacturer instructions. The results of IGRA based on tuberculins were interpreted using the cut-off point of sample to positive ratios (S/P) recommended by the manufacturer: ((Optical density (OD) of PPD-B – OD of PPD-A)/(OD mean kit positive control (CP) – OD mean kit negative control (CN))) × 100. A sample was considered positive if S/P ≥ 16%. The results of IGRA based on EC, were calculated as follows: S/P = ((OD of EC – OD of PBS)/(OD CP – OD CN)) × 100. A sample was considered positive if the sample S/P ≥ 16%.

4.2.4. Flow cytometry and intracellular IFN-γ staining

Flow cytometry analysis was performed at week 59 (3 weeks after revaccination), and in order to evaluate IFN-γ^+ T-cell memory subsets expanded after revaccination and one year after vaccination. Firstly, the blood from the 30 goats was collected using the BD vacutainer CPT with sodium citrate (BD, Franklin Lakes, NJ, U.S.A.). Peripheral blood mononuclear cells (PBMC) were then isolated from blood samples following the BD vacutainer CPT instructions. Isolated PBMC were stimulated in 24-well plates (2×10^6 cells/well) with PPD-B at a final concentration of 10 μg/mL in RPMI 1640 cell culture medium (Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 10% foetal calf serum (Sigma-Aldrich), non-essential amino-acids (Sigma-Aldrich), 5×10^{-5} M 2-mercaptoethanol, 100 U/mL penicillin and 100 μg/mL streptomycin sulphate. Cells were incubated at 37 °C with 5% CO_2, 95% humidity for 6 h. Then, brefeldin A (Sigma-Aldrich, Steinheim, Germany) was added at a final concentration of 10 μg/mL, and cells were incubated for a further 16 h.

Stimulated PBMC were counted, and 3×10^5 cells/well were dispensed in 96-well plates. Cells were stained with mouse monoclonal antibodies (mAb) CC8

(IgG2A, conjugated to FITC), which recognizes bovine CD4, and mAb IL-A116 (IgG3, conjugated to RPE), which recognizes bovine CD45RO (both from Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.). CD4 and CD45RO stained cells were then fixed and permeabilized with Leucoperm™ (Bio-Rad), according to the manufacturer's instructions, and incubated with mAb CC302 (IgG1, conjugated to Alexa Fluor® 647, Bio-Rad), which recognizes bovine IFN-γ, for 30 min at room temperature. Finally, cells were re-suspended in 200 µL PBS with 1% paraformaldehyde, stored at 4 °C in the dark, and analyzed within 24 h.

Stained cells were analyzed by flow cytometry in a MACSQuantify™ instrument (Miltenyi Biotec, Bergisch Gladbach, Germany). The gating strategy is shown in Figure 4.1.

4.2.5. Skin tests

In order to evaluate the effect of vaccination on the skin test, tuberculin-based and DIVA skin tests were carried out in all experimental goats from each group. Skin tests were carried out 59 weeks after vaccination for the BCG group and 3 weeks after revaccination for the BCG-BCG group. First, the proximal zone of both sides of the neck was shaved, and basal skinfold thickness (BST) was measured with a caliper. Then, the single intradermal cervical tuberculin test (SIT) was performed by inoculating 100 µL of PPD-B (CZ Veterinaria, 25,000 IU/mL) at the right side of the neck using a Dermojet® syringe. At the left side of the neck, 100µL of a tetravalent fusion recombinant protein (TFP, Lionex) was intradermically inoculated to all animals (100 µg/mL). The TFP contained four MTBC-specific antigens (Rv3615c, Rv3020c, ESAT-6, and CFP-10), and was used as DIVA skin test reagent. Finally, 72 h after inoculation, skinfold thickness was measured and compared to BST. An animal was considered as positive if the difference between the BST and the 72 h post-inoculation

skinfold thickness was ≥4 mm or clinical signs were seen (i.e., swelling, oedema, exudation, or necrosis), as a doubtful if this difference was ≥2 mm and <4 mm, and negative if the difference was <2 mm.

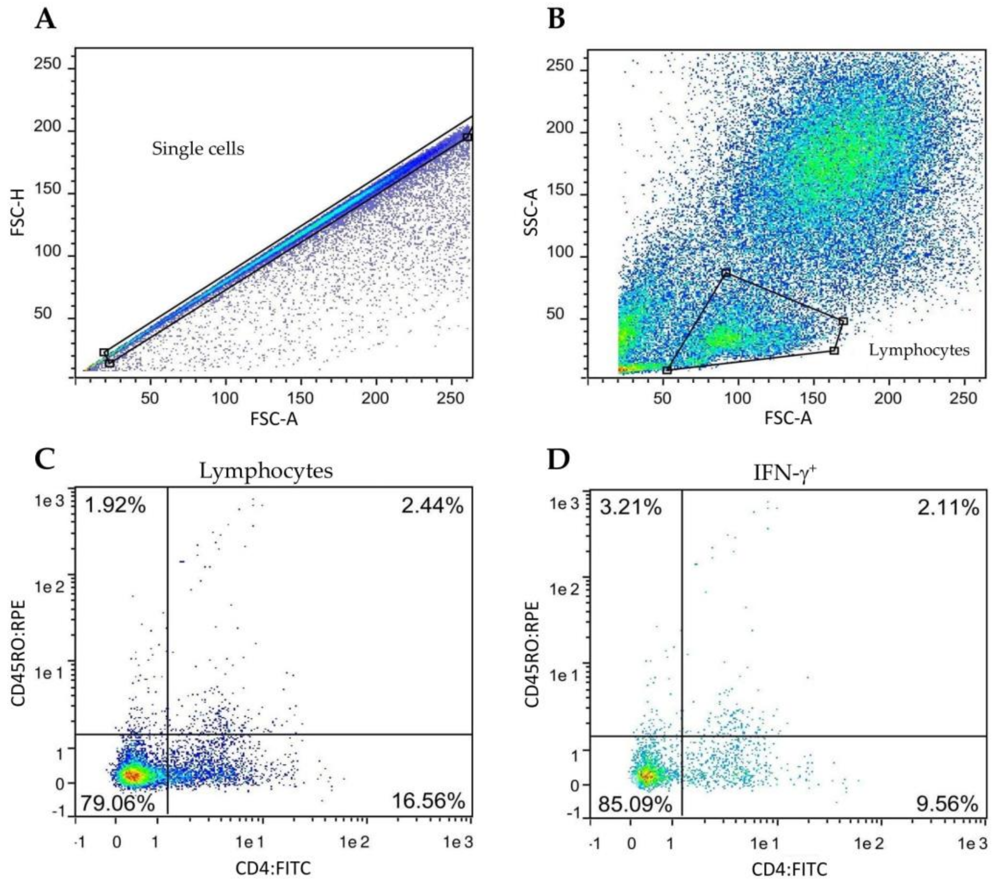


Figure 4.1. Gating strategy for the determination of frequency of the IFN-γ positive lymphocyte subsets. Peripheral blood mononuclear cells (PBMC) from all goats were cultured in the presence of *M. bovis* tuberculin (PPD-B). (**A**, **B**) Singlet lymphocytes were identified based on the degree of cellular differentiation determined by forward scatter (FSC) and side scatter (SCC). (**C**) Representative frequencies of the CD4/CD45RO cell populations. (**D**) Representative frequencies of intracellular IFN-γ staining of cells gated from prelabelled $CD4^{+}CD45RO^{+}$.

4.2.6. *Post-mortem* examination

In order to assess the efficacy of BCG revaccination against *M. caprae* challenge, a complete necropsy was carried out at week 72 (week 9 post-challenge). First, goats were euthanized by intravenous injection of an overdose of sodium pentobarbital. Then, pulmonary lymph nodes (LN), i.e., cranial and caudal mediastinal LN and tracheobronchial LN, were carefully removed, avoiding piercing of the pleura. Pulmonary LN were sliced at 3-mm intervals, and diameters of TB lesions were measured for further calculation of the approximate volume of gross lesions. The volume of lesions in the LN was calculated by using the formula of the most similar geometrical morphology of each lesion (sphere, cylinder, cone or prism). Afterwards, whole pulmonary LN were stored at −20 °C for further MTBC DNA burden assessment.

Whole lungs were carefully removed and filled with 10% buffered formalin (Pérez de Val et al., 2011), and analyzed 30 days later by computed tomography following procedures previously described (Balseiro et al., 2017). Briefly, whole lungs were scanned using a 16-slice multi-detector CT scanner (Brivo CT-385, General Electric Healthcare, Madrid, Spain). The volume of lungs and total volume of TB lesions were then calculated employing the volume rendering, using different density patterns (mineralized, solid and cavitary lesions), and multi-planar 2-D and 3-D reconstructions. The volume of mineralized lesions was calculated using 100–300 Hounsfield units.

Other viscera with gross TB-like lesions were collected and fixed in formalin for histopathological confirmation by Hematoxylin/Eosin staining.

4.2.7. *M. caprae* DNA burden assessment by PCR

Mediastinal and tracheobronchial LN were thawed and sliced with sterile scissors and subsequently homogenized in 10 mL of sterile distilled water using a homogenizer (Masticator®, IUL Instruments, Barcelona, Catalonia, Spain). Homogenates of LN for each goat were pooled, and an aliquot of 200µL was inactivated at 75 °C for 1 h. In parallel, an aliquot of *M. caprae* strain at 10^8 CFU/mL, the same used for the challenge inoculum preparation, was also inactivated and then serially diluted ten-fold in order to establish titered standards. Afterwards, DNA from homogenates and the standards was extracted using the DNA EXTRACT VK kit (Vacunek, Derio, Spain) and amplified using a commercial MTBC-specific qPCR kit (TBC-VK kit, Vacunek), both steps according to the manufacturer's procedures. Amplification was performed in a 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, U.S.A.). A standard curve was established using Ct values of standards (ten-fold dilutions of already tittered *M. caprae* inoculum strain), and *M. caprae* CFU genomic equivalents were calculated by the extrapolation of Ct value obtained for each DNA sample

Statistical analyses were carried out using StatsDirect v3.1.1. Kruskal–Wallis test was used for comparisons of ELISA S/P values and cytokine levels between groups and between sampling points. Significance level was set at $p \leq 0.05$.

4.2.8. Data analysis

A randomized block design was used for data analyses conducted after *M. caprae* challenge (blocking was used to remove the eventual box effects). Differences in the volume of TB lesions in the lungs among groups were assessed by two-way ANOVA followed by a one-tailed post-hoc Tukey test.

The volume of lesions in LN and volume of mineralized lesions in lungs were Box-Cox transformed to meet a normal distribution of data, and comparisons among groups were also analyzed by two-way ANOVA followed by a post-hoc Tukey test. Comparisons among treatment groups of bacterial loads inferred by qPCR (previously adjusted to consider the blocking factor by obtaining residuals from box effect with a linear regression model), IFN-γ levels obtained by IGRAs prior to challenge, and frequency of cell subsets obtained by flow cytometry were performed by non-parametric Kruskal–Wallis test followed by a post-hoc Wilcoxon rank-sum test. Mean rectal temperatures, mean body weight changes, and IFN-γ levels against PPD-B and EC after challenge were analyzed by a linear mixed-effect model (LMER), with the box factor as a random effect, in order to take into account missing data from two euthanized animals at week 72 of the experiment. Pairwise comparisons among groups were performed with the function "pairs" from the package "emmeans" from R. A *t*-test was used to compare IFN-γ levels of revaccinated animals 8 weeks after the first vaccination and 8 weeks after revaccination. In addition, Spearman and Pearson correlations were used to evaluating the association between different populations of memory or effector T-cells and post-mortem parameters (i.e., bacterial load measured by qPCR, the volume of lesions in LN, and total volume of lesions) and the association of post-mortem parameters with IFN-γ levels at weeks 68, 70 and 72, the body weight changes at weeks 3, 4 and 5 post-challenge, and rectal temperatures at weeks 3, 4 and 5 post-challenge.

The analysis was carried out using RStudio Team (2019). RStudio: Integrated Development for R. (RStudio, Inc., Boston, MA, U.S.A.).

4.3. RESULTS

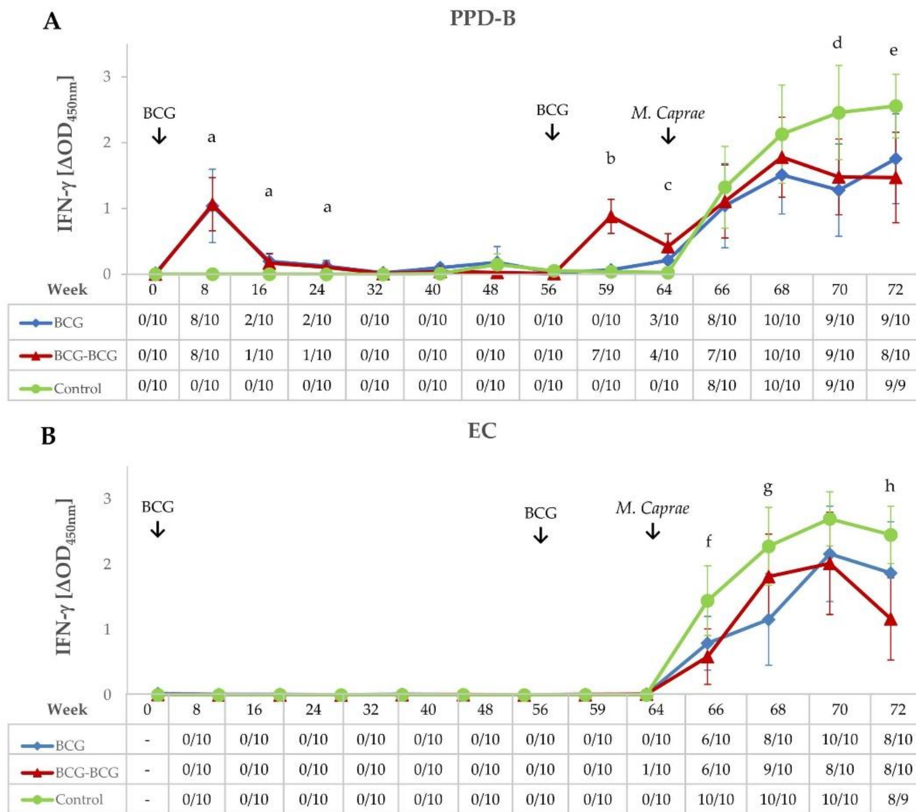
4.3.1. Immunological responses after vaccination and revaccination

IFN-γ release after ex vivo stimulation of whole blood with PPD-B and EC antigen cocktail was measured throughout the experiment (Figures 4.2A and 4.2B, respectively). Both vaccinated groups showed higher secretion of IFN-γ in response to PPD-B at eight weeks post-vaccination (wpv) compared to the control group ($P < 0.01$), and responses decreased appreciably at 16 wpv. At week 32, responses returned to pre-vaccination levels, with all animals remaining negative to IGRA until 56 wpv. Animals in the BCG-BCG group were inoculated at week 56 with BCG as indicated in Materials and Methods. Whole blood IFN-γ responses to PPD-B in the BCG-BCG group increased three weeks after revaccination (59 wpv); these IFN-γ responses were significantly higher compared to non-revaccinated animals (BCG and control groups) ($P < 0.001$). Eight weeks after revaccination (64 wpv), antigen-specific secretion of IFN-γ in the BCG-BCG group was significantly lower than that detected at eight weeks after primary vaccination (mean ΔOD: 0.422, 95% CI: 0.226–0.618; and mean ΔOD: 1.065, 95% CI: 0.660–1.470, respectively, $P < 0.01$, Figure 4.2A), whereas no animals positive for EC-IGRA were found after vaccination and/or revaccination (Figure 4.2B).

Analysis of T-cell subsets and potential secretion of IFN-γ was performed by flow cytometry at 59 wpv (three weeks after revaccination). Revaccination of goats resulted in an expansion of all IFN-γ^+ T-cell subsets after stimulation of PBMC with PPD-B (Figure 4.3A). Higher frequencies of $CD4^+CD45RO^+IFN\text{-}\gamma^+$ and $CD4^-CD45RO^+IFN\text{-}\gamma^+$ T-cell subsets (hypothetical IFN-γ-producing memory T-cells) were observed in the BCG-BCG group compared to BCG and control groups ($P < 0.05$), and the proportion of $CD4^+CD45RO^-$ IFN-γ^+ T-cells

was also higher compared to the BCG group (P < 0.05). Within revaccinated goats, whole blood PPD-B-specific IFN-γ release at 59 wpv correlated directly with the frequency of $CD4^+CD45RO^+IFN\text{-}\gamma^+$ populations (Spearman ρ = 0.68, P < 0.05), whereas no association between the frequency of the other $IFN\text{-}\gamma^+$ cell populations and the levels of IFN-γ released were found (Figure 4.3B).

Delayed-type hypersensitivity (DTH), after PPD-B and TFP intradermal inoculations, was also measured in all goats at three weeks post-revaccination (59 wpv). Individual skin thickness increase and qualitative results of SIT and TFP-skin test are shown in Figure 4.4. Five out of ten and four out of ten single-dose BCG-vaccinated animals were positive and doubtful to SIT, respectively, while 2/10 animals were doubtful to the TFP-skin test. All revaccinated animals were positive to SIT, and 4/10 were positive and 2/10 were doubtful to TFP-skin test. One animal from the control group was positive to both skin tests, and three other animals were doubtful for SIT or TFP-skin tests.



Week	0	8	16	24	32	40	48	56	59	64	66	68	70	72
BCG	0/10	8/10	2/10	2/10	0/10	0/10	0/10	0/10	0/10	3/10	8/10	10/10	9/10	9/10
BCG-BCG	0/10	8/10	1/10	1/10	0/10	0/10	0/10	0/10	7/10	4/10	7/10	10/10	9/10	8/10
Control	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	8/10	10/10	9/10	9/9

Week	0	8	16	24	32	40	48	56	59	64	66	68	70	72
BCG	-	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	6/10	8/10	10/10	8/10
BCG-BCG	-	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	1/10	6/10	9/10	8/10	8/10
Control	-	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	10/10	10/10	10/10	8/9

Figure 4.2. IFN-γ responses after bacillus Calmette–Guerin (BCG) vaccination, revaccination, and *M. caprae* challenge. (**A**, **B**) Mean IFN-γ levels released after stimulation whole blood with *M. bovis* tuberculin (PPD-B) and ESAT-6/CFP-10 (EC) DIVA reagent, respectively. Tables on horizontal axes show IFN-γ release assay (IGRA) qualitative results for each time point. BCG (blue): single BCG-vaccinated group, BCG-BCG (red): BCG revaccinated group, Control (green): unvaccinated group. Kruskal–Wallis test with post-hoc Wilcoxon test: (a) BCG and BCG-BCG compared to Control, $P < 0.01$; (b) BCG-BCG compared to BCG and Control, $P < 0.001$; (c) BCG and BCG-BCG compared to Control, $P < 0.001$; LMER with pairwise comparisons; (d) BCG and BCG-BCG compared to Control, $P < 0.05$; (e) BCG and BCG-BCG compared to Control, $P < 0.05$ and $P < 0.01$, respectively; (f) BCG-BCG compared to Control, $P < 0.05$; (g) BCG compared to Control, $P < 0.05$, (h) BCG-BCG compared to Control, $P < 0.01$.

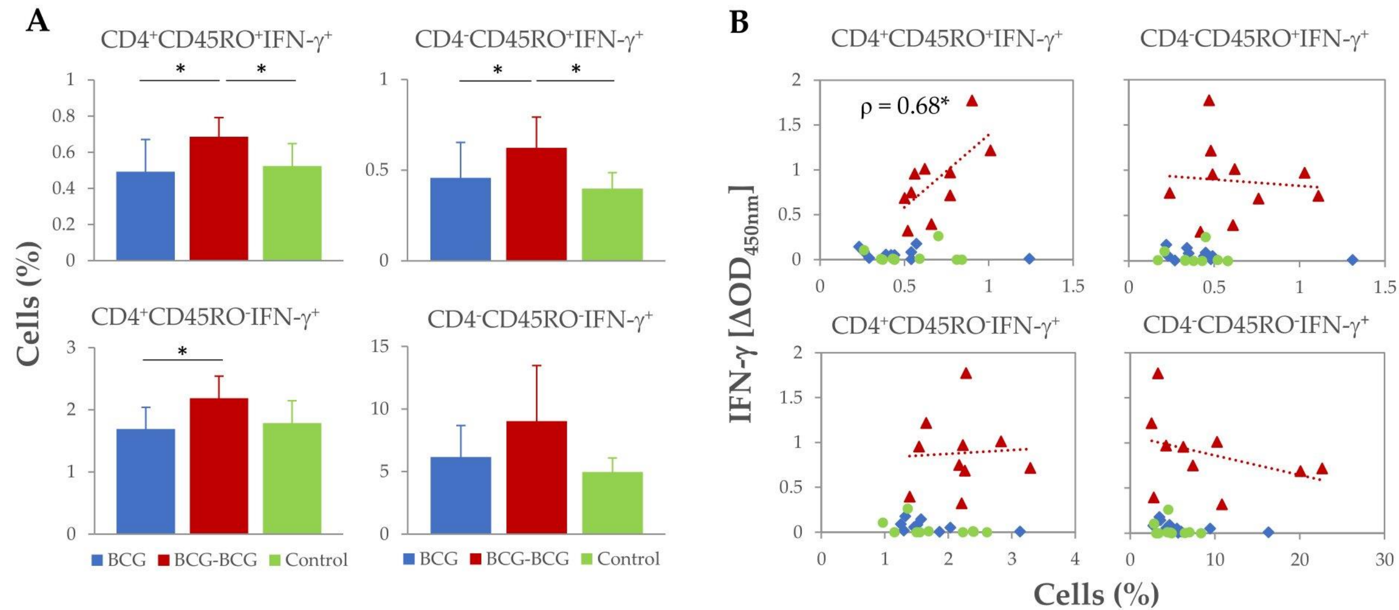
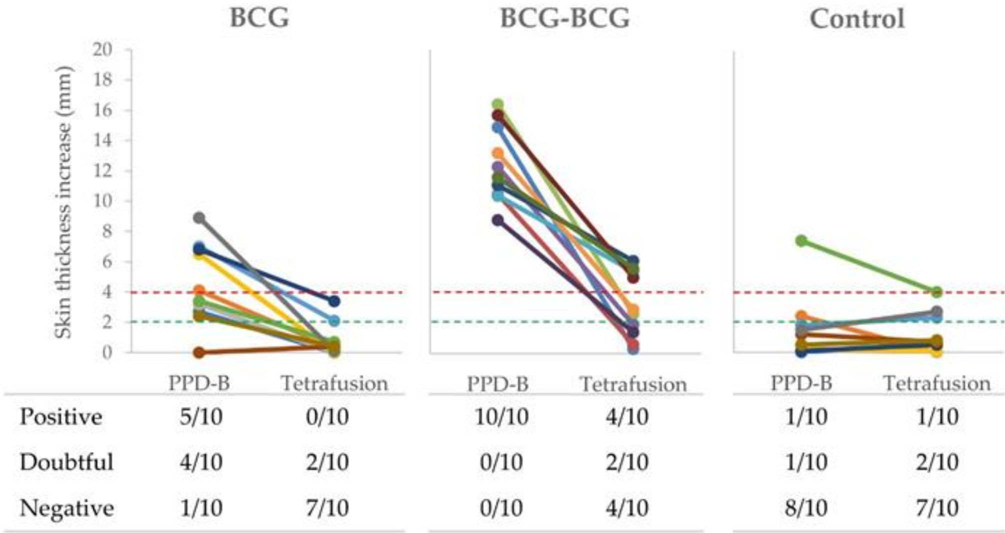


Figure 4.3. Proportion of *M. bovis* tuberculin (PPD-B)-specific IFN-γ$^+$ T-cell subsets isolated from peripheral blood at week 59 (3 weeks after revaccination). (**A**) Frequency (%) of IFN-γ$^+$ lymphocyte subsets. * $P < 0.05$, Kruskal–Wallis test with post-hoc Wilcoxon test. (**B**) Correlation of IFN-γ+ lymphocyte subsets frequencies with IFN-γ released after whole blood stimulation with PPD-B at week 59. * $P < 0.05$ (Spearman $\rho = 0.680$).



	BCG PPD-B	BCG Tetrafusion	BCG-BCG PPD-B	BCG-BCG Tetrafusion	Control PPD-B	Control Tetrafusion
Positive	5/10	0/10	10/10	4/10	1/10	1/10
Doubtful	4/10	2/10	0/10	2/10	1/10	2/10
Negative	1/10	7/10	0/10	4/10	8/10	7/10

Figure 4.4. Skin tests. Individual skinfold thickness using *M. bovis* tuberculin (PPD-B) and a Tetrafusion protein (ESAT-6/CFP-10/Rv3615c/Rv3020). BCG (single BCG-vaccinated group), skin tested one year after vaccination; BCG-BCG (revaccinated group after one year of first vaccination), skin tested three weeks after revaccination; Control: unvaccinated group. Red dashed line: threshold for positive results in the skin test. Green dashed line: threshold for doubtful results in the skin test. In the table, qualitative results of skin tests are presented per group.

4.3.2. IFN-γ responses after *M. caprae* challenge

Goats were challenged at 64 wpv and remained infected for nine weeks (from 64 to 72 wpv). After the challenge, in vitro whole blood PPD-B-specific IFN-γ responses increased in all groups and reached a plateau in both vaccinated groups from four weeks post-challenge (68 wpv) onwards, while responses remained high in the control group until the end of the experiment, being statistically higher when compared to BCG and BCG-BCG groups ($P < 0.05$) at seven and nine weeks post-challenge (wpc), respectively (Figure 4.2A). Similar kinetics were observed for EC-specific IFN-γ responses, however a remarkable decrease in the BCG group and even more in the BCG-BCG group were found at 9 wpc. The increase in EC-specific IFN-γ levels (Figure 4.2B) in the control

group was statistically significant ($P < 0.05$) compared to the BCG group at 5 wpc and the BCG-BCG group at 3 and 9 wpc ($P < 0.05$ and $P < 0.01$, respectively).

4.3.3. Clinical signs and body condition *post*-challenge

Goats were monitored for clinical signs of TB after the challenge. Twenty-six, out of 30 animals, showed a sporadic or persistent cough from 4 wpc until the end of the experiment. By the end of the experiment (72 wpv, 9 wpc), severe clinical signs (i.e., dyspnoea, lethargy or anorexia) were observed in 5/10, 5/10, and 5/9 goats in the BCG, BCG-BCG, and control groups, respectively. Dyspnoea and apathy were first observed at 5 wpc in one animal of the control group, which was euthanized one week before the end of the experiment due to ethical endpoint criteria.

Revaccinated animals showed a significantly lower mean rectal temperature ($P < 0.05$) compared to control and BCG groups at 3 and 4 wpc, respectively (Figure 4.5A). Additionally, the BCG-BCG group showed significant mean body weight gains at 3, 5 and 7 wpc compared to the control group ($P < 0.05$). Mean body weight changes were also higher in the BCG group than in the control group, but were not statistically significant (Figure 4.5B).

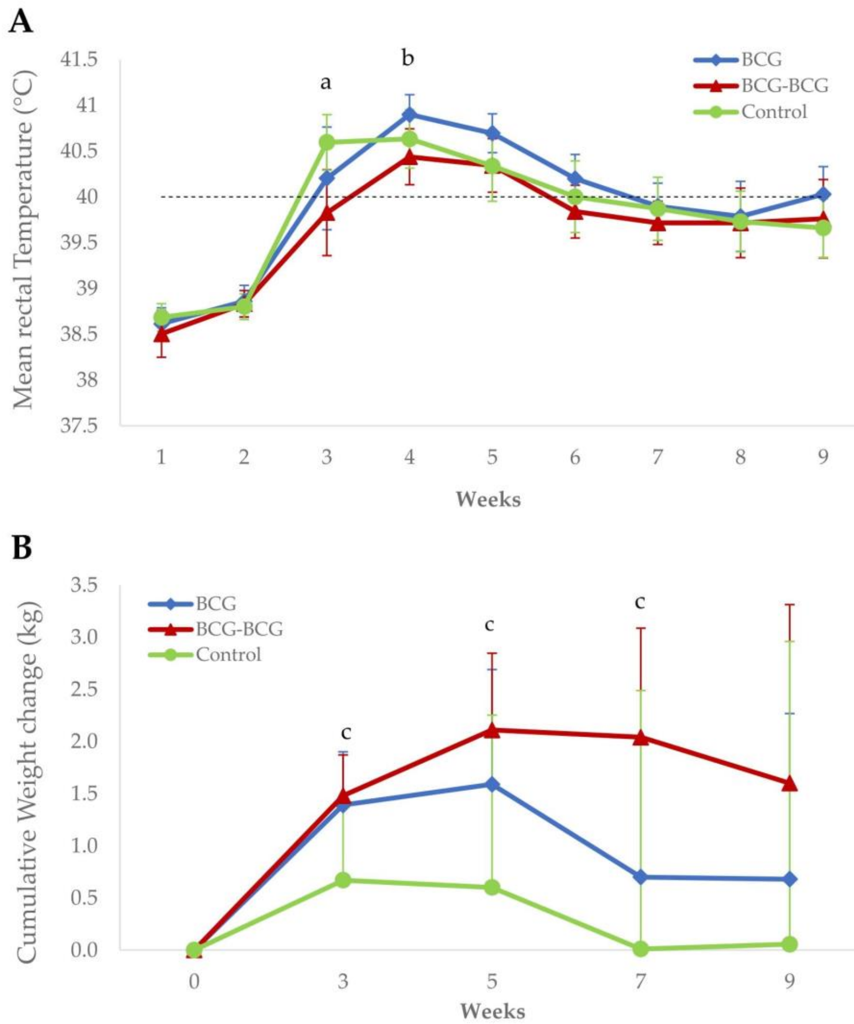


Figure 4.5. Clinical signs after *M. caprae* challenge. (**A**) Mean rectal temperatures per group. BCG (blue): single BCG-vaccinated group, BCG-BCG (red): BCG revaccinated group, Control (green): Unvaccinated group. Dashed line: fever threshold (40 °C). (a) BCG-BCG compared to Control, $P < 0.05$; (b) BCG-BCG compared to BCG, $P < 0.05$. (**B**) Mean cumulative weight changes per group. (c) BCG-BCG compared to Control, $P < 0.05$; LMER with pairwise comparisons.

4.3.4. Post-mortem findings

Animals in the revaccinated group showed a lower mean volume of TB lesions in pulmonary LN, compared to the control ($P < 0.05$, Figure 4.6A). Bacterial burden, as measured by log CFU genome equivalents, was also lower in the BCG-BCG group compared to unvaccinated controls ($P < 0.05$, after adjustment by box effect, see supplementary data, Figure 4.6B). However, the mean volume of TB lesions in the lungs was slightly lower in BCG (698 cm^3, 95% CI: 573–823) and BCG-BCG (698 cm^3, CI: 453–943) compared to the control group (728 cm^3, CI: 490–966), but this difference was not statistically significant (Figure 4.6C). Vaccinated groups also showed a lower mean volume of mineralization in TB lung lesions (BCG: 41 cm^3, 95%CI: 16–65; BCG-BCG: 41 cm^3, 95% CI: 19–64) compared to the control group (76 cm^3, 95%CI: 34–118, $P < 0.1$, Figure 4.6D). Remarkably, the total volume of lung lesions was not correlated with the volume of mineralization in the lungs ($r = -0.019$).

The volume of lesions and bacterial DNA load in LN were directly correlated with increased rectal temperature from 3 to 5 wpc (range in which the fever peak was detected) and inversely correlated with body weight increase after challenge (Table 4.1). Neither post-mortem nor clinical parameters were individually associated with IFN-γ-producing cell frequencies measured before challenge (see Supplementary Materials).

Extra-pulmonary lesions were identified in spleen, liver, kidney, and mesenteric lymph nodes in seven BCG and BCG-BCG goats, and in 9 unvaccinated controls (Table 4.2)

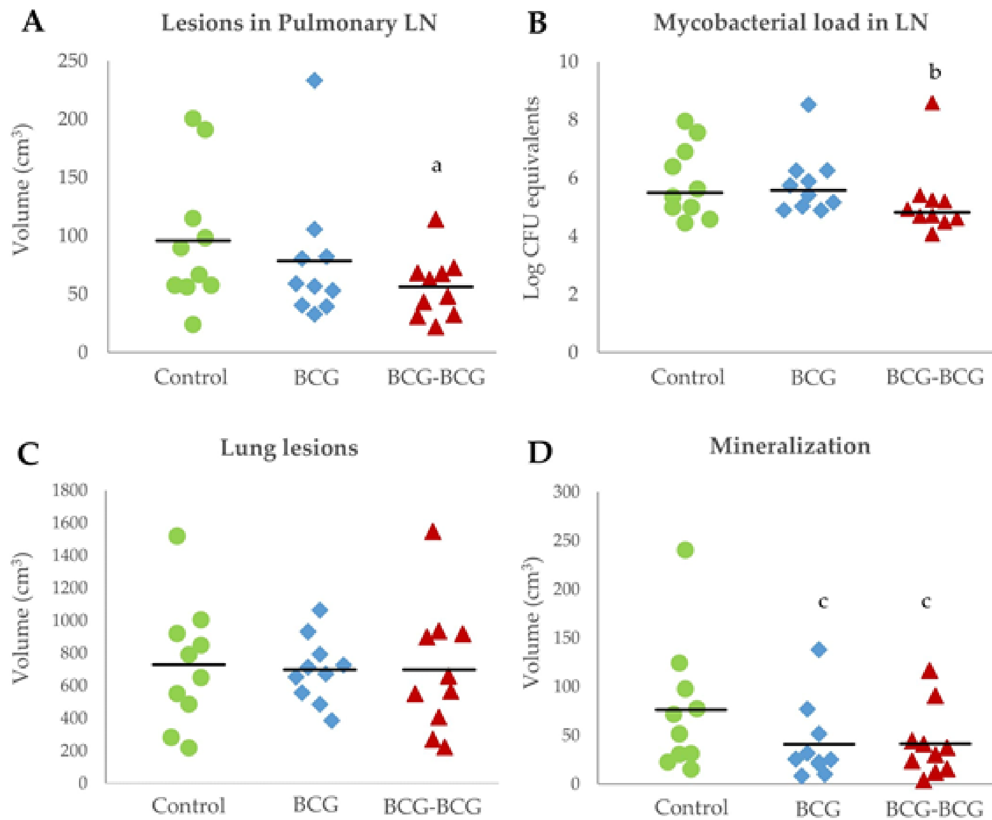


Figure 4.6. Postmortem analyses. (**A**) Volume of tuberculous lesions in pulmonary lymph nodes (LN). BCG (blue): single BCG-vaccinated group, BCG-BCG (red): BCG revaccinated group, Control (green): unvaccinated group. (a) BCG-BCG compared to Control, $P < 0.05$ (two-way ANOVA with post-hoc Tukey test). (**B**) Mycobacterial load in LN measured by qPCR. (b) BCG-BCG compared to Control, $P < 0.05$, Kruskal–Wallis test with Wilcoxon post-hoc test. (**C**) Volume of TB lesions in lungs. (**D**) Volume of mineralization in lung lesions. (c) BCG and BCG-BCG compared to Control, $P < 0.1$, two-way ANOVA with post-hoc Tukey test.

Table 4.1. Individual association between clinical and post-mortem parameters.

Post-Mortem Parameter	Body Weight Change				Rectal Temperature		
	W 3	W 5	W 7	W 9	W 3	W 4	W 5
M. caprae CFU equivalents	−0.061	−0.221	−0.234	−0.3035	0.234	0.27	0.336
Vol Lesions in LN	−0.414 *	−0.511 **	−0.576 ***	−0.542 **	0.444 *	0.428 *	0.435 *
Vol Lung Lesions	−0.319	−0.482 **	−0.587 ***	−0.692 ***	0.277	0.456 *	0.548 **

Values are Pearson correlation coefficients (r). W: week. CFU: Colony forming units, LN: Pulmonary lymph nodes * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 4.2. Extrapulmonary lesions.

Animals with Extrapulmonary Lesions		Localization of Lesions				
		Spleen	Liver	Ln Ms [1]	Kidney	Ln RF [2]
BCG	7/10	5	2	4	2	0
BCG-BCG	7/10	6	2	4	1	4
Control	9/10	8	4	8	4	1

[1] Mesenteric lymph node. [2] Retropharyngeal lymph nodes.

4.4. DISCUSSION

This study was focused on the effects of BCG revaccination and the duration of immunity afforded by a single-dose vaccination in goats. The results showed that T-cell effector and memory immunity induced by BCG was undetectable one year later, but revaccination with a second dose of BCG one year after primary vaccination, induced cell-mediated responses again at a similar magnitude to that detected at eight weeks after the first immunization and elicited detectable T-cell memory immunity. Moreover, protection after *M. caprae* challenge in re-vaccinated animals was greater than in animals that were not re-vaccinated, as determined by pulmonary lymph node pathology and bacterial DNA load, degree of lung mineralization, fever, and body weight measurements. This is similar to what has been observed in calves vaccinated with BCG at two to four weeks of age, and revaccinated at two years of age, and then challenged with *M. bovis* six months after the second vaccination, whereas single-dose vaccinated animals were not protected (Parlane et al., 2014). Besides, a field trial in BCG-vaccinated goats has shown an increase in TB incidence one year after vaccination (Arrieta-Villegas et al., 2020a), suggesting a loss of protective immunity of BCG after this period of time and the need for a revaccination strategy.

The reduction in bacterial load in pulmonary LN without a reduction in the volume of lung lesions in vaccinated animals is consistent with the findings of a previous study in BCG-vaccinated and *M. caprae*-challenged goats eight weeks later (Arrieta-Villegas et al., 2018). However, in the present study, the *M. caprae* load in pulmonary LN was approached by measuring DNA equivalents instead of bacterial culture to avoid the negative impact on bacterial counts of viability losses after the congelation and thawing of samples (Vidal et al., 2017). In addition, quantification by qPCR avoids miscounting due to mycobacterial

aggregation and allows the inclusion of known dilutions of the same *M. caprae* strain used for the challenge as a DNA standard, ensuring an accurate equivalent for bacterial counts (Pathak et al., 2012).

Interestingly, vaccinated goats showed a greater reduction in lung mineralized lesions, and this parameter was not directly correlated with the total volume of lung lesions, as observed in a previous study conducted in BCG-vaccinated and *M. caprae*-challenged goats (Arrieta-Villegas et al., 2018). Hence, despite an extensive lung inflammatory response, BCG vaccination could contain the progress of granulomatous lesions to advanced developmental stages, characterized by necrosis and mineralization (Wangoo et al., 2005). Indeed, the lower proportion of necrotized and mineralized granulomas were previously found in BCG-vaccinated and revaccinated cattle (Parlane et al., 2014).

However, in the present study, 70% of animals in the BCG and BCG-BCG groups showed extra-pulmonary lesions, most of them in the spleen and/or liver, indicating hematogenous dissemination of the infection, whereas in previous experiments, lesions in BCG-vaccinated goats were mainly restricted to respiratory tissues (Arrieta-Villegas et al., 2018; Roy et al., 2019; Vidal et al., 2017). In the experiments described above, animals were young and were exposed to *M. caprae* at 0 to 2 months after vaccination; in contrast, in the present study, animals reached adulthood by the time of challenge (approx. 70 weeks old). Thus, the differences in the capacity of BCG to contain the extrapulmonary dissemination of the infection might be explained by sensitization of vaccinated animals with other mycobacteria when the immunity of the primary vaccination waned. Indeed, BCG protection was reduced in cattle naturally pre-sensitized to environmental mycobacteria (EM) (Buddle et al., 2002). In this regard, it has been suggested that pre-exposure to EM either masks or blocks the effect of BCG vaccination (Andersen and Doherty, 2005).

The fact that four unvaccinated control goats reacted positively to the skin test performed before the challenge, could indicate an exposure of experimental goats to EM during the year that they remained in the experimental farm.

Clinical markers could also be used as indicators of vaccination outcomes. The results in the present study are in concordance with significant reductions in rectal temperatures and body weight increases observed in BCG-vaccinated goats after challenge with *M. caprae* compared to non-vaccinated goats and sheep (Arrieta-Villegas et al., 2018; Balseiro et al., 2017).

Understanding of the underlying mechanisms which confer protection is an essential pre-requisite for the development of markers of protection and evaluations of vaccine efficacy. As previously observed in cattle (Parlane et al., 2014), BCG revaccination induced similar levels of PPD-B-specific IFN-γ secreted in peripheral blood compared to those induced by primary vaccination, rather than inducing a classical secondary immune response. Moreover, the significant reduction in IFN-γ after eight weeks of revaccination compared to the IFN-γ levels after eight weeks from the first immunization could be due to the contact with animals with EM. Indeed, in mice orally pre-sensitized with different strains of *Mycobacterium avium*, the IFN-γ responses after BCG vaccination were significantly reduced (Young et al., 2007). On the other hand, a reduction in IFN-γ responses after revaccination could be expected because the goats were adults by the time of revaccination. In fact, it has been demonstrated that specific IFN-γ responses against BCG rapidly decrease with age (Whittaker et al., 2018). However, the strong antigen-specific IFN-γ responses induced by revaccination were not associated with an appropriated protective response, as observed in cattle revaccinated with BCG six weeks after neonatal vaccination (Buddle et al., 2003b).

It has been previously reported that the presence of different subsets of memory T-cells elicited by vaccination provided long-term protection in mice and humans (Andersen and Smedegaard, 2000; Sallusto et al., 1999). In cattle, the presence of $CD4^{+}CD45RO^{+}IFN\text{-}\gamma^{+}$ and $CD8^{+}CD45RO^{+}IFN\text{-}\gamma^{+}$ T-cells has been associated with a reduction in the mycobacterial load (Maggioli et al., 2015). Similarly, in the present study, PPD-B-specific IFN-γ-producing memory T-cells, $CD4^{+}CD45RO^{+}IFN\text{-}\gamma^{+}$ and $CD4^{-}CD45RO^{+}IFN\text{-}\gamma^{+}$, were highly proliferative in revaccinated goats.

Remarkably, ex vivo antigen-specific production of IFN-γ determined by IGRA was only significantly correlated with the proportion of $CD4^{+}CD45RO^{+}$ IFN-γ-producing T-cells in revaccinated goats. Similar findings were observed in BCG-vaccinated and *M. bovis*-infected cattle (Blunt et al., 2015). The recirculation of antigen-specific IFN-γ-producing memory T-cells has been associated with the containment of mycobacterial replication and/or sterilization of the lesion in the site of the infection (Kipnis et al., 2005).

The durability of the antigen-specific memory T-cell response is also required for the development of effective prophylactic strategies against TB. In this study, BCG-induced immunity waned at 59 wpv because no expansion of antigen-specific IFN-γ-producing memory T-cells were observed in goats that received a single dose of BCG. Besides, IFN-γ secreted ex vivo was undetectable from 32 wpv onwards. The persistence of BCG is necessary to induce protective immune responses (Weir et al., 2008). In this sense, a previous study in BCG-vaccinated goats demonstrated the complete removal of viable BCG bacilli in the injection site before 24 wpv. It was associated with a decrease in antigen-specific IFN-γ responses (Pérez de Val et al., 2016). Long-term studies conducted in cattle showed variable whole blood PPD-B-specific IFN-γ detectable responses ranging from less than 50 to over 90 weeks after

BCG vaccination (Parlane et al., 2014; Thom et al., 2012). Nonetheless, in the latter case, the authors observed a significant decrease in PPD-B-specific IFN-γ-secreting central memory T-cells at 24 months post vaccination (p.v.) compared to 12 months p.v. These results were associated with a decrease in protection against *M. bovis* challenge (Thom et al., 2012).

To the best of our knowledge, the present study is the first evaluating the duration of the immunity of BCG vaccination in goats. In the light of the results obtained, it is possible that immune protection of BCG in goats waned before one year, as it occurs for other diseases, i.e., clostridial diseases, in which protective immunity decays before then in sheep or cattle, and thus, two or three vaccinations per year are recommended (Tizard, 2020; Uzal et al., 1998). Therefore, revaccination after six months of the first BCG immunization or other schedules needs to be further evaluated in goats. However, it is important to take in account that BCG is not a fully protective vaccine and, as observed with the *Mycobacterium avium* sbsp *paratuberculosis* vaccine, vaccination might be considered as part of the measures for control of the disease in farms (Lacasta et al., 2015).

Although single-dose BCG-vaccinated goats showed negative results to IGRA from 32 wpv to the challenge time point, DTH was detected in 50% or 90% of single-dose vaccinated goats at 59 wpv by SIT using the conservative or stringent criteria, respectively. Therefore, BCG did not cause interferences on TB diagnosis by DIVA-based IGRA, as previously demonstrated in goats (Arrieta-Villegas et al., 2018; Pérez de Val et al., 2016), neither in tuberculin-based IGRA seven months after vaccination but compromised the SIT for a minimum of one year. In addition, 20% and 60% of BCG and BCG-BCG-vaccinated goats reacted positively to TFP-ST using the stringent criteria, and cross-reactive responses were also detected in unvaccinated controls. A cocktail

including peptides of the four antigens included in TFP was proven highly specific in BCG-vaccinated goat kids (Bezos et al., 2015b), but showed divergent sensitivity results in *M. bovis*-infected cattle (Jones et al., 2012; van der Heijden et al., 2017). The lack of specificity found herein does not support the suitability of TFP as a skin test DIVA reagent. In fact, either Rv3615c or Rv3020c are not absent in BCG genome, but the first one cannot be expressed due to the deletion of Esx1 system in BCG (Millington et al., 2011). Probably, the presence of Rv3020c in TFP is the causative factor of diagnostic interferences in vaccinated goats. Thus, this antigen could be removed from diagnostic reagents. Additionally, a newly defined skin test containing ESAT-6, CFP-10 and Rv3615c peptide cocktail improved the skin test for the diagnosis of TB in cattle and showed DIVA capability (Srinivasan et al., 2019).

Finally, during the present study, the main limitation encountered was that the animals, despite being housed in a controlled TB free experimental farm, maintained in the same pen without grazing, and with regular health checking, contact with environmental mycobacteria could not be avoided. Moreover, we cannot rule out the effects of underexposure to sunlight (i.e., deficiency in vitamin D) or other key factors that may have had an effect in mounting an appropriate immune response. Thus, even if the dose for *M. caprae* challenge was similar to that used for previous challenges, the model of adult goats that developed a large volume of lesions in the lungs and vaccinated goats could not properly contain the infection as observed in extrapulmonary lesions. Therefore, future experimental studies in adult goats might better control the aspects mentioned above or use lower doses for the challenge.

In conclusion, BCG vaccination of goat kids did not provide significant levels of protection against *M. caprae* challenge 64 weeks later, and lifespan for immunity elicited by vaccination was lower than 59 weeks. Moreover, BCG-

vaccinated goats showed negative results to the tuberculin-based whole blood IGRA from 32 weeks after vaccination. In contrast, BCG revaccination 56 weeks after primary vaccination was shown to induce an increase in the proportion of antigen-specific IFN-γ-producing memory T-cell phenotypes. BCG re-vaccination was also shown to afford a higher degree of protection against TB in terms of reduced pathology, bacterial DNA load and clinical signs after *M. caprae* infection. Further studies will establish the most suitable moment for BCG revaccination and will determine the durability of revaccination-induced immunity.

CHAPTER 5

Study III

Efficacy of parenteral vaccination against tuberculosis with Heat-Inactivated *Mycobacterium bovis* (HIMB) in experimentally challenged goats

(HIMB VAC)

5.1. INTRODUCTION

Goats are natural hosts of both *M. caprae* and *M. bovis,* becoming a potential a source of TB infection for other epidemiologically related species such as cattle (Napp et al., 2013) or sheep (Vidal et al., 2018). Therefore, the lack of an official TB control program in goats could jeopardize the eradication efforts made in cattle (Vordermeier et al., 2014).

In this scenario, vaccination of goats may be a useful long-term tool to reduce TB prevalence in goat herds. *M. bovis* Bacillus Calmette-Guérin (BCG) is the only vaccine licensed for humans and badgers (Chambers et al., 2014) and previous studies in different animal experimental models had shown heterogeneous results (Cardona and Williams, 2017). In experimentally challenged goats, BCG afforded protection by reduction of pulmonary disease severity and preventing extra-pulmonary dissemination (Pérez de Val et al., 2013, 2012b). Moreover, as a live-attenuated vaccine, BCG stability in environmental conditions could be limited and an eventual transmission to non-vaccinated animals cannot be excluded (Palmer et al., 2009).

The Heat-Inactivated *Mycobacterium bovis* (HIMB), is a new vaccine candidate that may rule out some constraints of live-attenuated vaccines (Beltrán-Beck et al., 2014). The efficacy of HIMB has already been evaluated under experimental conditions in cattle (van der Heijden et al., 2017), sheep (Balseiro et al., 2017), red deer (Thomas et al., 2017) and wild boar (Garrido et al., 2011) and, under field conditions in wild boar (Díez-Delgado et al., 2017), yielding variable results.

The aim of this study was to evaluate the efficacy of parenteral HIMB vaccination (injected i.m. and s.c) in comparison with parenteral BCG

vaccination in *M. caprae* experimentally challenged goats, by studying cell-mediated and humoral immune responses after vaccination and challenge, and TB lesion volume reduction. Additionally, the effects of vaccination on IFN-γ release assay (IGRA) based TB diagnostic were evaluated.

5.2. MATERIAL AND METHODS

5.2.1. Animals and experimental design

Twenty-four *Murciano-granadina* goat kids, aged from 4 to 7 weeks and acquired from a farm with no history of TB, were selected based on negative results to IGRA (ID Screen® Ruminant IFN-γ, ID.vet, Grabels, France) and all animals were accommodated in a pen of an experimental farm. Animals were randomly distributed in 3 experimental groups of 4 males and 4 females each. Then, distribution was corrected by weight, in order to have homogenous characteristics in each group. One group of 8 animals was subcutaneously vaccinated with a live attenuated *M. bovis* BCG vaccine (BCG group), another group of 8 animals (HIMB group) was parenterally vaccinated with the heat-inactivated *M. bovis* vaccine (4 were vaccinated subcutaneously and 4 intramuscularly with the purpose to investigate adverse reactions) and, finally, the last 8 animals remained unvaccinated (Control group). At seven weeks post vaccination (p.v.) goat kids were placed in biosafety level (BSL) 3 containment facilities at IRTA-CReSA, in two boxes, one for unvaccinated animals and another for both BCG and HIMB vaccinated animals. Finally, one week later (8 weeks p.v.), the experimental challenge with *M. caprae* was performed.

Goat kids were vaccinated at week 0. Blood samples were taken from jugular vein in heparinized blood tubes at weeks 0, 3, 5, 8, 11, 13, 15 and 17 of the

experiment. Clinical signs of TB were monitored after the experimental infection and animals were weighed at weeks 0, 8, 11, 13, 15 and 17. Finally, rectal temperature was measured weekly p.c.

5.2.2. Ethics statement

All animal procedures used during this experiment were approved by the Animal Welfare Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya (Procedure Number 8697), and in conformity with European Union Laws for protection of experimental animals (2010/63/EU).

5.2.3. *M. bovis* BCG vaccine

M. bovis BCG Danish 1331 strain (ATCC, Ref.35733 ™) vaccine stock was prepared as described previously (Pérez de Val et al., 2013). Then, BCG was diluted in sterile Phosphate Buffered Saline (PBS) to reach a suspension of 10^6 colony forming units (CFU)/ml, and 0.5 ml of the suspension (5×10^5 CFU) was subcutaneously inoculated at the right axilla.

5.2.4. Heat-inactivated *M. bovis* (HIMB) vaccine

The *M. bovis* strain (SB0339) used was first isolated from a naturally infected wild boar on Coletsos medium. The vaccine was prepared as described by Balseiro *et al*. (Balseiro et al., 2017). The inactivated *M. bovis* suspension was adjuvated with Montanide™ ISA 50V2 (Seppic, Paris, France) to form a water in oil emulsion in a proportion 1:1 and contained approximately 10^7 CFU of heat-treated bacteria per dose (1 ml). Animals were injected subcutaneously, at the right axilla, or intramuscularly, at the right semitendinosus muscle.

5.2.5. *M. caprae* inoculum preparation and experimental challenge

The field strain *M. caprae* SB0416 (www.Mbovis.org) used for the inoculum was subcultured in Middlebrook 7H9 medium and titrated in 7H11 plates (BD diagnostics, Sparks, USA) as described previously (Pérez de Val et al., 2013). For challenge, an aliquot was used for preparing the inoculum by diluting it with sterile PBS to attain a final suspension of 2×10^4 CFU/ml of *M. caprae*.

Goat kids were premedicated by an intramuscular injection with a cocktail of 0.05 mg/kg of acepromazine maleate (Equipromacina®) and 0.2 mg/kg of butorphanol tartrate (Torbugesic®). After sedation, they were intravenously anesthetized with propofol (Propofol Lipuro®) at 4 - 6 mg/kg and midazolam (Dormicum®) at 0.2mg/kg. Anesthetized animals were endobronchially challenged with a 0.5 ml of *M. caprae* inoculum (each animal received 10^4 CFU) as previously described (Pérez de Val et al., 2011).

5.2.6. Whole-blood IFN-γ assay (IGRA)

Blood samples were collected at weeks 0, 3, 5, 8, 11, 13, 15 and 17, and were processed as described by Pérez de Val et al., (Pérez de Val et al., 2016). Shortly, 3 aliquots of 900 µl of whole blood were added into 3 wells of 96-well cell culture plates (Eppendorf Ibérica, Madrid, Spain), two wells were subsequently stimulated with M. bovis (PPD-B) and M. avium (PPD-A) tuberculins (CZ Veterinaria, Porriño, Galicia, Spain), both at a final concentration of 20 µg/ml and PBS (Sigma-Aldrich, Steinheim, Germany) was added in the other well as the unstimulated control. In addition, 225 µl of whole blood were stimulated with a mixture of ESAT-6 and CFP-10 (EC) recombinant proteins (Lionex, Braunschweig, Germany), used at a final concentration of 10 µg/ml each. Samples were incubated at 37 ºC with CO2 overnight. Finally,

plasma was collected and analyzed by ruminant IFN-γ ELISA (ID.vet) following the manufacturer instructions. ELISA was read at 450 nm using a spectrophotometer (Biotek Power Wave XS). The interpretation of tuberculin-based IGRA results was performed according the two cut-off points of sample-to-positive ratios (S/P) recommended by the manufacturer, i.e. ((Optical density (OD) of PPD-B – OD of PPD-A) / (OD mean kit positive control (CP) – OD mean kit negative control (CN))) × 100. A sample was considered as positive if S/P ≥ 35% (conservative criterion) or ≥ 16% (stringent criterion). In addition, EC-specific IGRA results were calculated as following: S/P = ((OD of EC – OD of PBS) / (OD CP – OD CN)) × 100. A sample was considered as positive if S/P ≥ 16%.

5.2.7. Serology

Plasma samples were analyzed in duplicate for antibodies against the cell-surface lipoprotein MPB83 (Lionex), specific for *M. tuberculosis* Complex (MTBC), using a homemade ELISA as described previously [19]. A sample was classified as positive when the ΔOD 450 nm (sample wells average $OD_{450\ nm}$ minus the blank well OD_{450nm}) was equal or higher than 0.2 (the optimal cut-off point previously determined by Pérez de Val et al. (Pérez de Val et al., 2017).

5.2.8. Post mortem examination

Goats were euthanized at week 17 (week 9 p.c.) by intravenous injection of a sodium pentobarbital overdose. A complete necropsy procedure was conducted, pulmonary (tracheobronchial, mediastinal cranial and caudal) lymph nodes (LN) were carefully removed and sliced, and then the diameters of each lesion were measured. The approximate volume of gross lesions was calculated using the

formula of the most similar geometrical morphology of each lesion (sphere, cylinder or prism). After slicing, whole pulmonary LN were frozen and stored for later bacterial culture. All remaining viscera were also examined and other extra-pulmonary tissues with presence of TB-like lesions were collected and subsequently fixed in 10% buffered formalin for histopathological confirmation by Hematoxylin/Eosin staining. Finally, the whole lungs were filled with formalin as previously described (Pérez de Val et al., 2011) and one month later, 20 lungs (6 from the control group, and 7 from each vaccinated group) were analyzed by computed tomography (CT).

5.2.9. Computed tomography

After fixation, the extension of the pathology in lungs was assessed by 16-slice multi-detector CT scanner (Brivo CT-385, GE Healthcare, UK) as previously described (Balseiro et al., 2017). Briefly, volume rendering (VR) was employed to calculate the whole volume of each lung. Different density patterns (calcified lesions, cavitary lesions and solid lesions) were used to settle down tuberculous lesions in lungs, and to determine its volume by 2D, 3D images and VR, using multiplanar reconstructions. Calcified lesions were selected by their Hounsfield units (range 80-300 HU) and the total volume of them was calculated.

5.2.10. Bacterial culture and count

Whole pulmonary LN of each animal were thawed, pooled, homogenized and decontaminated as previously described (Pérez de Val et al., 2011). Four ten-fold serial dilutions of tissue homogenates in sterile PBS were performed and 100µl of each dilution were plated on Middlebrook 7H11 medium (Ref.: I01S01687820, BD diagnostics). All the cultured plates were incubated at 37 ºC

for 28 days. Finally, CFU were counted and the total bacterial burden in LN of each animal was estimated. MTBC colonies were confirmed by multiplex PCR, as described by Wilton et al. (Wilton and Cousins, 1992).

5.2.11. Data analysis

Non parametrical Kruskal Wallis test, followed by pair-wise comparisons with the non-parametric one-tailed Wilcoxon rank sum test with Bonferroni correction was used to assess differences among groups in mean rectal temperature, weight increase, bacterial load (log_{10} CFU transformed counts) and pathological variables. For antigen-specific IFN-γ responses and MPB83-IgG responses, the same statistical tests were performed with two-tailed significance. Differences in the frequency of extra-pulmonary TB lesions among groups were assessed using a Fisher exact test. Statistical significance was established when P-value < 0.05. Statistical analysis was performed with Deducer for R package V2.15.0 (R Foundation for Statistical Computing, Vienna, Austria).

5.3. RESULTS

5.3.1. Clinical signs and body condition

No adverse reactions to vaccinations were observed at the site of vaccine injection. Neither clinical signs nor remarkable changes in body conditions were observed after vaccination and prior to challenge. After challenge, one animal of HIMB group did not recover from anesthesia. Clinical signs appeared in some animals at 5 weeks p.c. and all unvaccinated animals showed clinical signs at the end of the experiment. The clinical sings mostly observed were cough and dyspnea, while some animals showed anorexia and/or lethargy at the last time

point. Table 5.1 shows the proportion of animals with clinical signs recorded after challenge. A goat from the control group with dyspnea, anorexia and lethargy was euthanized for ethical reasons at week 16.

A peak of mean rectal temperature (above 40 ºC) was detected at week 12 (4 weeks p.c.) in control and HIMB groups when compared to BCG group (P = 0.007 and P = 0.031, respectively). In control group, the mean rectal temperatures remained above 40 ºC from week 12 until the end of the experiment, whereas mean rectal temperatures in HIMB group showed a mild decrease after week 12. BCG group showed the lowest mean rectal temperatures throughout the experiment, reaching the maximal mean rectal temperature at week 11, without attaining 40 ºC (See Figure 5.1A).

The mean body weight increase for each group after challenge is shown in Figure 5.1B. BCG vaccinated group showed weight gain in all time points after challenge, however differences were not statistically significant when compared to the other groups. Unvaccinated group did not show weight gain from 3 weeks p.c. onwards, while HIMB vaccinated group started to show weight gain after week 13 (5 weeks p.c.).

5.3.2. Interferon-γ responses after vaccination and challenge

The mean IFN-γ responses before and after challenge for each treatment group are represented in Figure 5.2. The IFN-γ response to PPD-B (Figure 5.2A) started to increase after vaccination in both vaccinated groups when compared to control group from week 3 to 8 (at week 3, P = 0.001 for both vaccinated groups; at week 5, P = 0.004 and P = 0.035; and at week 8, P < 0.066 and P < 0.008, for BCG and HIMB group, respectively). All BCG vaccinated and 7 out of 8 HIMB vaccinated (3 s.c. and 4 i.m.) animals showed detectable PPD-B-

specific IFN-γ responses after vaccination. The other subcutaneously HIMB vaccinated animal did not show any detectable response from vaccination to *M. caprae* challenge. Three weeks after challenge (week 11), all animals showed high IFN-γ responses to PPD-B, although they were slightly higher in the BCG group, being statistically significant when compared to HIMB group at week 13 ($P = 0.044$) and to both control and HIMB groups at week 17 ($P = 0.031$ and $P = 0.044$, respectively, Figure 5.2A). After vaccination, IFN-γ to EC was undetectable among groups, but after challenge, all animals responded to EC, although no differences were observed (Figure 5.2B).

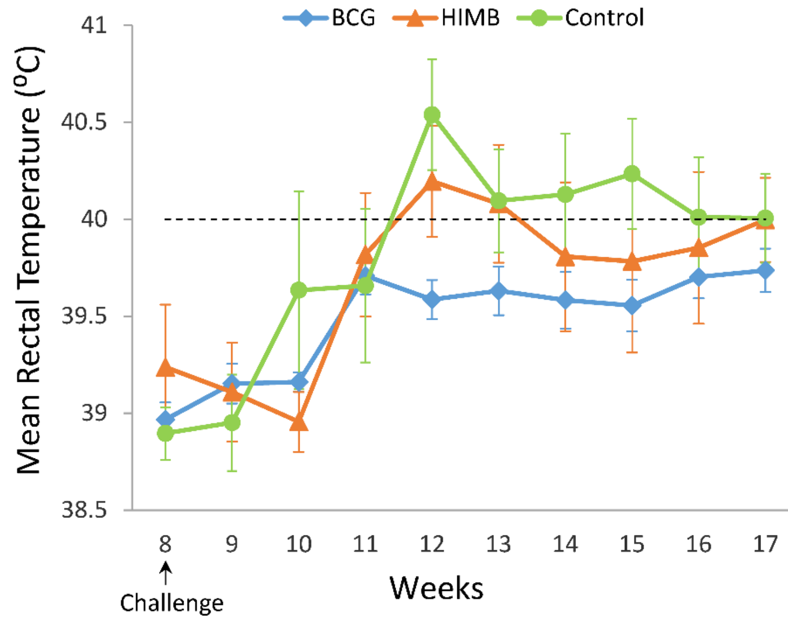
Table 5.1. Number of animals with clinical signs after *M. caprae* challenge

	Week				
Group	**8**	**11**	**13**	**15**	**17**
Control	0/8	0/8	5/8	6/8	8/8[a]
BCG	0/8	0/8	4/8	4/8	4/8
HIMB	0/7	0/7	2/7	6/7	4/7[b]

Animals were challenged at week 8. All animals with clinical signs showed cough and dyspnea.

[a] One goat was anorexic, another lethargic and one with both signs was euthanized at week 16.

[b] Two goats were anorexic.



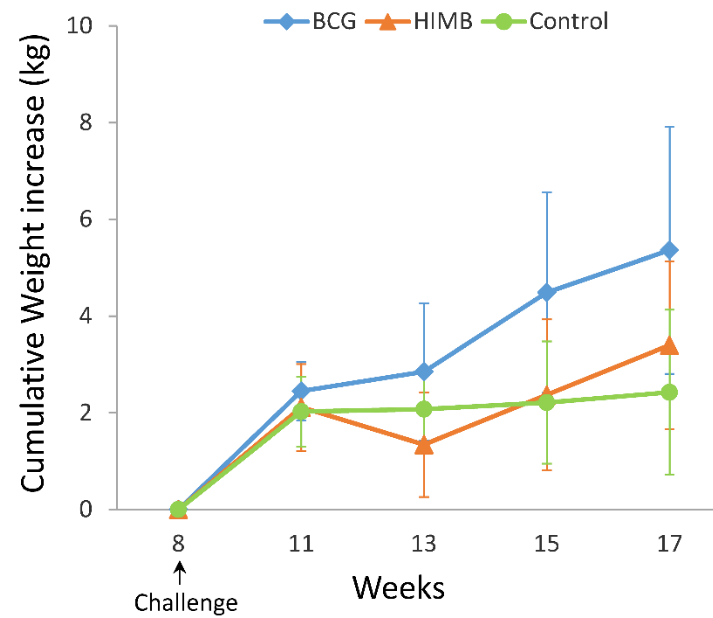


Figure 5.1. Clinical signs after *M. caprae* challenge. (**A**) Rectal temperature. Results are expressed as mean rectal temperature (ºC) ± 95% confidence interval (CI). Horizontal dashed line shows the threshold used for defining fever (40 ºC). (**B**) Body weight increase. Results expressed as increase of weight (kg) ± 95% CI from the week of challenge. Groups: control (n = 8), BCG vaccinated (n = 8) and HIMB vaccinated (n = 7) animals.

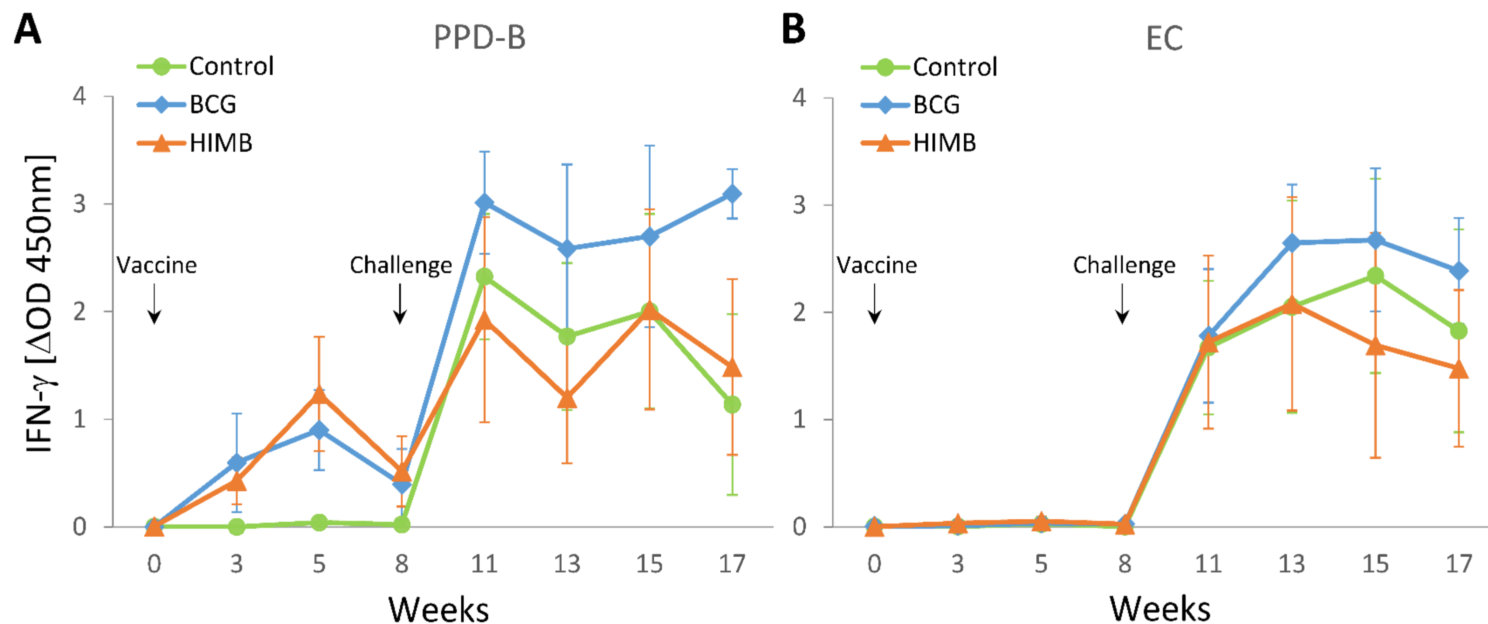


Figure 5-2. Antigen specific IFN-γ responses after vaccination and *M. caprae* challenge**.** The graphic shows the levels of IFN-γ measured by ELISA. Results are expressed as $\Delta OD_{450mn} \pm 95\%$ CI. (**A**) Response against Bovine tuberculin (PPD-B). (**B**) Response against EC antigen cocktail (ESAT-6 / CFP-10). Groups: Control (n = 8), BCG (n = 8), HIMB (n = 7).

Qualitative results of the IGRA to both PPD-B and EC are shown in Table 5.2. After vaccination, in both vaccinated groups positive animals to PPD-B were detected (positivity varied according to the criterion used) and no positive animals to EC were observed regardless of treatment group. All animals showed positivity to both reagents p.c., except at the last week when some animals from control group were negative to PPD-B, especially when the conservative criterion was used.

Table 5.2. Number of positive goats to each IFN-γ release assay

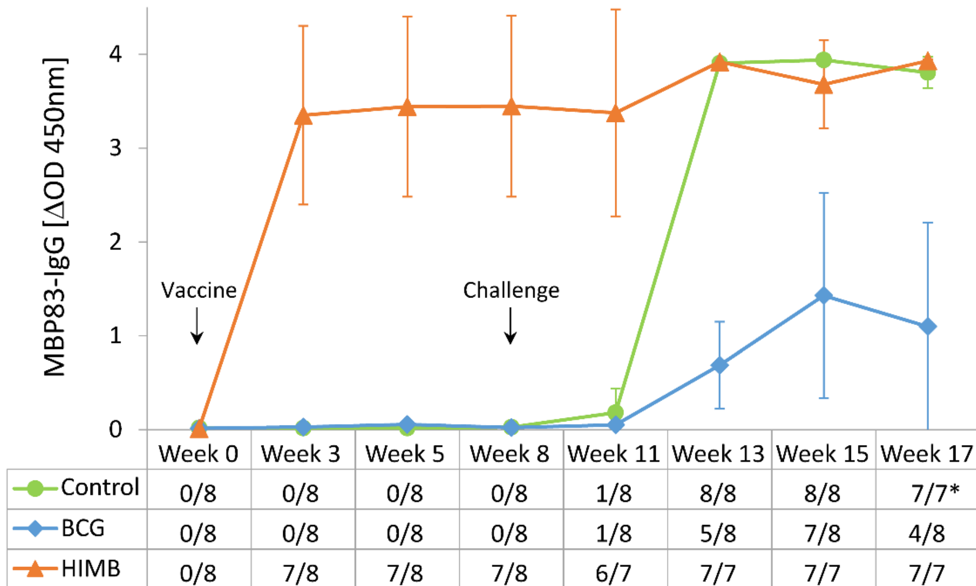
		Week							
Antigen	**Group**	**0**		**3**		**5**		**8**	
		C	S	C	S	C	S	C	S
PPD-B[b]	Control	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
	BCG	0/8	0/8	2/8	5/8	4/8	6/8	1/8	5/8
	HIMB	0/8	0/8	1/8	2/8	4/8	4/8	3/8	3/8
EC[c]	Control	0/8		0/8		0/8		0/8	
	BCG	0/8		0/8		0/8		0/8	
	HIMB	0/8		0/8		0/8		0/8	
		Week							
		11		**13**		**15**		**17**[a]	
		C	S	C	S	C	S	C	S
PPD-B[b]	Control	8/8	8/8	8/8	8/8	8/8	8/8	5/7	6/7
	BCG	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8
	HIMB	6/7	6/7	7/7	7/7	6/7	7/7	7/7	7/7
EC[c]	Control	8/8		8/8		8/8		8/7	
	BCG	8/8		8/8		8/8		8/8	
	HIMB	7/7		7/7		6/7		7/7	

Animals were vaccinated at week 0 and challenged at week 8.
[a] One goat of the Control group was euthanized at week 16.
[b] PPD-B, *M. bovis* purified protein derivative; two criteria were used for positivity: C, Conservative (S/P ≥ 35%), and S, Stringent (S/P ≥ 16%).
[c] EC, ESAT-6/CFP-10 protein mixture; the criterion for positivity was S/P ≥ 16%.

5.3.3. Humoral responses after vaccination and challenge

The mean MPB83-specific IgG levels (ΔOD) measured by ELISA, before and after challenge, and qualitative results of serology are shown in Figure 5-3. Three weeks after vaccination, mean IgG levels of MPB83 dramatically increased in HIMB vaccinated group (at week 3, $P = 0.021$ when compared to both BCG and control group; at week 5, $P = 0.030$ and $P = 0.008$; and at week 8, $P = 0.021$ and $P = 0.031$, when compared to BCG and control group, respectively). Nonetheless, one subcutaneously HIMB vaccinated animal did not show any detectable serological response to MPB83 after vaccination, while the rest of HIMB vaccinated animals showed strong responses at a similar level. Five weeks after challenge (week 13), all animals presented serological responses against *M. caprae*. In control group, levels of MPB83-IgG sharply raised and in HIMB group, a slight boost of serological response was observed. However, the BCG group showed the lowest MPB83-IgG levels p.c. compared to both control and HIMB groups (at week 13, $P < 0.003$ and $P < 0.001$, at week 15 $P < 0.004$ and $P < 0.018$, and at week 17, $P < 0.007$ and $P = 0.006$, when compared to control and HIMB groups, respectively).

With regard to qualitative analysis of humoral response to MPB83, HIMB vaccinated animals exhibited positive results after vaccination, except for one animal. In contrast, control and BCG group did not show any positive animal before challenge. Five weeks after challenge, in control and HIMB groups all animals were positive and remained so until the end of the experiment. On the other hand, in BCG group the number of positive animals fluctuated during the p.c. period.



	Week 0	Week 3	Week 5	Week 8	Week 11	Week 13	Week 15	Week 17
Control	0/8	0/8	0/8	0/8	1/8	8/8	8/8	7/7*
BCG	0/8	0/8	0/8	0/8	1/8	5/8	7/8	4/8
HIMB	0/8	7/8	7/8	7/8	6/7	7/7	7/7	7/7

Figure 5.3. Antibody responses to MPB83 after vaccination and challenge. The graphic shows the MPB83-IgG levels measured by ELISA. Results are expressed as ΔOD_{450mn} ± 95% CI. Groups: Control (n = 8), BCG (n = 8), HIMB (n = 7). The table in the horizontal axis represents the qualitative results of the test (No. of seropositive goats/total goats).*One goat of the control group was euthanized at week 16.

5.3.4. Post-mortem findings

All goats presented extensive lung TB lesions at necropsy. The assessment of TB lesions and pathological parameters are shown in Figure 5.4. When compared to the control group, volume of pulmonary LN lesions in BCG and HIMB vaccinated groups were significantly lower ($P < 0.001$ and $P = 0.00186$, respectively), volume of lung lesions was also lower, but yet not statistically significant ($P = 0.075$), with also lower volume of mineralization in lungs ($P < 0.002$ and $P = 0.052$, respectively). However, when the ratio lesion volume / lung volume was assessed, only BCG group showed a slightly lower ratio than the control group ($P = 0.05$). Regarding the mineralization volume / lesion volume ratio, no differences were observed among groups. However, both BCG

and HIMB vaccinated groups showed lower mineralization volume / lung volume ratios than the control group ($P < 0.004$ and $P < 0.079$, respectively). For the pathological parameters described above, no statistical differences were detected between vaccinated groups.

The total volume of lesions in lungs and pulmonary LN in the BCG group (median: 290 cm^3, IQR: 411-178) and in HIMB group (median: 449 cm^3, IQR: 468-377) were significantly lower than in the control group (median: 625 cm^3, IQR: 734-537, $P = 0.024$, $P = 0.042$; respectively), whereas no significant differences were observed between vaccinated groups ($P = 0.778$).

TB lesions were predominant in lungs and pulmonary LN in the three groups. However, the number of animals with extra-pulmonary lesions was significantly lower in both BCG and HIMB vaccinated groups, 1/8 and 2/7, respectively, while unvaccinated animals showed 7/8 animals with extra-pulmonary lesions ($P = 0.005$ and $P = 0.024$ respectively; Table 5.3).

The bacterial load in pulmonary LN in the unvaccinated group (median: 4.56 log_{10} CFU, IQR: 4.96-4.30) was slightly higher than in BCG and HIMB vaccinated groups (median: 4.12 log_{10} CFU, IQR: 4.50-3.91, and median: 3.95 log_{10} CFU, IQR: 4.63-3.86) but no statistical differences were detected among groups (Figure 5.5).

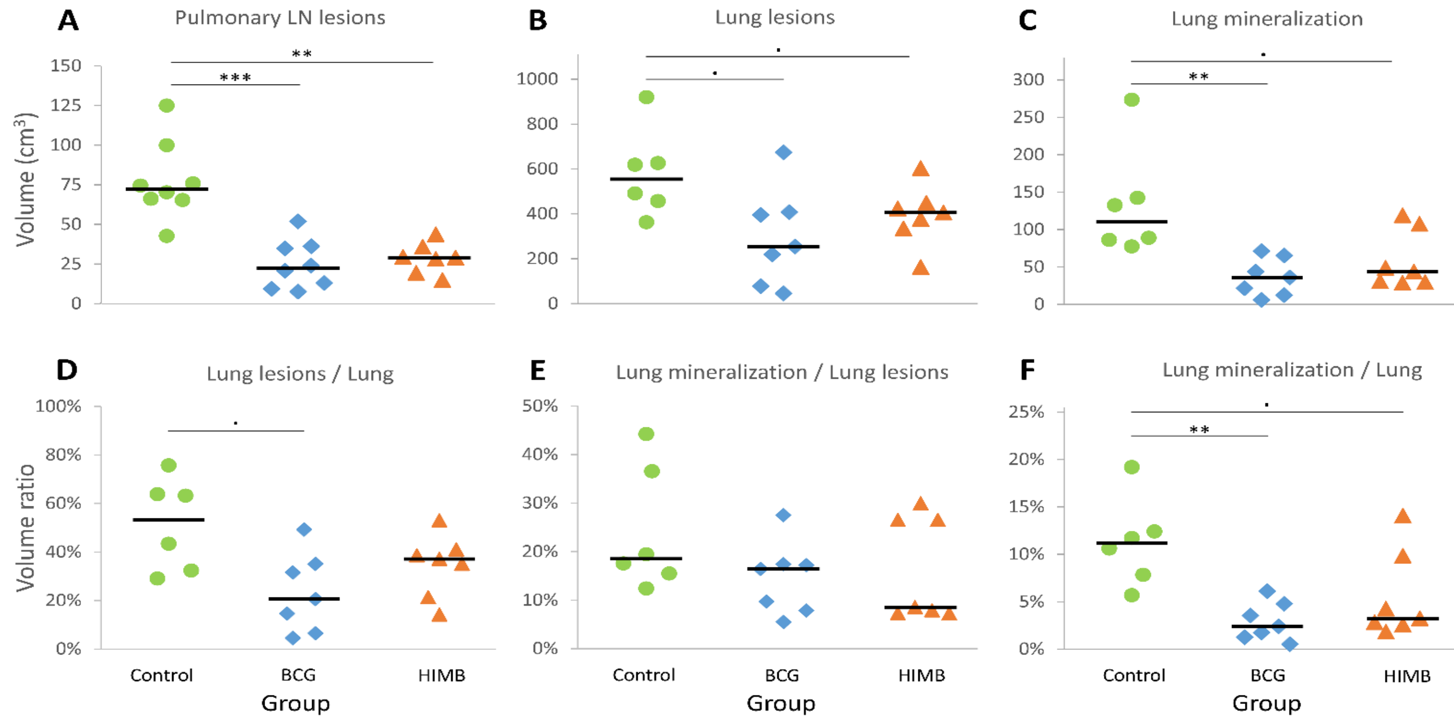


Figure 5.4. Quantitative pathological results. (**A-C**) Individual volumes of TB lesions expressed in cm^3. (**D-F**) Individual ratios between volumes expressed in %. (**A**) Total volume of lesions in pulmonary lymph nodes (LN). Groups: Control (n = 8), BCG (n = 8) and HIMB (n = 7). (**B**) Total volume of lung lesions. (**C**) Total volume of mineralized lesions in lungs. (**D**) Ratio of total volume of lung lesions / volume of the whole lung. (**E**) Ratio of total volume of lung mineralization / total volume of lung lesions. (**F**) Ratio of total volume of lung mineralization / volume of the whole lung. Groups (**B-F**): Control (n = 6), BCG (n = 7) and HIMB (n = 7). Horizontal lines represent median values. · $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Kruskal-Wallis test with the *post hoc* Wilcoxon rank sum test with Bonferroni correction.

Table 5.3. Distribution of extrapulmonary lesions

Group	No. of animals with extra-pulmonary lesions	RF LN	MS LN	Liver	Spleen	GS LN	RH LN
Control	7/8	1/8	5/8	1/8	2/8	1/8	1/8
BCG	1/8**	0/8	0/8	0/8	1/8	0/8	0/8
HIMB	2/7*	1/7	1/7	0/7	0/7	0/7	0/7

LN: Lymph node. RF: Retropharyngeal LN (not confirmed by histopathology). MS: Mesenteric LN. GS: Gastrosplenic LN, RH: Retro-Hepatic LN. * $P < 0.05$, ** $P < 0.01$, Fisher exact test.

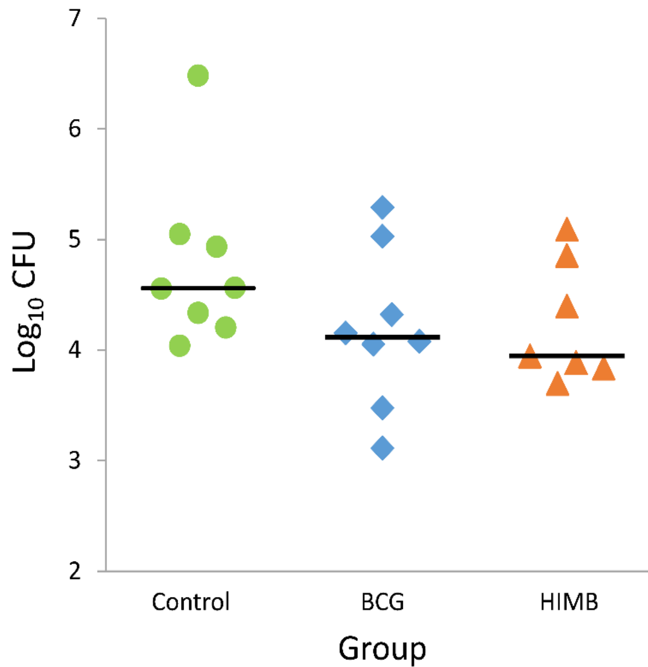


Figure 5.5. Bacterial load in pulmonary lymph nodes. Results are expressed as $\log_{10}$ CFU for each group. Groups: Control (n = 8), BCG (n = 8) and HIMB (n = 7). Horizontal lines represent median values.

5.4. DISCUSSION

In this study, the efficacy of a heat inactivated *M. bovis* vaccine in a goat model was evaluated in comparison to BCG vaccinated and control unvaccinated goats. The results indicated that parenteral HIMB vaccination of goats confers protection, mainly in terms of volume lesions reduction in both lungs and pulmonary LN, being comparable with BCG vaccinated animals. In accordance to these results, previous studies showed similar degree of protection in orally and parenterally vaccinated wild boar (Garrido et al., 2011), using a slightly different HIMB inactivation procedure (80 ºC for 30 min. instead of 83-85 ºC for 45 min.), and in orally vaccinated red deer (Thomas et al., 2017) using the same HIMB inactivation procedure than in the present study. On the contrary, HIMB vaccination through the oral route did not show protection in *M. caprae* experimentally challenged lambs (Balseiro et al., 2017).

Even though all goats showed TB lesions in the thoracic cavity irrespectively of the treatment group, BCG and HIMB vaccinated groups showed a significant reduction of mean volume of TB lesions in lungs and in pulmonary LN. These results are in accordance to those previously observed in HIMB vaccinated red deer (Thomas et al., 2017) and wild boar (Garrido et al., 2011), that showed a reduction in the percentage of lung lobe affectation compared to control group. Furthermore, a significant reduction of the presence of TB lesions in extra-pulmonary tissues was observed in both vaccinated groups when compared to the unvaccinated group. These results are consistent with previous BCG vaccination studies in goats, under experimental (Pérez de Val et al., 2012b) and field conditions (Vidal et al., 2017). In contrast, HIMB orally vaccinated but not BCG orally vaccinated red deer showed a reduction of presence of extra-pulmonary lesions (Thomas et al., 2017).

Besides, no adverse reactions at the inoculation point (in either subcutaneous or intramuscular delivery) were observed after HIMB vaccination, as previously reported in parenterally vaccinated wild boar (Beltrán-Beck et al., 2014; Díez-Delgado et al., 2017). As expected, no adverse reactions were observed after subcutaneous vaccination of goats with BCG, in consistency with those previously reported in a BCG safety study in goats (Pérez de Val et al., 2016).

The lower *M. caprae* burden in pulmonary LN of vaccinated groups compared to unvaccinated animals, suggests a reducing effect of vaccines in the mycobacterial drainage from lungs to pulmonary LN. Previous studies indeed demonstrated a significant reduction of bacterial load in lungs and pulmonary LN in parenterally BCG vaccinated badgers (Lesellier et al., 2009) and goats (Pérez de Val et al., 2013, 2012b), respectively. In fact, these badgers were challenged endobronchially with a similar dose than the one in the present study (~10^4 CFU), but goats were challenged with a lower dose of *M. caprae* (10^3 CFU). In contrast, in a high dose *M. bovis* (10^6 CFU) challenge conducted in red deer using the intratracheal route (Thomas et al., 2017), no differences in bacterial load in pulmonary LN were observed among groups (HIMB and BCG orally vaccinated animals and unvaccinated animals). All these findings suggest that the challenging dose may influence the bacterial burden found in LN, thus interacting with the vaccine effect.

In HIMB vaccinated and unvaccinated goats, a peak of fever was registered at 4 weeks p.c. and was consistent with a weight gain cessation observed in both groups one week after. Similar results were described in HIMB orally vaccinated lambs (Balseiro et al., 2017). On the other hand, BCG vaccinated animals did not show fever neither weight losses, as previously described in BCG vaccinated goats (Pérez de Val et al., 2013) and lambs (Balseiro et al., 2017).

In the present study, HIMB and BCG vaccines interfered on the diagnosis of TB using the IGRA with standard tuberculins. Thus, some vaccinated animals (2/8 and 1/8, from BCG and HIMB vaccinated animals, respectively) were positive to the tuberculin-based IGRA after vaccination, when assessed with the conservative criterion. Positivity increased when the stringent criterion was used, mainly in BCG group (5/8) and, in lower number of cases, in the HIMB group (2/8). IFN-γ responses against tuberculins were previously observed after parenterally HIMB vaccination in goats (Roy et al., 2017), cattle (Jones et al., 2016; van der Heijden et al., 2017), and wild boar (Garrido et al., 2011).

As expected, EC-specific IFN-γ responses were not detected after BCG vaccination as previously described in goats (Pérez de Val et al., 2016, 2013, 2012b). The BCG genome does not contain the genes codifying for ESAT-6 and CFP-10, but this genomic region is not deleted in the virulent *M. bovis* strain from which HIMB vaccine was originally obtained. Interestingly, no detectable IFN-γ responses to EC protein mixture were observed after HIMB vaccination, as previously described in orally vaccinated lambs (Balseiro et al., 2017) and cattle (Jones et al., 2016). On the contrary, parenterally HIMB vaccinated cattle were positive to EC protein mixture and EC peptide cocktail-based IGRAs (Jones et al., 2016). In another study, no detectable responses to single ESAT-6 protein were detected in cows subcutaneously vaccinated with HIMB, whereas, slight responses were observed when single CFP-10 protein was used (van der Heijden et al., 2017). However, in other studies, experimentally challenged goats showed low IFN-γ responses to ESAT-6 protein compared to EC peptide cocktail (Pérez de Val et al., 2012b), and experimentally challenged lambs showed similar IFN-γ responses to both EC protein mixture and peptide cocktail (Balseiro et al., 2017). This lead to speculate that these antigens may be present in HIMB vaccine but at an undetectable concentration, or are present in an

altered form induced by the vaccine inactivation (van der Heijden et al., 2017). The results of the present study suggest that the EC protein mixture might be the most useful DIVA reagent in HIMB vaccinated goats, although further longer and larger-scale studies are required to confirm these results.

With regard to the antibody dynamic, HIMB vaccinated animals showed a rapid and strong seroconversion against MPB83 after vaccination (3 weeks p.v.). Similar results were previously found in parenterally HIMB vaccinated wild boar (Garrido et al., 2011) and cattle (van der Heijden et al., 2017). By contrast, MTBC-specific antibody responses were not detected after oral vaccination of lambs (Balseiro et al., 2017) and wild boar (Garrido et al., 2011). After challenge, HIMB vaccinated animals remained with high levels of MPB83-IgG, reaching the saturation levels of the test, thus, the effect of challenge on serological responses could not be evaluated in this group. Interestingly, the control group showed a rapid and strong seroconversion (3 weeks p.c.) in contrast to previous studies in goats (Pérez de Val et al., 2011). This could be due to the high *M. caprae* dose used in the present study. Finally, in BCG vaccinated animals lower MPB83-IgG levels were observed, in concordance to previous studies in goats (Pérez de Val et al., 2012b) and cattle (Lyashchenko et al., 2004).

HIMB induced similar cell-mediated and humoral immune responses in 7 out of 8 animals irrespectively of the parenteral vaccine delivery route (s.c. or i.m.). Intriguingly, one animal of the HIMB group (s.c.) did not show neither humoral nor cell-mediated responses after vaccination. Since this animal was only 4 weeks old at the vaccination point (2-3 weeks younger than the rest of experimental animals), this lack of response might be explained by a poorer capability of the immunological system of this animal to respond properly to the

vaccination. This suggests that age of vaccination is an important variable that should be taken into consideration in future studies with HIMB vaccine.

Finally, at the end of the study, some control goats became less responsive or unresponsive to tuberculins, being negative to qualitative diagnose, which might suggest an exhaustion of cell-mediated specific response. Unresponsiveness due to exhaustion of cell mediated response in controls had already been observed in goat trials with tuberculosis, although unresponsiveness was observed at week 14 p.c. and challenge was performed with 10^3 of *M. caprae* (Pérez de Val et al., 2011).

In conclusion, the results provide evidence that parenteral vaccination of goats with HIMB can be as protective against TB infection as BCG vaccination. Moreover, since it is an inactivated vaccine, HIMB is more stable and environmentally safer under field conditions than live attenuated BCG. Thus, HIMB vaccine may be an improved tool for goat TB vaccination programs. Further studies are required using other experimental conditions (namely, lower bacterial dose for challenge, different administration routes with larger number of animals, longer follow-up) and interaction with other environmental factors in field trials.

CHAPTER 6

Study IV

Evaluation of P22 antigenic complex for the Immuno-diagnostics of tuberculosis in BCG vaccinated and unvaccinated goats

(P22 BCGDIAG)

6.1. INTRODUCTION

The cornerstone of an efficient caprine TB eradication program is the diagnosis. The Spanish bovine TB eradication program effectiveness is highly dependent on the routine tuberculin skin testing (Napp et al., 2019). Current bovine TB testing is based on the single intradermal tuberculin test (SIT) and single intradermal cervical comparative tuberculin test (SICCT), and the interferon-gamma release assay (IGRA). However, in goats under certain epidemiological contexts, those diagnostic tests have some drawbacks in terms of sensitivity (Se) and specificity (Sp) (Bezos et al., 2014b, 2012a).

Another concern for TB diagnostics is the vaccination against *M. avium* subsp *paratuberculosis* (MAP), which has been largely implemented in small ruminants, to prevent the development of clinical disease (Bastida and Juste, 2011). Nevertheless, even though MAP vaccines are authorized (e.g., Gudair ® vaccine), it has been demonstrated that paratuberculosis (PTB) vaccination interferes with skin tests (STs) and IGRA used for TB diagnosis (Chartier et al., 2012; Pérez de Val et al., 2012a). In addition, the efficacy of *M. bovis* bacille Calmette-Guérin (BCG) vaccine has also been assessed in goats during the last decade in different vaccination trials (Bezos et al., 2017; Pérez de Val et al., 2016, 2013; Roy et al., 2019; Vidal et al., 2017). Even though these trials showed that BCG conferred certain protection to experimentally and naturally infected goats, it was evidenced that vaccination interfered with current TB diagnostic tests (Bezos et al., 2015b; Pérez de Val et al., 2016).

To overcome diagnostic interferences due to BCG vaccination, defined antigens to differentiate infected from vaccinated animals (DIVA) have been developed (Pérez de Val et al., 2012a; Vordermeier et al., 2016); nevertheless, those antigens have shown lower sensitivity compared to tests based on standard

tuberculins (Vordermeier et al., 2001). Recently, a new multi-protein complex called P22, obtained from purified protein derivative of *M. bovis* (PPD-B) by affinity chromatography, has been developed (Infantes-Lorenzo et al., 2017), yielding high sensitivity and variable specificity, depending on the animal species and epidemiological contexts (Infantes-Lorenzo et al., 2019a). To date, this antigen has been tested to detect humoral response against MTBC in different species (Bezos et al., 2018; Casal et al., 2017; Infantes-Lorenzo et al., 2018, 2019b; Thomas et al., 2019a, 2019b); however, there is a lack of information regarding its performance for cell-mediated immunity (CMI)-based diagnostics.

The aim of this study was to evaluate the performance of different cell-mediated and humoral immunodiagnostic tests, based on the P22 antigenic complex, for the diagnosis of TB in goats under different epidemiological and control scenarios.

6.2. MATERIALS AND METHODS

6.2.1. Herds and experimental design

A total of 222 goats from 3 herds were included in the study (Table 6.1): 77 infected goats (infection was confirmed *post-mortem* by gross lesions, histopathology or mycobacterial culture, or both) from a TB positive herd of murciana-granadina goats (herd A); 77 goats belonging to an officially TB-free herd of alpine goats (herd B) that were vaccinated against paratuberculosis (PTB) with Gudair (CZ Vaccines, Porriño, Spain), around two years before sampling; and 68 goats from another TB-free herd (4erd C) of Blanca de Rasquera autochthonous breed, that were vaccinated against PTB (Gudair®) and

against TB with *M. bovis* BCG Danish 1331 strain (ATCC, Ref. 35733) as described previously (Pérez de Val et al., 2013). In herd C, 50% of goats were vaccinated with BCG and Gudair ® 9 to 10 months before sampling, and the remaining 50% were vaccinated more than one year before. STs, IGRAs and IgG enzyme-linked immunosorbent assays (ELISA) were carried out in the 77 infected goats, as well as in 138, 142 and 142 non-infected goats, respectively (Table 6.1).

Two TB control scenarios were hypothesized in order to study the performance of each diagnostic test: the conventional (TB unvaccinated) scenario, using data from herds A and B, and the BCG vaccinated (TB-VAC) scenario, using data from herds A and C. Se was calculated using data from herd A, and Sp was calculated using data from herds B and C depending on TB control scenario (Table 6.2).

Table 6-1. Herds and treatment distribution of tested animals

				No. of animals tested		
Herd	**TB status**	**BCG[1]**	**Gudair®[2]**	**ST**	**IGRA**	**ELISA**
A	Positive	No	No	77	77	77
B	Free	No	Yes	77	74	74
C	Free	Yes	Yes	61	68	68

[1] BCG: Bacilli Calmette-Guérin *Mycobacterium bovis* vaccine. [2] Gudair ® vaccine: Vaccine against Paratuberculosis (*Mycobacteium avium* subspecies *paratuberculosis*).

Table 6-2. TB control scenarios distribution of tested animals

				No. of animals tested		
Control scenario	**Herds**	**BCG**[1]	**Gudair®**[2]	**ST**	**IGRA**	**ELISA**
Conventional[a]	A+B	No	Yes	154	151	151
TB-VAC[b]	A+C	Yes	Yes	138	145	145

[a] Conventional scenario: composed by TB unvaccinated goats.[b] TB-VAC Scenario: TB negative animals from herd C were vaccinated with BCG and TB positive animals from herd A were not vaccinated. [1] BCG: Bacilli Calmette-Guérin *Mycobacterium bovis* vaccine. [2] Gudair ® vaccine: Vaccine against Paratuberculosis (*Mycobacteium avium* subspecies *paratuberculosis*).

6.2.2. Ethics statement

All animals included in this study belonged to commercial farms and were not experimental animals. All sampling and handling procedures were carried out by authorized veterinarians according to standard farm methods and in conformity with Spanish legislation (Royal Decree 2611/1996 and amendments) and European Union laws for the protection of animals used for scientific purposes (2010/63/EU). Test and slaughter of positive animals, as well as *post-mortem* sampling to confirm the disease, were conducted according to the regulations defined by the Catalan Government (Resolution AAM/1314/2014).

6.2.3. Antigens

M. tuberculosis var. *bovis* (PPD-B) and *M. avium* (PPD-A) tuberculins (2500 IU/ml) were obtained from CZ Vaccines and used at concentrations recommended by the Spanish Ministry (MAPA, 2019). The protein complex P22 was produced by immunopurification of PPD-B (CZ Vaccines) as described previously (Infantes-Lorenzo et al., 2017) and prepared at a concentration of 500

µg/ml (unpublished data). DIVA reagent based on a cocktail of recombinant ESAT-6 and CFP-10 proteins (500 µg/ml) (Cockle et al., 2002), as well as the recombinant MPB83 (MPT83) protein (500 µg/ml) (Vordermeier et al., 1999), were purchased from Lionex (Braunschweig, Germany).

6.2.4. Skin tests (ST)

SIT was performed by intradermal inoculation of 0.1 ml of PPD-B in the left-hand side of the neck by using a Dermojet® syringe (Akra Dermojet, Pau, France). In the same way, SICCT was performed by intradermal inoculation of 0.1 ml of PPD-B and PPD-A, both in the left-hand side of the neck, at the proximal and distal part of the neck, respectively. Besides, 0.1 ml of P22 (at 500 µg/ml) were inoculated in the right-hand side of the neck. The increase in skinfold thickness (SFT) was measured just before the inoculation and after 72 h. Severe interpretations of SIT and SICCT were performed, as previously described in the manual of the Spanish bovine TB eradication program (MAPA, 2019). Briefly, positive criterion for SITT: SFT PPD-B > 2 mm (severe); and for SICCT: positive to SIT and SFT PPD-B - SFT PPD-A > 1 mm (severe) or presence of clinical signs in the PPD-B inoculation site. P22 Single and comparative skin tests (sP22-ST and cP22-ST) were interpreted using the same criteria as SIT and SICCT, respectively, i.e., considering SFT P22 and SFT P22 - SFT PPD-A measures, respectively.

6.2.5. Whole-blood Interferon gamma release assays (IGRA)

Blood samples were collected from the jugular vein prior to ST performance using heparinized tubes and were processed as described previously (Pérez de Val et al., 2016). Shortly, blood samples were stimulated with PPD-B, PPD-A

and P22 at a final concentration of 20 μg/ml, and with DIVA reagent (ESAT-6/CFP-10) at 20 μg/ml, while PBS was added as unstimulated control. Samples were incubated at 37±1 ºC with 0.5% CO_2 overnight. Finally, plasma supernatant was collected and analyzed by ELISA (BOVIGAM®, Thermo Fisher Scientific, Waltham, MA, USA) and read at 450 nm using a spectrophotometer (Biotek Power Wave XS). The interpretation of tuberculin-based IGRA (STAND-IGRA) results was performed according to the cut-off point recommended by the manufacturer, i.e., the criterion for positivity: PPD-B OD – PBS OD ≥ 0.05 and PPD-B OD > PPD-A OD. Similarly, cP22-IGRA was considered positive when P22 OD – PBS OD ≥ 0.05 and P22 OD > PPD-A OD, whereas sP22-IGRA and DIVA-IGRA were considered positive when P22 OD – PBS OD ≥ 0.05 and DIVA OD – PBS OD ≥ 0.05, respectively.

6.2.6. Antibody detection tests

Plasma samples were analyzed for antibody detection by using two in-house indirect ELISA, one for detecting MPB83 antigen, performed and interpreted as described previously (Pérez de Val et al., 2017), and another one for detecting P22, performed as described previously (Infantes-Lorenzo et al., 2019a). P22-ELISA was interpreted as follows: ELISA percentage (E%) = [mean sample OD/(2 x mean negative control OD)]x100. A sample E% <100% was classified as negative, and a sample E% ≥100% was classified as positive.

6.2.7. Post-mortem examination

Seventy-seven goats from the positive herd (herd A) were euthanized after STs reading by intravenous injection of a sodium pentobarbital overdose. A complete necropsy procedure was conducted for TB lesions examination.

Lesions were collected and immediatelyfixed in 10% buffered formalin for histopathological confirmation by Hematoxylin/Eosin staining. Mediastinal and tracheobronchial lymph nodes (LN) were removed and stored at -20 °C for bacterial culture.

6.2.8. Bacteriology

Whole pulmonary LN of each animal were thawed, pooled, homogenized, and decontaminated as previously described (35), and plated on Middlebrook 7H11 medium (BD diagnostics, Sparks, MD, USA). Then, cultured plates were incubated at 37 ºC for 28 days. Finally, plates were read, and colonies were confirmed as MTBC by multiplex PCR (Glas et al., 2003).

6.2.9. Data analysis

The specificity was calculated in TB free farms (herd B and herd C), using the formula Sp = True negatives / (True negatives + False positives). The sensitivity (Se) was calculated in the TB infected farm by the formula Se = True positive / (True positive + False negative). Clooper-Pearson 95% confidence intervals were calculated for Sp and Se. Differences in diagnostic results, between tests, were evaluated by the McNemar test. Moreover, agreement between tests was calculated by Cohen's Kappa coefficient (*k*), and interpreted as follows: < 0.00 poor, 0.00-0.20 slight, 0.21-0.4 fair, 0.41-0.60 moderate, 0.61-0.80 substantial and 0.81-1.00 almost perfect. The diagnostic performance of each test was calculated using the Diagnostic Odds Ratio (DOR) (Risalde et al., 2017). All statistical tests and 95% confidence intervals were calculated using the Epitools calculator (Sargento, ESG, 2018. Epitools Epidemiological Calculators. Ausvet. Pty Ltd, Australia Available in www.epitools.ausvet.com.au).

6.3. RESULTS

The results of Se of herd A and Sp of herds B and C, are summarized in Table 6.3. The TB positive status of all animals from herd A was confirmed by positive mycobacterial culture and/or positive lesions in histopathological analysis.

6.3.1. Skin tests

The Se of the cP22-ST was the lowest among tests, but the Sp in herd B was the highest, being identical to the Sp of the SICCT, and a 6 percent point (p.p.) and 8 p.p. more specific than the SIT and the sP22-ST, respectively. Regarding the herd C, the cP22-ST and the sP22-ST displayed similar Sp, being significantly more specific than the SIT (31 p.p. of increase, $P < 0.001$ and 30 p.p. of increase, $P = 0.005$ for cP22-ST and sP22-ST, respectively) and the SICCT (18 p.p. of increase, $P = 0.0026$ and 17 p.p. of increase, $P = 0.0094$ for cP22-ST and sP22-ST, respectively).

Table 6.3. Sensitivity (Se) and specificity (Sp) of diagnostic tests.

Diagnostic test	TB + farm (A)		Unvaccinated (B)		BCG vaccinated (C)	
	N[9]	Se (95% CI)[10]	N	Sp (95% CI)[11]	N	Sp (95% CI)[11]
sP22-ST[1]	77	87% (77-94)	77	92% (84-97)	61	97% (89-100)
cP22-ST[2]	77	74% (63-83)	77	100% (95-100)	61	98% (91-100)
SIT[3]	77	94% (85-98)	77	94% (85-98)	61	67% (54-79)
SICCT[4]	77	91% (82-96)	77	100% (95-100)	61	80% (68-89)
sP22-IGRA[5]	77	91% (82-96)	74	95% (87-99)	68	84% (73-92)
cP22-IGRA[6]	77	86% (76-93)	74	96% (89-99)	68	85% (75-93)
STAND-IGRA[7]	77	77% (66-86)	74	100% (95-100)	68	96% (88-99)
DIVA-IGRA[8]	77	71% (60-81)	74	100% (95-100)	68	100% (95-100)
P22-ELISA	77	74% (63-83)	74	93% (85-98)	68	96% (88-99)
MPB83-ELISA	77	75% (64-84)	74	92% (83-97)	68	94% (86-98)

[1]sP22-ST: single P22 skin test, [2]cP22-ST: comparative P22 skin test, [3]SIT: Single intradermal tuberculin test, [4]SICCT: Single intradermal cervical comparative tuberculin test. [5]sP22-IGRA: single P22 IGRA test, [6]cP22-IGRA: comparative P22 IGRA test, [7]STAND-IGRA: standard tuberculin IGRA test, [8]DIVA-IGRA: Differentiating Infected from Vaccinated animals (ESAT-6/CFP-10 peptide cocktail) IGRA test. [9]Number of animals tested, [10]Clopper – Pearson 95% confidence interval for Se. [11]Clopper – Pearson 95% confidence interval for Sp.

6.3.2. Serological tests

In terms of Sp and Se, diagnostic results of P22-ELISA were similar to diagnostic results of MPB83-ELISA. In herd A, the MPB83-ELISA detected 10 TB+ animals more than the P22-ELISA, and the P22-ELISA detected 9 TB+ animals more than the MPB83-ELISA, and the agreement between tests was

considered fair although statistically significant ($k = 0.35$, $P = 0.001$). In herd B, diagnostic results of Sp showed a moderate but significant agreement between ELISA tests (k = 0.51, $P < 0.001$), but in herd C, no agreement was observed ($k = -0.05$, $P = 0.33$).

6.3.3. Complementarity of diagnostic tests

Combined interpretation of P22-based tests was evaluated. Results of Sp and Se of complementarity of diagnostic tests are shown in Table 6.4. In general, complementarity between tests yielded an overall rise of Se with a variable reduction in the Sp.

The combination of cP22-ST + P22 ELISA improved the Se in 20 p.p. and displayed a similar Sp in both herds B and C, being the combined interpretation with the best results in all situations. The combination of SICCT + P22 ELISA showed similar results of Se and Sp in herd B. In herd C, the latter combination detected ten false-positive more than the cP22-ST + P22-ELISA, reducing its Sp in 16 p.p., and with diagnostic results significantly different between tests ($P = 0.004$). The combination of cP22-ST+cP22-IGRA improved the Se and Sp in herd B at a similar level than the combined interpretations above described, but in herd C, the Sp was reduced in 11 p.p. respect to the cP22-ST + P22-ELISA test.

The combination of current diagnostic tests, e.g., SIT and SICCT, with other diagnostic tests, increased the Se but not the Sp, except for the SICCT + STAND-IGRA. The latter combination improved the Se in a 4 p.p. and 18 p.p., compared to the SICCT and the STAND-IGRA alone, respectively, and maintained the Sp in herd B but not in herd C (reduction of 16 p.p. compared to the STAND-IGRA alone). In herd A, the combined results of MPB83-ELISA +

P22-ELISA improved the Se in 12 and 13 p.p. respect to the MPB83-ELISA and the P22-ELISA alone, respectively, and maintained the Sp in herd B, and in herd C, showed a mild reduction of Sp (4 p.p. and 6 p.p. of reduction respect to the MPB83-ELISA and the P22-ELISA alone, respectively). Other combinations of tests did not improve the Se and the Sp, as did the aforementioned combined interpretations.

6.3.4. Performance of diagnostic tests

The results of DOR to assess the diagnostic performance for each test are represented in Figure 6.1. In general, a reduced DOR in TB-VAC scenario was observed compared to the Conventional one (0.47, 95% CI: 0.28-0.654, of mean reduction in log DOR). In Conventional context, SICCT + STAND-IGRA (3.38, 95% CI: 2.35-4.41), SICCT alone (3.16, 95% CI: 2.36-3.97), SICCT + cP22-IGRA (2.94, 95% CI: 1.12-4.76) and SICCT + sP22-IGRA (2.81, 95% CI: 1.08-4.54) showed the best performances (Figure 6.1A). In TB-VAC context, the best performances were observed in DIVA IGRA (2.53, 95% CI: 1.98-3.8), cP22-ST

Table 6.4. Sensitivity and specificity (Sp) combined results of P22-based diagnostic tests.

Diagnostic tests	TB + farm (A)		Unvaccinated (B)		BCG vaccinated (C)	
	N[8]	Se (95% CI)[9]	N	Sp (95% CI)[10]	N	Sp (95% CI)[10]
SIT[1] + sP22-IGRA[2]	77	97% (91-100)	73	89% (80-95)	61	59% (46-71)
SIT + cP22-IGRA[3]	77	97% (91-100)	73	90% (81-96)	61	61% (47-73)
SIT + P22-ELISA	77	96% (89-99)	73	89% (80-95)	61	66% (52-77)
SICCT[4] + sP22-IGRA	77	97% (91-100)	73	95% (87-98)	61	67% (54-79)
SICCT + cP22-IGRA	77	97% (91-100)	73	96% (88-99)	61	67% (54-79)
SICCT + P22-ELISA	77	95% (87-99)	73	95% (87-98)	61	79% (66-88)
sP22-ST[5] + sP22-IGRA	77	95% (87-99)	73	88% (78-94)	61	82% (70-91)
sP22-ST + P22-ELISA	77	94% (85-98)	73	88% (78-94)	61	93% (84-98)
cP22-ST[6] + sP22-IGRA	77	95% (87-99)	73	95% (87-98)	61	84% (72-92)

[1]SIT: Single intradermal tuberculin test, [2]sP22-IGRA: single P22 IGRA test, [3]cP22-IGRA: comparative P22 IGRA test, [4]SICCT: Single intradermal cervical comparative intradermal tuberculin test, [5]sP22-ST: single P22 skin test, [6]cP22-ST: comparative P22 skin test; [8] Number of animals tested; [9] Clopper – Pearson 95% confidence interval for Se; [10] Clopper – Pearson 95% confidence interval for Sp.

Table 6-4. Sensitivity and specificity (Sp) combined results of P22-based diagnostic tests (continuation).

Diagnostic tests	TB + farm (A)		Unvaccinated (B)		BCG vaccinated (C)	
	N[8]	Se (95% CI)[9]	N	Sp (95% CI)[10]	N	Sp (95% CI)[10]
cP22-ST[6] + P22-ELISA	77	94% (85-98)	73	95% (87-98)	61	95% (86-99)
sP22-IGRA[2] + STAND-IGRA[7]	77	92% (84-97)	74	95% (87-99)	68	84% (73-92)
P22-ELISA + sP22-IGRA	77	95% (87-99)	74	89% (80-95)	68	79% (68-88)
P22 ELISA + cP22- IGRA[3]	77	95% (87-99)	74	91% (81-96)	68	81% (70-89)
P22-ELISA + MPB83-ELISA	77	87% (77-94)	74	92% (83-97)	68	90% (80-96)
P22-ELISA + STAND-IGRA	77	90% (81-95)	74	93% (85-98)	68	91% (82-97)
SIT[1] + STAND-IGRA	77	95% (87-99)	73	93% (85-98)	61	67% (54-79)
SICCT[4] + STAND-IGRA	77	95% (87-99)	73	100% (95-100)	61	80% (68-89)

[1]SIT: Single intradermal tuberculin test, [2]sP22-IGRA: single P22 IGRA test, [3]cP22-IGRA: comparative P22 IGRA test, [4]SICCT: Single intradermal cervical comparative intradermal tuberculin test, [5]sP22-ST: single P22 skin test, [6]cP22-ST: comparative P22 skin test, [7]STAND-IGRA: standard tuberculin IGRA test. [8] Number of animals tested; [9] Clopper – Pearson 95% confidence interval for Se; [10] Clopper – Pearson 95% confidence interval for Sp.

+ P22 ELISA (2.44, 95% CI: 0.97-3.92) and sP22-ST + P22 ELISA (2.31, 95% CI: 0.95-3.67) (Figure 6.1B).

6.4. DISCUSSION

Efficient and accurate diagnosis is of paramount importance for the success of eradication programs based on test and slaughter strategy. Here, the performance of new P22 antigenic complex-based cell-mediated and humoral tests for the diagnosis of TB in goats was assessed under different epidemiological and TB control scenarios.

Recently, the P22 antigenic complex has been evaluated for the detection of IgG in ELISA tests, in different species: cattle goat, sheep, pigs, and wild boar, (Bezos et al., 2018; Casal et al., 2017; Infantes-Lorenzo et al., 2019a; Thomas et al., 2019a), red deer (Thomas et al., 2019b), badgers (Infantes-Lorenzo et al., 2019b), alpacas and llamas (Infantes-Lorenzo et al., 2018). In the present study, the performance of the P22 antigenic complex for diagnostic tests based on CMI, namely, skin tests and IGRA, was evaluated for the first time in goats. Indeed, the use of P22 for IGRA tests has only been reported in red deer experimentally infected with *M. bovis* (Risalde et al., 2017).

The combined interpretation of tests leads to a substantial improvement of Se at the expense of variable loss of Sp. As expected, in the conventional context, the SICCT alone or combined with the STAND-IGRA (Bezos et al., 2014b, 2012a, 2012b), showed the best performances by DOR analysis.

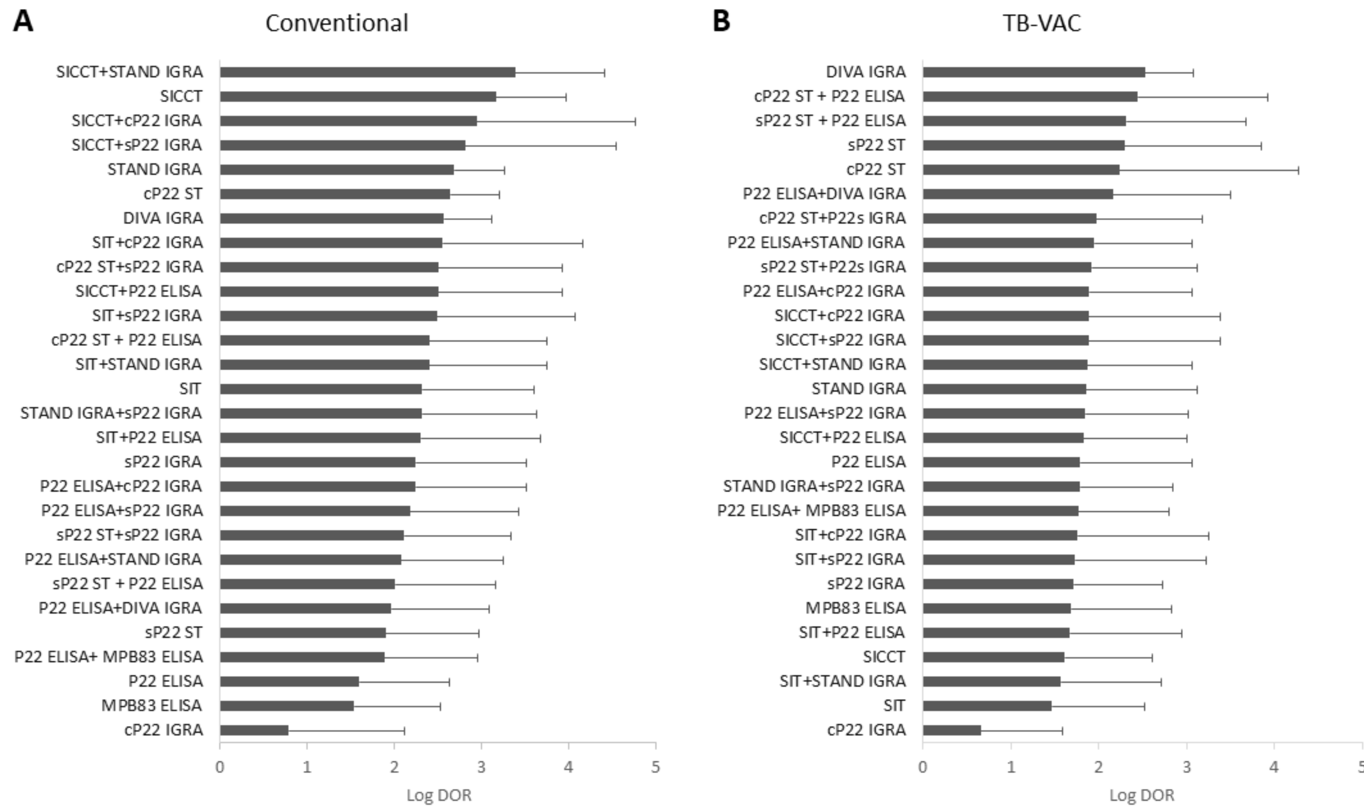


Figure 6-1. Diagnostic tests performance measured by Diagnostic odds ratio (DOR). **A**. Conventional (unvaccinated scenario). **B**. TB-VAC scenario, animals vaccinated with *M. bovis* Bacille Calmette-Guérin (BCG). sP22-ST: single P22 skin test, cP22-ST: comparative P22 intradermal skin test, SIT: Single intradermal tuberculin test, SICCT: Single intradermal cervical comparative tuberculin test. sP22-IGRA: single P22 interferon-gamma release assay (IGRA) test, cP22-IGRA: comparative P22 IGRA test, STAND-IGRA: standard tuberculin IGRA test, DIVA-IGRA: Differentiating Infected from Vaccinated animals (ESAT-6/CFP-10 peptide cocktail) IGRA test.

The performances of tuberculin-based tests were followed by the combinations of SICCT with the P22-IGRAs, which increased Se at the cost of a certain loss in Sp. Moreover, the combination of cP22-ST+ P22-ELISA clearly increased the Se with the benefit of a minimal decrease of Sp, showing similar results than the combination of SICCT + P22-ELISA. These findings are in concordance with previous studies of P22-ELISA and tuberculin-based skin testing. In cattle, the combination of SIT+ P22-ELISA showed an improvement of Se of 30 p.p. and 6 p.p. compared to the SIT and the P22-ELISA alone, respectively (25). In another study conducted in goats (39), the same combination improved the Se of the SIT and the P22-ELISA in 19 p.p. and 9.5 p.p., respectively. Also, in the same study in goats, the combination SICCT + P22-ELISA improved the Se of the SICCT in a 24 p.p. These results confirmed the benefits of the strategic use of serological and CMI-based diagnostic tests in parallel to maximize the Se in infected settings.

In the TB-VAC context, the combination of P22-ELISA with the two P22-based skin tests showed similar performances than the DIVA-IGRA. However, the latter showed considerably lower Se than the combinations of P22-ELISA with P22-based skin tests (reduction in 23 to 24 p.p.). Previous studies reported the excellent Sp (Pérez de Val et al., 2016) and the lack of Se (Bezos et al., 2011a) of DIVA-IGRA, although the DOR analysis tended to overestimate the Sp in this study. The Se of vaccine-associated diagnostic tests is an essential requirement for the development of an integral vaccination strategy (Vordermeier et al., 2014), and the combination of cP22-ST + P22-ELISA showed an efficient and innovative diagnostic approach in the TB-VAC context, showing the highest combined Se and Sp values (94% and 95%, respectively).

Concerning the use of the ST in solitary, the P22-based skin tests showed lower Se compared to both the SIT and the SICCT tests, although previous studies in

dairy goat flocks, with larger samples and different epidemiological situations, have shown lower Se for SIT (65%, 95% CI: 63.3-68.2) (Bezos et al., 2014b) and SICCT (44.5%, 95%CI: 35-55) (Buendía et al., 2013). However, the Se of the cP22-ST (74%, 95% CI: 63-83) was similar to Se observed in two previous studies using DIVA skin tests (based on the peptide cocktails ESAT-6, CFP-10 and Rv3616c) developed for the diagnosis of TB in cattle: 76%, 95% CI: 59-93 (Srinivasan et al., 2019) and 75%, 95% CI: 47.7-97.7 (Jones et al., 2012). In the latter, the addition of the Rv3020c peptide improved the Se to reach 87.5% (95% CI: 61.7-98.5), being similar to the Se of sP22-ST (87%, 95% CI: 74-94) obtained in the present study. On the other hand, in BCG vaccinated animals, the Sp of SIT and SICCT decreased dramatically (27 p.p. and 20 p.p. of reduction, respectively) whereas the Sp of sP22-ST and cP22-ST remained high (97% and 98%, respectively). These findings again highlight the suitability of P22-based skin tests as TB vaccine-associated diagnostic candidates, although improvements to increase the Se should be necessary.

Moreover, herd PTB status and MAP vaccination may also affect the interpretation of the results. MAP infection was not reported in farms B and C, and no recent clinical history of PTB was observed by the veterinarians. Despite this, vaccination against MAP is a common practice in small ruminants in Spain (Bastida and Juste, 2011), and diagnostic interferences due to MAP vaccination on TB diagnosis cannot be ruled out in these two MAP vaccinated herds. In this sense, strong reactions to PPD-A were observed at skin testing, but the results of comparative tests (cP22-ST and SICCT) showed higher Sp compared to their respective single skin tests (i.e., sP22-ST and SIT). These findings indicate that some degree of cross-reactivity due to MAP vaccination was still maintained. Similarly, interferences of MAP vaccination on TB diagnosis, mainly in CMI-

based diagnostic tests, were previously observed in MAP vaccinated goat (Pérez de Val et al., 2012a; Roy et al., 2018a).

Surprisingly, the P22-based IGRAs, particularly the sP22-IGRA, showed higher Se compared to STAND-IGRA, and even higher compared to DIVA-IGRA. However, the Se of sP22-IGRA was similar to that previously observed by the STAND-IGRA (92%, 95%CI: 84-96) in other studies conducted in goats (Bezos et al., 2018). The results of Se of the cP22-IGRA in the present study were also similar to those previously observed in experimentally *M. bovis*- infected red deer (Risalde et al., 2017). However, a slight loss of Sp in the P22-IGRAs was detected compared to STAND-IGRA. Even so, the Sp was within ranges (95-100%) described for STAND-IGRA in previous studies (Bezos et al., 2012a, 2012b; Roy et al., 2018a). This mild reduction in Sp could be explained by the high concentration of P22 used for stimulation of whole blood (20μg/ml), and by the fact that P22 complex contains 21 proteins also present in *M. avium* (Infantes-Lorenzo et al., 2017), which can cause cross-reactivity with MAP vaccination and/or infection. Indeed, the interference of MAP vaccination on STAND-IGRA has been previously observed in adult MAP-vaccinated goats (Chartier et al., 2012; Pérez de Val et al., 2012a; Roy et al., 2018a). The Sp of P22-IGRAs considerably decreased in BCG vaccinated herds compared to that previously described for the STAND-IGRA (Pérez de Val et al., 2016). Overall, the results of sP22-IGRA suggest that this test could be a potentially valuable tool for TB eradication in endemic areas, although further studies to determine the optimal concentration of P22 are required to improve its Sp with a minimal loss of Se.

Serological diagnostic is a cost-effective alternative for TB diagnostics. However, the Se of antibody-based diagnostic tests were generally lower compared to tests based on CMI (Bezos et al., 2014a; de la Rua-Domenech et

al., 2006). In the present study, the Se of P22-ELISA was slightly lower than in previous studies in goats and cattle (Casal et al., 2017; Roy et al., 2020b). This loss of Se might be explained by the fact that animals from herd A were not vaccinated against MAP nor subjected to frequent intradermal testing, factors that could enhance humoral responses against MTBC antigens (O'Brien et al., 2017). Interestingly, the Se was significantly enhanced when using P22 and MPB83 ELISAs in parallel. Thus, even though MPB83 is a major component of the P22 complex, specific IgG of some infected animals were only detectable using the MPB83 purified recombinant protein alone, while others were only detected using the P22 complex, which contains additional serodominant epitopes (Infantes-Lorenzo et al., 2017).

Finally, Sp of the P22-ELISA reached considerably higher Sp in MAP vaccinated (and BCG unvaccinated, i.e., herd B) goats (93%) compared to previously found in Spanish (78%) and Norwegian MAP vaccinated goats (58%) (Infantes-Lorenzo et al., 2019a). In the latter study, besides MAP vaccination, MAP coinfection and/or contact with environmental mycobacteria were not discarded as a source of diagnostic interference. Interestingly, in the present study, the Sp was also high in BCG and MAP vaccinated goats (96%), suggesting that BCG vaccination does not induce antibody responses that cause interference on the diagnosis by the P22-ELISA. The absence of antibody responses consistent with the fact that the BCG Danish strain used for vaccination express low levels of MPB83 and MPB70 (Charlet et al., 2005), which are the most abundant proteins of the P22 antigenic complex (Infantes-Lorenzo et al., 2017). Moreover, tuberculin skin testing after 42 days of MAP or BCG vaccination, caused a boosting effect on humoral responses against tuberculin antigens, resulting in false-positive cattle for an MPB83-based ELISA (Coad et al., 2013). Here, minimal or no boosting effects of MAP/BCG

vaccination due to skin testing were observed on the P22-ELISA. Indeed, goats from herd B were sampled around two years after vaccination against MAP, and ST was performed once or twice after MAP vaccination. Also, 34/68 goats from herd C were vaccinated with BCG and Gudair® at 9-10 months before the sampling, whereas the rest of the animals were vaccinated more than one year before, and no ST was performed since. Based on the results herein, the P22-ELISA seemed to be a useful ancillary diagnostic tool, either in BCG or MAP vaccination context, although it should be confirmed in further studies with larger sized herds.

In conclusion, this study reinforces the applicability of the P22 antigen complex as a complementary instrument for TB diagnostic in goats under different control scenarios. The P22 serological diagnostic is a cost-effective alternative, and combined interpretation with skin tests, either with PPD-B or P22, showed promising results. Moreover, the use of P22 antigenic complex in CMI based diagnostic tests showed encouraging results, being suitable for further research on the improvement of TB diagnostics.

CHAPTER 7

General Discussion

Animal tuberculosis is still an important issue for human and animal health, entailing important economic losses for farmers and governments, owing to the increase in mortality, reductions in milk and meat production, and costs of interventions for eradication policies (Azami and Zinsstag, 2018). With this in mind, effective TB control strategies need to be adapted to local contexts and need to be embraced by institutions and stakeholders.

In goat livestock industries from developing countries and some European regions, eradication of TB by test and slaughter of positives remains unaffordable (Ayele et al., 2004). Under this perspective, the present thesis intended to provide insights on the use of vaccination and associated diagnostics as an integrated strategy for the control of TB in goat herds.

The results of the first study (BCG LONFIELDT, Chapter 3) showed that long-term BCG vaccination of goat kids for replacement may be the best prospect in terms of cost-benefit to address the control of TB in highly infected goat farms. Although BCG protective immunity was reduced after one year of vaccination, the results of the second study (BCG REVAC, Chapter 4) showed that revaccination enhanced vaccine-induced immunity, thus paving the way for the use of BCG as a strategy for boosting immunity. On the other hand, the third study (HIMB VAC- Chapter 5) showed that a Heat-Inactivated *Mycobacterium bovis* vaccine conferred similar protection than BCG, becoming an interesting alternative for use in field conditions. Finally, the fourth study (P22 BCGDIAG, Chapter 6) addressed the vaccine-associated diagnostics by evaluating the performance of an antigen cocktail conceived as a DIVA reagent and providing insights on the usefulness of the P22 antigenic complex for TB diagnostics under BCG and paratuberculosis vaccination, as well as non-vaccination, contexts.

The results of the first study (BCG LONFIELDT) suggest that in highly TB prevalent goat herds, a long-term BCG vaccination strategy in replacement goat kids contributes to a progressive reduction of TB incidence and prevalence. Although BCG does not provide full protection against TB, BCG vaccination contributes to the containment of TB infection in the herd by reducing the development of lesions and the excretion of Mycobacteria in vaccinated animals (Ameni et al., 2018, 2010; Lopez-Valencia et al., 2010; Nugent et al., 2018b, 2017; Vidal et al., 2017). This blanket vaccination strategy would be considered as the first step enabling a decrease in prevalence, subsequently allowing the eradication of TB in highly prevalent goat herds, as it has been suggested for cattle herds and wildlife in which the eradication of TB by test and slaughter is not feasible (Buddle et al., 2018; Waters et al., 2012).

As the protection conferred by BCG is incomplete, vaccination should be considered as a part of a plan for control of TB in goat herds. Hence, the contribution of other factors to the control of TB was analyzed in the first study. For this purpose, the mathematical multilevel linear mixed effect model was useful to detect risk factors for TB infection in our particular conditions. The risk analysis detected two significant risk factors: the maintenance of unvaccinated positive animals in the herd, which were adults, and of TB positive vaccinated adult goats. The protection conferred by vaccination of adult goats seems to be lower than that of goat kids. Therefore, our findings are in line with previous observations in humans and buffaloes, where BCG vaccination of adults did not afford substantial protection (Andersen and Doherty, 2005; de Klerk et al., 2010). As a consequence, in blanket BCG vaccination programs against goat TB, adult infected individuals should be prioritized to be removed as soon as possible, even those that become infected after vaccination. Furthermore, in chronically TB infected goat herds, some adult individuals are

likely to be infected and develop anergy to tests based on CMI (skin test and IGRA). This phenomenon could be common in herds that are not subjected to frequent TB testing, because TB infection seems to progress faster in goats (Domingo et al., 2009), subsequently exhausting CMI responses. Moreover, adult individuals are more likely to have been in contact with environmental mycobacteria or are usually infected by helminths, which may cause impaired results in diagnostic tests (Cadmus et al., 2020; Claridge et al., 2012) and reductions in the efficacy of BCG (Brandt et al., 2002; Cadmus et al., 2020; Verma et al., 2020). Besides, the mathematical model showed that infected BCG vaccinated goat kids did not represent a risk for TB infection in terms of increase of incidence in vaccinated animals. Probably goat kids display a better containment of TB infection, reducing onward transmission to other goats. Consequently, according to our results, for the management of goat herds that would use BCG vaccination to control TB, the most suitable strategy would be to systematically vaccinate all goat kids entering the farm. The adult goats detected as infected at the first screening and adult goats vaccinated (TB negative at the first screening) subsequently detected as TB infected, should be prioritized for being progressively discarded, as it has been previously suggested in cattle (Ameni et al., 2010), because these animals may act as a source of TB for vaccinated animals.

The results of the first study (BCG LONFIELDT) draw attention to the fact that the BCG vaccination of goat kids is part of the program for control of TB in herds, but in itself is not sufficient. Similarly, the long-term strategy of vaccination that has been used to fight against ovine paratuberculosis in Australia stressed that not all farms responded in a similar way to MAP vaccination (Dhand et al., 2016). Therefore, besides vaccination, the compliance of other management measures, such as culling positive animals and strategies

to reduce infection of young animals, is required to reach a significant reduction of prevalence in the long term (Barkema et al., 2018). In our particular conditions, other management practices that could improve the efficacy of BCG vaccination programs were not evaluated. Future investigations of BCG vaccination in goat herds should be addressed to evaluate the impact of other control strategies, such as:

- Segregation of goat kids after birth: This practice in cattle herds is presumed to reduce the risk of transmission of pathogens from cows to calves by direct contact or through ingestion of milk or colostrum. In cattle, the most important route of TB transmission is close and persistent contact with infected individuals (Goodchild and Clifton-Hadley, 2001; Menzies and Neill, 2000). Thus, the segregation of goat kids in a separated facility may avoid persistent contact with aerosols or contaminated environments from infected adults, reducing the risk of TB early in life and before BCG vaccination. However, proper ingestion of colostrum or milk might be ensured in order to ensure the goat kids' survival.

- Pasteurization of colostrum: Different studies have demonstrated the presence of *M. bovis* in colostrum and milk samples from cattle (Ben Kahla et al., 2011; Daza Bolaños et al., 2017; Ereqat et al., 2013; Serrano-Moreno et al., 2008), being a route of transmission that need to be considered for TB control. Similarly, in goats the excretion of MTBC in colostrum could be expected and pasteurization may help to inactivate the mycobacteria, avoiding very early TB transmission to goat kids.

- Feed goat kids with milk substitute or pasteurized milk: it has been demonstrated that in a TB infected farm, feeding calves with milk

substitute for 90 days, reduced the risk of TB exposure of calves by 40% (Garro et al., 2011). However, the risk of infection due to the consumption of raw milk was 19%. These findings highlight that these practices may help to reduce the risk of TB. However, it has to be part of a set of measures for the control of TB infection in goat kids.

- Age of BCG vaccination: in the studies performed in the present thesis, all animals were vaccinated between 1 to 2 months of life and efficacy against *M. caprae* infection was observed in both field and experimental contexts. In humans, it has been demonstrated that delaying vaccination for 10 weeks after birth was related to improved immune responses (Kagina et al., 2009). However, as mentioned before, in field conditions and in high TB prevalence settings, contact with environmental mycobacteria or MTBC early in life are issues difficult to rule out. In this respect, experimental and field studies of neonatal BCG vaccination have shown that neonatal BCG vaccination was protective against *M. bovis* challenge (Ameni et al., 2010; Buddle et al., 2003b; Hope et al., 2011, 2005; Lopez-Valencia et al., 2010; Thom et al., 2012). Similarly, in humans neonatal BCG vaccination reduced the risk of TB infection (Mangtani et al., 2014), being the most used policy to fight TB in countries with high incidence. Therefore, the efficacy of neonatal BCG vaccination of goats might be explored in future studies, in order to determine the appropriate time for the first immunization.

On the other hand, in the first (BCG LONFIELDT) and the second (BCG REVAC) studies, a decrease of protective immunity of BCG was observed after one year of vaccination, in concordance with previous findings in cattle (Parlane et al., 2014; Thom et al., 2012). Particularly, in goats, according to data of the second study (BCG REVAC), immunity wanes before one year because no

protection to challenge with *M. caprae* was obtained after 64 weeks of BCG vaccination. In contrast, BCG revaccination after one year of the first BCG immunization induced bPPD specific memory cellular immune responses and was protective against *M. caprae* challenge. Therefore, these findings might have implications for the implementation of vaccination programs for the control of TB disease in goats. Although previous reports have suggested that repeated immunizations with BCG in humans did not show enhanced protection against TB infection (Ahmad et al., 2013; Fine, 1996), our observations suggest that BCG could be considered as a candidate for enhancing immunity against TB when it has waned, in concordance with recent observations in humans (Nemes et al., 2018; Whittaker et al., 2018) and cattle (Parlane et al., 2014). The second study (BCG REVAC) opens up the possibility to implement annual BCG revaccination for the improvement of potential TB vaccination schedules in goats. Besides, future studies should assess the duration of protective immunity of revaccination and the suitability of subsequent annual BCG revaccinations.

The third study (HIMB VAC) provided insights on the efficacy of the Heat-Inactivated *Mycobacterium bovis* vaccine candidate for the control of TB infection in goats. Our findings suggest that parenteral vaccination with HIMB is safe, highly immunogenic, afford protection at a similar level to BCG and does not interfere with IGRA DIVA diagnostics. The approach of inactivated vaccines could solve problems from live attenuated vaccines such as stability at room temperature, low risk of spread in non-targeted species, and return to virulence (Chambers et al., 2016). Hence, HIMB vaccine could be suitable for use in field conditions for goats, as it has been used for oral immunization of free-ranging wild boar piglets (Díez-Delgado et al., 2018), conferring protection against TB in managed populations. However, it is well known that inactivated vaccines confer a short protective immunity (Chambers et al., 2016). In this

context, one study of homologous boosting with HIMB after one month has been performed in goats, showing that the protective immunity was maintained at least 5 months after the first dose (Roy et al., 2018b) and in wild boar the same homologous regime by oral immunization was also protective (Díez-Delgado et al., 2019). Consequently, the use of the inactivated HIMB vaccine could be a suitable strategy to implement under field conditions to protect goat herds from TB infection. Besides, it would be necessary to assess the duration of the immunity of HIMB vaccine, as well as the usefulness of homologous or heterologous boosting vaccinations (or even revaccination), that could allow the maintenance of the protective immunity.

The overall findings highlight that the duration of immunity of vaccines against TB is limited and strategies to maintain adequate levels of protection need to be investigated. In goats and cattle, experimental studies have shown that boosting BCG with subunit vaccines, adenoviral vectored vaccines, and DNA vaccines have shown better protection against TB than BCG alone (Buddle et al., 2011; Dean et al., 2014; Pérez de Val et al., 2013, 2012b). However, the duration of immunity of these boosting strategies has not been yet assessed in order to establish a vaccination schedule to warrant protective immunity in the long term. Furthermore, one of the most challenging issues in veterinary vaccines is their production and distribution because of the large number of doses that need to be administered and distributed. The approach used for the conception of the HIMB vaccine, which is a *Mycobacterium bovis* isolated from a wild boar inactivated by heat and immersed in an oil adjuvant (Balseiro et al., 2017), would pave the way for the development of auto-vaccines against MTBC, reducing the dependency on the production of BCG, which has been suffered from shortages since 2013 (Harris et al., 2016), and could also reduce economic costs due to licenses or patents.

With this in mind and data obtained from studies of the present thesis, besides BCG revaccination after one year, another strategy to maintain protective immunity against TB could be a protocol based on first immunization with a live attenuated vaccine, followed by a heterologous prime-boost and/ or revaccination with a killed or inactivated vaccine. This type of vaccination strategy has been successfully tested in cattle for prevention of bovine viral diarrhea (Reber et al., 2006; Walz et al., 2017, 2015). Under this basis, potential protocols that could be tested are:

- BCG for fist parenteral immunization, and parenteral revaccination with HIMB or an adjuvanted MTBC inactivated autovaccine one year after.
- Parenteral homologous prime-boost with HIMB after one month, followed by annual revaccinations.

The main drawback of vaccination against TB, with BCG or whole-cell inactivated vaccines, is its interference with current diagnostic tests based on tuberculins. Therefore, associated vaccine diagnostic tests, allowing differentiation of infected from vaccinated animals, are necessary for the successful implementation of vaccination programs. On this basis, the present thesis provided insights on the usefulness of DIVA tests under experimental and field conditions in goats.

All the studies of the present thesis endorse the suitability of IGRA DIVA (based on ESAT-6 and CFP-10 protein cocktail - EC) for diagnostics of TB in BCG vaccinated goats, in experimental conditions and in herds with high TB prevalence, in concordance to previous studies in goats and cattle that had shown that the Se of the EC IGRA DIVA was similar to that of the IGRA based on tuberculins under field conditions (Bezos et al., 2011a; Sidders et al., 2008). Although it has been assumed that IGRA DIVA tests based on ESAT-6 and

CFP-10 had a lower sensitivity than IGRA based on tuberculins (Buddle et al., 2003a; Pollock et al., 2000), the fourth study (P22 BCGDIAG) showed a slightly lower Se of the EC-based DIVA-IGRA compared to the IGRA based on tuberculins. Taking into account that the latter is not applicable under vaccination settings, the most reasonable strategy for diagnostics would be DIVA antigens. In this context, the addition of the Rv3516c antigen for EC IGRA DIVA diagnostics would improve the Se without losing Sp in TB goat vaccination settings, as it has been demonstrated in experimental studies in BCG and MAP vaccinated goats (Bezos et al., 2015b; Pérez de Val et al., 2012a). Indeed, a IGRA DIVA test based on ESAT-6, CFP-10 and Rv3516c was useful for the follow up of TB infection in BCG vaccinated cattle under high prevalent settings (Retamal et al., 2021; Srinivasan et al., 2019). Moreover, the inclusion of these three antigens for skin test DIVA diagnostics in BCG vaccinated cattle and goat kids have demonstrated encouraging results (Bezos et al., 2015b; Srinivasan et al., 2019). In concordance, future larger field studies, under different epidemiological situations, particularly in chronically and highly TB prevalent goat herds, must address the benefits of the inclusion of Rv3516c for IGRA and skin test DIVA diagnostics in goats.

Overall, our findings support the usefulness of BCG vaccination coupled with DIVA diagnostics as a strategy for the eradication of TB in goat herds, in a similar way as it has been used for the eradication of viral diseases in animals (Pasick, 2004). However, an important concern for the implementation of blanket vaccination programs for the eradication of the TB in goats is its cost-benefit for farmers and governments. In this regard, previous simulation studies in cattle suggests that economic feasibility of BCG vaccination at a large-scale is highly dependent on DIVA diagnostics performance (Conlan et al., 2015), as well on other factors inherent to each farm that may have an impact on the

disease management (Bennett and Cooke, 2006). Accordingly, as observed in the first (BCG LONFIELDT) and the second study (REVAC), IFN-γ responses against tuberculins in BCG vaccinated animals are not detectable one year after vaccination, and taking into account that tuberculins are less expensive than DIVA reagents a dual diagnostic strategy could be considered to optimize the cost-effectiveness of the vaccination program. The dual approach would be based on the use of IGRA DIVA tests during the first year after vaccination of goat kids, and then, after one year of vaccination, use of tuberculin-based IGRA tests to detect TB infection. Moreover, another aspect influencing the cost-effectiveness of a vaccination plan is its duration in the long-term, which would depend on the prevalence to attain to implement a systematic cull of TB positive animals, economically feasible for stakeholders. Therefore, future studies need to address costs of TB vaccination programs, considering the initial TB prevalence and management situations from farms, which may enlarge or shorten the duration of the program.

The fourth study (P22 BCGDIAG) provided useful insights on the use the P22 antigenic complex for diagnostic tests based on CMI, which has not been yet extensively tested under field conditions in goats, and was indeed developed as an alternative antigen for immunodiagnostics of TB (Infantes-Lorenzo et al., 2017). With regard to the results obtained, the P22 could be used for the diagnostics in BCG vaccinated settings, potentially being a cost-effective alternative to DIVA antigens. In fact, the fourth study (P22 BCGDIAG) provided knowledge on the parallel interpretation of TB diagnostics tests using the P22 antigenic complex to maximize the Se under different epidemiological situations. In conventional epidemiological contexts, the approach of parallel interpretation of serology and tuberculin skin test has already been tested showing significant improvements of the Se (Bezos et al., 2018; Casal et al.,

2017; Machado Carneiro et al., 2021). Therefore, we applied the same diagnostic approach using serological and comparative skin tests based on P22 antigenic complex (P22-ELISA and cP22-ST) in goats with both BCG and MAP vaccination. Our results suggest that the interference of both tests with vaccination is minimal. Therefore, this strategic use of diagnostic tests, which has never been tested under BCG vaccination settings, was highly performant in terms of Se and Sp. P22 ELISA has been successfully tested in different animal species for TB diagnose (Bezos et al., 2018; Casal et al., 2017; Infantes-Lorenzo et al., 2018, 2019a, 2019b; Thomas et al., 2019a). Antibody-based testing in TB has different advantages: it allows the detection of chronically infected individuals anergic to skin tests (Casal et al., 2017; Coad et al., 2008; Waters et al., 2017), it has lower economic costs and lower demand in terms of timing for transport of samples to the laboratory. Besides, skin tests are easy to perform under field conditions and our results suggest that the cP22-ST displays a very low interference in BCG +MAP vaccinated goats after 9 to 12 months of vaccination, contrary to skin tests based on tuberculins, where interferences with BCG could extend to one year, as it has been observed in study 2 (BCG REVAC) and in concordance with previous findings in cattle (Parlane et al., 2014). Another less invasive approach, that could be useful under field conditions, is the collection of milk samples to perform the serological diagnose with P22, which displays a similar Se to serum samples (Roy et al., 2020b). Overall, the parallel interpretation of P22-ELISA from milk or serum samples and cP22-ST could be an applicable alternative for TB testing under field BCG vaccination settings, if proper DIVA tests would not be available. However, this diagnostic strategy needs to be further assessed under larger BCG vaccination programs for control of TB, and particularly the earliest after vaccination of goat kids.

On the other hand, the IGRA P22 showed high sensitivity under a MAP vaccination context, particularly using the single interpretation, being similar or even more sensitive than the IGRA with bPPD in previous field assays (Bezos et al., 2011b, 2018). However, MAP vaccination induced a slight reduction in specificity of IGRA P22, being necessary further research to stablish a P22 concentration for whole blood stimulation offering a better compromise between specificity and sensitivity of IGRA test. These results may endorse further research on the validation of the IGRA P22 for TB diagnostics, especially for highly prevalent goat herds.

The overall findings of the present thesis would contribute to pave the way for the use of vaccination and vaccine associated diagnostics for control and future eradication of TB in goat herds under different epidemiological situations.

CHAPTER 8

General Conclusions

1. BCG vaccination of replacement goat kids and TB-negative goats contributes to the yearly reduction of TB incidence in goat herds, progressively reducing the overall TB prevalence in high prevalence herds.

2. An integrative approach based on BCG long-term vaccination of replacement batches in a row, in parallel with other management measures such as test and slaughter of positive animals, could be a suitable strategy to improve the control of goat TB in high prevalence herds.

3. BCG vaccination reduces the risk of TB infection in immunized goat kids. On the contrary, the infected adults pose a risk of TB spread within the herd even the ones vaccinated in adult age and later infected. Therefore, in future vaccination programs against TB, BCG vaccination of replacement goat kids should be prioritized. The usefulness of blanket vaccination of adult goats negative to TB current diagnostic tests at the first screening should be further investigated, in view of the reduced protection of the BCG vaccine in these animals.

4. One year after BCG vaccination, specific immune responses against the vaccine waned, particularly those from memory T-cells. This decline in detectable immune responses come along with a reduction of protective efficacy to *M. caprae* experimental challenge. Our findings highlight the need of revaccination or other boosting vaccinations to maintain the protective immunity against TB in goats in the long-term.

5. The lifespan of detectable IGRA responses elicited by BCG vaccination of goats lasts less than one year. On the contrary, after one year, skin test positivity to single intradermal tuberculin test is still present in 50% of

vaccinated animals. Therefore, in BCG vaccination programs diagnostic tests should remain based on IGRA using DIVA reagents (ESAT-6/CFP-10 antigen cocktail) at least during the first 6 months after vaccination. The diagnostics by skin test using tuberculins is not recommended during the first year after BCG vaccination.

6. The revaccination with BCG of goats one year after first immunization was protective in terms of reduction of TB pathology, mycobacterial load and clinical signs after experimental challenge with *M. caprae*. Revaccination after one year could be considered as a feasible strategy to maintain protective immunity against TB infection in goat herds.

7. The immunization of goats with HIMB confers similar protection than BCG vaccination, contributing to the reduction of thoracic and extrapulmonary TB lesions and the reduction of mycobacterial load in lymph nodes. HIMB could be a more stable and environmentally safer alternative to BCG to be implemented in the field.

8. Parenteral vaccination with either BCG or HIMB does not interfere with diagnosis of goat TB using the DIVA ESAT-6/CFP-10 antigen cocktail. The results obtained in both experimental and field conditions endorse the use of these DIVA antigen cocktail as a suitable option for TB diagnosis under vaccination settings with these vaccines.

9. The use of P22 ELISA and cP22-ST in parallel is an effective strategy, in terms of sensitivity and specificity, for the diagnosis of goat TB under both BCG and MAP vaccination settings, at least after 9 months of both vaccinations. In addition, the parallel interpretation of P22 ELISA and cP22-ST could be a suitable alternative for diagnosis under vaccination settings when DIVA tests show a lack of sensitivity.

CHAPTER 9

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