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New perspectives on bovine tuberculosis diagnostics and control based on experimental infections

Memoria del trabajo realizado en el Departamento de Sanidad Animal de NEIKER- Instituto Vasco de Investigación y Desarrollo Agrario, para optar al grado de Doctor por la Universidad del País Vasco.

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PRESENTACIÓN DE LA TESIS

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LIST OF ABBREVIATIONS

16S rRNA	16S ribosomal ribonucleic acid
AFB	Acid fast bacilli
CFUs	Colony forming units
CMI	Cell-mediated immune response
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EFSA	European Food and Safety Authority
IL	Interleukin
INF-γ	Interferon-gamma
ITS	Internal Transcribed Spacer
LTBI	Latent tuberculosis infection
MAC	*Mycobacterium avium* complex
MHC	Mayor Histocompatibility Complex
MTC	*Mycobacterium tuberculosis* complex
NK	Natural killer
OIE	World Organization for Animal Health
OTF	Officially Tuberculosis Free
PAMP	Pathogen associated molecular pattern
PCR	Polymerase chain reaction
PPD	Purified Protein Derivative
PRR	Pathogen-recognition receptor
TNF-α	Tumor necrosis factor-alpha
WHO	World Health Organization

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2. OBJECTIVES

Tuberculosis is an infectious disease caused by bacteria belonging to the *Mycobacterium tuberculosis* complex. According to recent data, tuberculosis remains currently as the leading infectious disease in terms of mortality rate among humans all around the world (World Health Organization, 2018). Although bovine tuberculosis can be caused by different mycobacteria (Domingo *et al.*, 2014), it is well known that *Mycobacterium bovis* (*M. bovis*) affects the largest number of hosts. Even though bovine tuberculosis has been described most frequently in cattle (Aagaard *et al.*, 2010) due to its greatest economic relevance, other domestic ruminant (Crawshaw *et al.*, 2008; Muñoz-Mendoza *et al.*, 2015) and non-ruminant species (Parra *et al.*, 2003; Sarradell *et al.*, 2015; Rocha *et al.*, 2017) as well as feral animals can be affected too (Anderson and Trewhella, 1985; Schmitt *et al.*, 1997; Serraino *et al.*, 1999). In addition *M. bovis* is categorized as a zoonotic microorganism since infection cases in humans have also been reported (Robinson *et al.*, 1988; Cosivi *et al.*, 1998). Therefore bovine tuberculosis is considered as a major objective for Animal and Public Health with heavy implications on economy and conservation.

In vivo diagnosis of the disease relies on the intradermal tuberculin test. This diagnostic technique is based on the intradermal injection of tuberculin purified protein derivatives and their capacity to elicit a delayed hypersensitivity in infected animals. However an accurate diagnosis of bovine tuberculosis may be influenced by the antigenic similarity of *M. bovis* with other non-tuberculosis mycobacteria such as *Map* (Seva *et al.*, 2014) or environmental mycobacteria (Humblet *et al.*, 2011; Jenkins *et al.*, 2018) as well as by the use of paratuberculosis vaccines (Garrido *et al.*, 2013). The latter has as the following consequence: vaccination of cattle against paratuberculosis is not allowed in countries carrying out bovine tuberculosis eradication programs.

Nowadays vaccination is the most effective measure to control paratuberculosis. Its effectiveness has been demonstrated repeatedly by different studies which

confirmed that its use diminishes the amount of bacteria shed in feaces, reducing the infectious level of the disease (Juste *et al.*, 2009; Alonso-Hearn *et al.*, 2012; Dhand *et al.*, 2016). In addition it has been proven that paratuberculosis vaccination restricts the extension of the lesions produced by *Map* (Juste *et al.*, 1994; Muñoz, 2014) as well as the colonization of intestinal tissues (Juste *et al.*, 1994; Arrazuria, Molina, *et al.*, 2016). However, despite all the benefits associated to vaccination, its use remains restricted in cattle.**Therefore the first objective of this thesis was to assess different strategies in order to avoid the interference caused by paratuberculosis vaccines on the tuberculosis official diagnostic techniques.** For that purpose new alternative interpretation criteria as well as new more specific antigens were studied.

In relation to the first objective, since paratuberculosis and bovine tuberculosis are caused by pathogens sharing similar antigenic composition, **the second goal of this thesis was to study the effect that *Map* vaccination could have on the establishment and evolution of bovine tuberculosis.**

Eradication of bovine tuberculosis has been a major objective for Animal and Public Health for almost a century. The implementation of eradications programs during the XXth century has helped to reduce the prevalence of bovine tuberculosis remarkably. However, eradication of the disease has been only achieved by a very low number of countries and most of them still have a residual infection rate. The airborne pathway is accepted as the most frequent transmission route in cattle (J Francis, 1947), being the respiratory system the principal target (Liebana *et al.*, 2008; Domingo *et al.*, 2014). On the other hand different wildlife species contribute to the maintenance and spread of the disease. In addition indirect transmission by ingestion of contaminated food and water by cattle has been described as the most important transmission pathway between wildlife and livestock. Therefore, in order to explain the appearance of reactive animals to the official diagnostic tests but not showing any

macroscopic lesions, **possible differences in the course of infection depending on the transmission route was the third objective of this set of experimental studies.**

3. LITERATURE REVIEW

3.1. MYCOBACTERIA

3.1.1. Taxonomy

Mycobacteria belong to the Mycobacteriaceae family within the Actinomycetales order (Stackebrandt and Ward-rainey, 1997). In 1882 Robert Koch was the first scientist to isolate a mycobacteria, the causative agent of tuberculosis (Koch, 1882), and named it *Bacterium tuberculosis*. However after Lehnmann and Neumann updated the taxonomic classification a few years later (Lehmann and Neumann, 1896) the genus *Mycobacterium* and the family Mycobacteriaceae were created and the species was renamed as *Mycobacterium tuberculosis* (*M. tuberculosis*).

The Mycobacteriaceae family contains only one genus, *Mycobacterium.* In the 80s and early 90s more than 70 species belonging to the *Mycobacterium* genus were described (Skerman *et al.*, 1980; Shinnick and Good, 1994). But, thanks mostly to the progress in molecular biology, it has been possible to identify other species using different techniques so that from the beginning of the 21st century mycobacteria have experienced an outstanding expansion that led to a list of more than 170 mycobacteria species (Forbes, 2017) .

Different types of classifications for the microorganisms within the *Mycobacterium* genus have been proposed. Mycobacteria have been classified not only after their pathogenicity and genotype. A study conducted in 1959 ranked the *Mycobacterium* members based on their growth and pigmentation in the presence or absence of light (Runyon, 1959). However the most widely used classification measures the capability of the bacteria within the *Mycobacterium* genus for being cultured and defines them as cultivable or hardly cultivable species. Cultivable species can be divided into two different groups: fast- and slow-growing bacteria. Fast-growing bacteria can lead to colony formation in less than seven days whereas slow growing species need more than a week.

Most pathogenic relevant mycobacteria species belong to the slow-growing bacteria group and almost all of them are classified into two main groups: *Mycobacterium tuberculosis* complex (MTC) and *Mycobacterium avium* complex (MAC) (Figure 1).

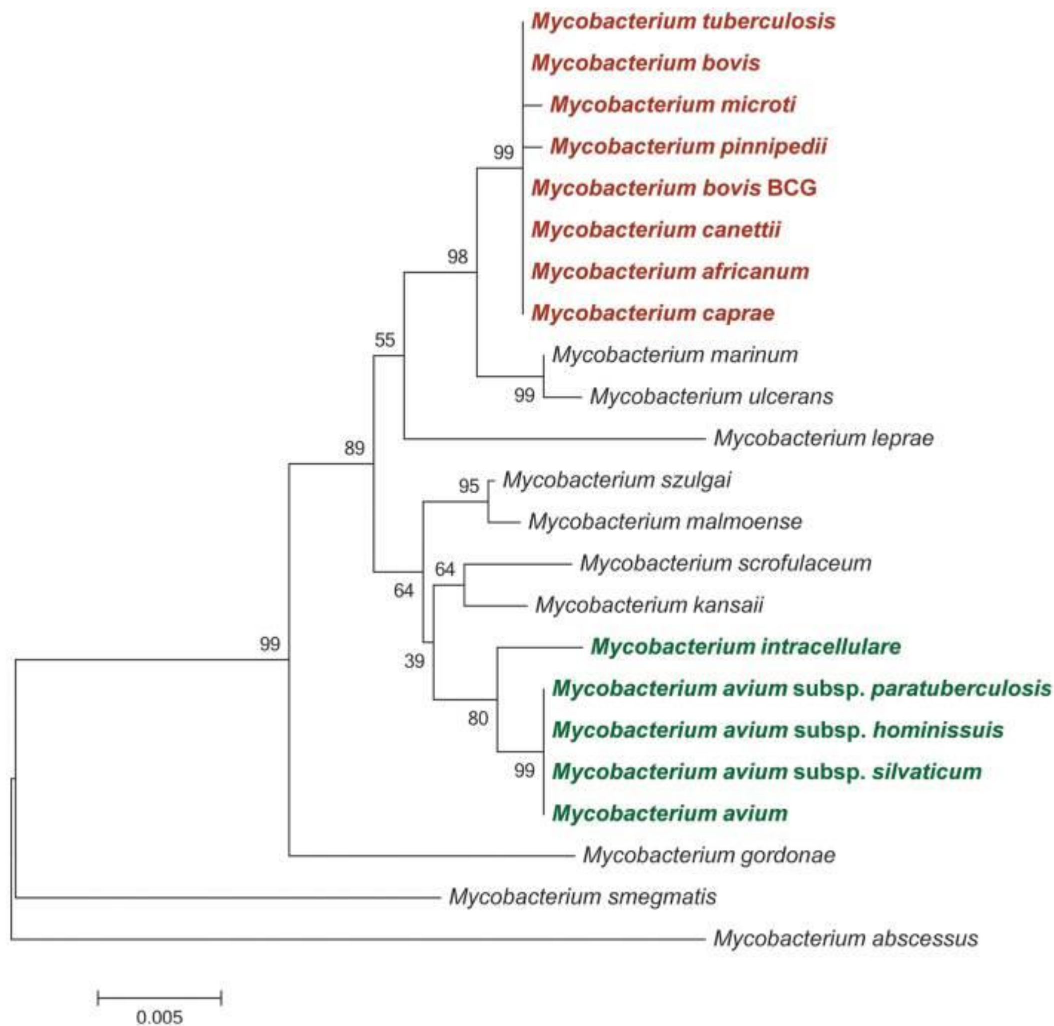

Figure 1. Phylogenetic tree of various mycobacteria species and subspecies. In red lettering: species from the *Mycobacterium tuberculosis* complex. In green lettering: species and subspecies from the *Mycobacterium avium* complex. Source: (Rue-Albrecht et al., 2014).

The MTC includes the causative agents of tuberculosis in mammals (Figure 1). *M. tuberculosis* and *Mycobacterium bovis* (*M. bovis*) are the main species causing tuberculosis in humans and animals. Mycobacteria constituting the MTC are remarkable genetically homogenous. They share more than 99.9% of their nucleotide level and 16S rRNA sequences (Boddinghaus *et al.*, 1990; Sreevatsan *et al.*, 1997),

which means that in strict taxonomic terms they should not be considered different species, but subs-species within a single species. However given that their host preferences, phenotypes and pathogenicity vary greatly, practical reasons especially regarding medical practice clarity sustain this exception (Brosch *et al.*, 2002; Rastogi and Sola, 2007; Wirth *et al.*, 2008).

M. tuberculosis is the best-known member of the *Mycobacterium* genus, affecting not only humans but also animals in contact with them. It is estimated that more than one third of the world human population is infected with the bacteria and remains in an asymptomatic state or latent tuberculosis infection (LTBI) (World Health Organization, 2017). It is also known that 5-15% of the LTBI people will end up developing the disease over the course of their lives (World Health Organization, 2017).

M. bovis has the widest MTC spectrum of host-tropism. The majority of them belong to mammal orders: from domestic and wild ruminants to rodents and insectivores, carnivores and even humans (Coleman and Cooke, 2001; Aranaz *et al.*, 2004; Aagaard *et al.*, 2010; Muñoz-Mendoza *et al.*, 2015; O'Reilly and Daborn, 2018) and is responsible for causing significant economic loses to the livestock sector with estimates of >50 million cattle infected worldwide (Waters *et al.*, 2012).

All members from the MAC are non-tuberculous bacteria. Because all of them share the same rRNA sequence, other genetic targets such as the Internal Transcribed Spacer (ITS) region are used for its classification (Turenne *et al.*, 2010). MAC uncovered the existence of a broad variety of environmental and animal-associated microorganisms with variable degrees of pathogenicity, host preference (including humans) and environmental distribution (Biet *et al.*, 2005; Rindi and Garzelli, 2014). Therefore, as the MTC, MAC acquires great relevance in veterinary (Thorel *et al.*, 2001) and human medicine (Biet *et al.*, 2005; Whiley *et al.*, 2012). *Mycobacterium avium* (*M. avium*) is the most clinically significant species for humans and animals

within the MAC and it is classified into four different subspecies (Figure 1): *Mycobacterium avium* subsp. parat*uberculosis* (*Map*), *Mycobacterium avium* subsp. *hominissuis, Mycobacterium avium* subsp. *silvaticum* and *Mycobacterium avium* subsp. *avium.*

3.1.2. Structural characteristics

Mycobacteria share common characteristics. They are aerobic (although some species manage to grow under reduced oxygen levels), non-spore-forming, non-motile, slightly curved or straight bacilli. The most remarkable feature, uniformly present and distinctive of the genus, is the lipid-rich cell envelope that confers these bacteria the property of resistance to decolorization when stained with carbol-fuchsin and decolorized with dilute hydrochloric acid. This cell envelope with high mycolic acid content confers the mycobacteria the ability to retain a pink color when stained with acid-fast stains such as Ziehl-Neelsen. As a result, the term “acid-fast bacilli” (AFB) is often associated with mycobacteria.

As mentioned above, several mycobacterial microorganisms are included within the M*ycobacterium* genus. However the present dissertation focuses only on the study of two of them that are more relevant in veterinary medicine, especially in cattle, *M. bovis* and *Map*, and on the consequences that vaccination against *Map* might trigger in the diagnosis and course of the disease after a *M. bovis* infection in the bovine host being pathogens so closely related to each other.

3.2. PARATUBERCULOSIS

Paratuberculosis is a chronic inflammatory infection of the intestine and it is caused by *Map*. There is a long subclinical period before the clinical manifestation of the infection appears upon a breakdown of the immune system. Symptoms include wasting, diarrhoea, reduced milk production and finally premature death of infected cattle causing great economic losses to the livestock sector.

3.2.1. Etiology

3.2.1.1. History and taxonomy

In the early 19th century, d'Aroval reported a type of intestinal disease with persistent diarrhea in some cattle. However its cause was unknown and it was not well described (Chiodini *et al.*, 1984). During decades it remained as just one uncharacterized disease of unknown etiology. Throughout that period the tubercle bacillus and the avian tubercle bacillus were identified and tuberculosis became a well-known disease in humans and cattle. Paratuberculosis was first described in 1895 in Germany by Johne and Frothingham. During a post-mortem examination of the intestinal tract of a cow with suspected intestinal tuberculosis, abundant AFB were found. However attempts to make the organism grow failed and its inoculation in guinea pigs did not cause tuberculosis. Thus, ruled out the tubercle bacillus as the cause of disease, the authors concluded that it should have been caused by the avian tubercle bacillus and proposed the name pseudotuberculous enteritis for the disease. A few years later, Bernard Bang differentiated this new pathology from the tuberculosis (Bang, 1906) and renamed it as paratuberculosis.

In 1912 Twort finally succeeded in isolating the causative agent and called it *Mycobacterium enteriditis chronicae pseudotuberculosis bovis johne* (Twort and Ingram, 1912). After figuring out that more species besides bovine could be affected, the agent was renamed as *Mycobacterium paratuberculosis* (Bergey, 1926) or *Mycobacterium johnei* (Francis, 1943). The actual name of *Mycobacterium avium* subsp. p*aratuberculosis* was assigned in 1990 by Marie Thorel and co-workers (Thorel *et al.*, 1990) in a revision of the group taxonomy and after Saxegaard and , (Saxegaard and Baess, 1988) had molecularly established that it was a subspecies of *M. avium.*

3.2.1.2. ***Map* general characteristics**

Map is a 1-2 µm long and 0.5 µm wide obligate intracellular bacillus. It is an aerobic, Gramm positive and nonmotile microorganism (Harris and Barletta, 2001; Manning and Collins, 2001). Like other mycobacterial species *Map* is an acid-fast bacterium and, as it shows a remarkably long average generation time (36 hours) (Elguezabal *et al.*, 2011), it belongs to the slow-growing mycobacteria group (Grange *et al.*, 1990). The high amount of time required for *Map's* colonies formation together with its inability to grow in absence of exogenous mycobactin are useful features for its characterization (Chiodini *et al.*, 1984). *Map* cannot produce mycobactin due to the *mbtA* gene deletion of its genome, responsible for encoding the first enzyme of the mycobactin biosynthesis pathway (Li *et al.*, 2005), thus it cannot grow on media were iron is not easily available like those based on egg. On the other hand *Map* mycobactin independent isolates have been reported in experiments using laboratory adapted strains (Morrison, 1965; Merkal and Curran, 1974; Gunnarsson and Fodstad, 1979). However it has been suggested that the mycobactin carried-over from previous cultures in medium supplemented with mycobactin could be responsible for these isolates (Lambrecht and Collins, 1992). Nevertheless lack of mycobactin dependence on primary isolates of non-iron-enriched media has been described too. However it seems very rare since it has only been described in a goat (Gunnarsson and Fodstad, 1979) and in a second experiment carried out with ovine samples (Adúriz *et al.*, 1995). Another characteristic which makes it easy to distinguish *Map* from other mycobacterial species is its possession of the insertion element IS900 (Collins *et al.*, 1989). IS900 consists of 1451 base pairs of which 66% are guanine + cytosine. There are 15–20 copies of the entire insertion element within the complete *Map*'s genome (Green *et al.*, 1989). Both discoveries took place in 1989 and provided the first definitive, non-subjective methods for the identification of *Map* and also led to the improvement of the diagnosis. However similar insertion elements or IS900-like sequences were

discovered in other mycobacteria (Cousins *et al.*, 1999; Englund *et al.*, 2002). A possible misdiagnosis of the disease led to the search of *Map* more specific sequences which could be single or multi copy-targets. Single copy-sequences such as the paratuberculosis specific elements: F57 (Poupart *et al.*, 1993), locus 251 (Rajeev *et al.*, 2005), or hspX (Ellingson *et al.*, 2005) are very interesting for quantification assays. Sequences with more than one copy like the ISMav2 (Strommenger *et al.*, 2001), ISMap04 or ISMap02 elements increase the chance to detect the pathogen.

Map cell envelope has structural common features with other mycobacteria. Same pathogen associated molecular patterns (PAMPs) of the cell envelope can be found in different species. The mannose capped lipoarabinomannan PAMP is expressed by *M. bovis*, *M. tuberculosis*, *Mycobacterium BCG* and *Mycobacterium leprae (M. leprae*) too (Prinzis *et al.*, 1993; Murray *et al.*, 2007). Because antigen preparations used in the paratuberculosis diagnostic techniques do not necessarily react exclusively to *Map,* causing false-positive and false-negative results, studies have been carried out to identify M*ap*-specific PAMPs. Recently a *Map*-specific cell surface lipopeptide (lipopeptide IIβ, 3) exhibiting high antibody binding activity in serum from Map infected cattle has been reported (Mitachi *et al.*, 2016). This could lead to an improvement of *Map* diagnostic accuracy.

3.2.2. Epidemiology

3.2.2.1. Host range

Due to *Map* economic impact on the livestock sector the study of paratuberculosis has being mainly focused on domestic ruminants: cattle, sheep and goats (Chiodini *et al.*, 1984) but in recent years the variety of hosts has been proven to be much broader. *Map* has been found in wild ruminants as: red deer (Nebbia *et al.*, 2000), roe deer (Robino *et al.*, 2003), fallow deer (Balseiro *et al.*, 2008), white tailed deer (Chiodini and Van, 1983), camels (Kramsky *et al.*, 2000) or alpacas (Miller *et al.*,

2000). Infection with *Map* has been also described in many wildlife and domestic monogastric species such as: rabbits (Arrazuria, Juste, *et al.*, 2016), horses (Cline *et al.*, 1991), donkeys (Dierckins *et al.*, 1990), dogs (Miller *et al.*, 2017), cats (Kukanich *et al.*, 2013), badgers (Beard *et al.*, 2001), wild boars (Álvarez *et al.*, 2005), hares (Salgado *et al.*, 2011), foxes (Beard *et al.*, 2001), wolves (Beard *et al.*, 2001), coyotes (Anderson *et al.*, 2007) as well as in different bird (Beard *et al.*, 2001; Daniels *et al.*, 2003) and rodent species (Daniels *et al.*, 2003).

3.2.2.2. Transmission

Map is primarily transmitted through the faecal-oral route by ingestion of food or water contaminated with mycobacteria present in the faeces of infected animals. Most of *Map* infections take place during the first days of life of the neonatal calves and are often associated with the fact that the young calves nurse on teats which have been contaminated with faeces of shedding animals (National Research Council, 2003; Barkema *et al.*, 2009). However *Map* can be transmitted vertically during pregnancy and lactation too, as it has been isolated from the uterus (Pearson and McClelland, 1955; Whittington and Windsor, 2009), fetal tissues (Lawrence, 1956), colostrum (Streeter *et al.*, 1995) and milk from clinical (Stabel *et al.*, 2014) and subclinically infected cows (Sweeney *et al.*, 1992; Streeter *et al.*, 1995). Furthermore it has been demonstrated that the amount of *Map* shed into milk and colostrum can be influenced by the clinical stages of infection and the different days of milk, being the advanced stages of the disease and the early lactation days the highest shedding periods (Stabel *et al.*, 2014).

It is widely accepted that resistance against *Map* infection increases with age (Taylor, 1953; Larsen *et al.*, 1975; Windsor and Whittington, 2010). *Map* bacilli target the small intestine where they are taken up by M cells and enterocytes, and subsequently engulfed by submucosal macrophages (Sigurethardottir *et al.*, 2004;

Ponnusamy *et al.*, 2012). Small intestine in neonatal calves is covered by organized lymphoid tissue. As animals get older, this lymphoid tissue starts to retract into small areas called Peyer's Patches reducing the chance of infection. Therefore, unless massive and repeated doses of *Map* are ingested, this represents a relatively low risk for adult cows but could be an important risk to younger replacement stock.

3.2.2.3. Distribution, prevalence and economic costs

Paratuberculosis is widely distributed around the world among dairy cattle (Nielsen and Toft, 2009; Corbett *et al.*, 2018). However a proper estimation of its prevalence is not easy to achievedue to different factors: 1) use of different diagnostic techniques makes results difficult to compare, 2) low sensitivity of these techniques for detection of subclinical infected animals causes false negative results and 3) early culling of animals showing clinical signs hinders a reliable final confirmation. Nevertheless a recent study, based on data from 48 countries all around the world, estimates that the global paratuberculosis prevalence is high (Whittington *et al.*, 2019). Results suggest that 20% of the herds and flocks in about half the countries participating in this assay were infected, with prevalence rates around 40% in some developed countries (Whittington *et al.*, 2019). Other studies estimate that the herd-level prevalence of *Map* infection is likely to be >30% (Donat *et al.*, 2014; Corbett *et al.*, 2018) or even >50% in most of the countries with a significant dairy industry (Barkema *et al.*, 2010).

Paratuberculosis causes great economic losses to the livestock sector as a result of: reduced milk production and slaughter values, increased premature and forced culling, reduced fertility and increased mortality rate as well as increased susceptibility to other diseases (Bakker *et al.*, 2000; Whittington and Sergeant, 2001). The exact economic costs of *Map* infection are difficult to estimate due to the absence of accurate prevalence data (National Research Council, 2003). In fact estimations of the net

economic impact of paratuberculosis on the U.S. dairy industry varies from US$ 200 to US$ 1500 million annually depending on the study (Ott *et al.*, 1999; Harris and Barletta, 2001). A more recent publication studied the financial impact of paratuberculosis on Scottish dairy farms (Shrestha *et al.*, 2018). The estimated results obtained after applying their chosen model match with earlier studies. Each Scottish farm affected by the disease suffers an estimate average loss of £31,940 annually which is similar to the estimate of £34,679 loss per infected farm per year published in a Dutch study (Groenendaal *et al.*, 2003). In addition the same Scottish study estimated the economic loss per cow as £185 on an paratuberculosis infected farm which exceeds the £112 estimated per cow in the United Kingdom (Stott *et al.*, 2005) but lies within the estimated range per cow in the United States (£46 to £192) (Ott *et al.*, 1999).

In addition the economic impact seems to be of increasing concern due to the apparent increase of the global prevalence, associated economic losses as well as the potential consequences for trade (Rideout *et al.*, 2003).

3.2.3. Diagnosis

Treatment against paratuberculosis in cattle is economically impracticable; therefore diagnostic techniques should be rapid and accurate enough to identify all affected animals regardless of their infectious status. Unfortunately none of the currently available techniques meets all the requirements individually. Current diagnostic tests, such as faecal culture test, faecal PCR and enzyme-linked immunosorbent assay (ELISA) show high sensitivity detecting animals shedding high levels of *Map*, but lower sensitivities detecting animals shedding low levels of *Map* (Whitlock *et al.*, 2000, 2007; Collins *et al.*, 2006).

Currently, the most commonly *in vivo* diagnostic techniques detecting the adaptive cellular or humoral immune response developed by the host in response to *Map* infection are the interferon-gamma (IFN-γ) release test and the serum antibody

indirect ELISA. The IFN-γ release test is the most widely in vitro immunological technique used to measure the cell type immune response. The estimated sensitivity for subclinical and clinical infected animals detection varies between 13% and 85% respectively and the specificity lies around 88% and 94% (Nielsen and Toft, 2008). The humoral immune response is generally measured with the serum ELISA. The sensitivity of the technique shows extreme values ranged between 17 and 87% in infected and infectious animals respectively (Nielsen and Toft, 2008). The specificity of the serum approach of the technique ranges between 40-100% whereas if milk is used the achieved values are higher (83%-100%) (Nielsen and Toft, 2008).

The most widely used *in vivo* techniques for the detection of the bacteria are faecal culture and PCR. Isolation of *Map* is nowadays the reference technique which also allows strain differentiation for epidemiologic purposes. However, there are two major disadvantages this technique has to face: 1) confirmation of negative results can take up to 20 weeks due to the slow growing nature of the agent and 2) isolated colonies require molecular confirmation. On the other hand detection of *Map* DNA in faecal samples by PCR has a much faster turnaround time than culture. Results can be obtained within 24 hours and this technique allows the detection of very small amounts of the etiological agent showing a sensitivity and specificity similar to the faecal culture (Collins *et al.*, 2006). However intermittent *Map* shedding in faeces during the subclinical phase of the infection affects the sensitivity of both diagnostic methods.

As already discussed in previous sections most of *Map* PCR detection tests are based on the detection and amplification of the IS900 sequence. This DNA insertion sequence has multiple copies per bacteria (15-20 copies). However it is not found exclusively in *Map,* it has been found in other environmental mycobacteria as well (Cousins *et al.*, 1999; Englund *et al.*, 2002). The main disadvantage of PCR is that detection of the microorganism does not necessarily represent infection risk because the cells might not be viable. In addition weak positive results in herds heavily infected

with *Map* should be interpreted carefully because they could be obtained due to the "pass through" of organisms recently ingested instead of proceeding from infected intestinal tissue.

Election of a diagnostic method will depend on whether the diagnostic efforts are aimed at detecting infection at individual- or at herd–level as well as on the available economic budget. In a recent study carried out by Whittington and co-workers diagnostic tests used by 22 countries within their control programs have been recorded (Whittington *et al.*, 2019) (Table 1).

Table 1. Types of diagnostic tests used in paratuberculosis control programs between 2012 and 2018 according to different livestock species. Data are the number of countries among the 22 countries with control programs, sorted by frequency of test. Adapted from source:(Whittington *et al.*, 2019)

Test	Cattle - dairy	Cattle - beef	Sheep	Goats	Camelids	Deer - farmed	Other	Not used
Serum ELISA	17	17	9	10	1	3	-	3
Faecal PCR - individual	18	17	13	12	6	9	3	4
Faecal culture – individual	13	13	9	9	6	9	3	5
Pathology	16	15	13	14	8	11	3	6

However, testing for *Map* is of very little value if no management changes within the herd or poor commitment to act on the test results are carried out.

3.2.4. Control

Paratuberculosis is really tough to control and even harder to eradicate because of a prolonged incubation period, during which animals can spread the agent without exhibiting signs of illness, poor sensitive diagnostic tests and prolonged survival of the organism in the environment (Kennedy and Benedictus, 2001).

Vaccination has proven to diminish the amount of bacteria shed and therefore reducing the infectious level of the disease (Juste *et al.*, 2009; Alonso-Hearn *et al.*, 2012). Even though current vaccines do not prevent new infections completely (Juste

et al., 1994; Windsor, 2006), vaccination has proven to diminish the amount of bacteria shed and therefore reducing the infectious level of the disease (Juste *et al.*, 2009; Alonso-Hearn *et al.*, 2012). Thus vaccination is an excellent strategy when no interference with diagnostic in official bovine tuberculosis programs is feared like in sheep, goats and camelids. Nevertheless in cattle paratuberculosis vaccination is not allowed and therefore control of the disease has to be primarily based on preventing new infections through cutting down the transmission rate by timely detection and culling of infectious animals. An objective such as eradication of the disease is really difficult to achieve and it is often impractical regarding the objectives of commercial farms. In contrast, preventing and reducing its spread and therefore reducing *Map* prevalence is a practical and more feasible objective for most types of farm operation (Rossiter and Burhans, 1996; Collins *et al.*, 2010).

For negative herds, the goal is to maintain their *Map* free status. In order to achieve it rigorous biosecurity measures must be carried out and herds must grow from within avoiding introduction from animals from other herds.

In infected herds, efforts to decrease the within-herd paratuberculosis prevalence require different fundamental approaches: prevent exposure of susceptible animals to the infectious agent (taking special care of the neonatal calves), identify and eliminate *Map*-infected animals from the herd, impede the entry of infected animals into the herd and improve resistance to *Map*. To achieve the overall aim, control of paratuberculosis is currently based on good management practices, identification and elimination of affected animals and vaccination. However over the last years and increasingly interest over the genetic susceptibility or resistance against *Map* infection in cattle has appeared. The use of genetic tests as a tool to select animals more resistant to *Map* infection may become very useful to control the disease in a near future (Juste *et al.*, 2018)

3.2.4.1. Good management practices

It is widely accepted and simulation studies have pointed out too (Groenendaal *et al.*, 2002; Kudahl *et al.*, 2007) that hygienic measures related to calf management are very important for achieving control of the disease since most new infections occur during the neonatal period.

Good management practices include: 1) avoiding contact of faeces with the animals (especially with the calves), food and water (Goodger *et al.*, 1996); 2) separating the calving area from the rest of the farm facilities (Kalis *et al.*, 2001); 3) detaching the newborns from their dams as soon as possible and restricting contact between them as well as with other adult animals by keeping them in separate facilities and pastures for at least the first 6 months of life (Goodger *et al.*, 1996); or, according to more recent studies, for the first 12 months of life (Windsor and Whittington, 2010); 4) isolating animals presenting clinical signs (Muskens *et al.*, 2003) and finally, 5) introducing only infection-free new animals and, if possible, coming from paratuberculosis-free farms.

Implementation of good management practices represents a critical point for achieving control of the disease. However if no other additional measure is taken, such as test and cull of affected animals, long periods of time should be required before any positive result can be observed. This fact is responsible for many farmers to lose interest in the control of the disease and so they quit the programs. Two of the major challenges in controlling the disease farmers must be aware of before introducing the chosen control measures are that: 1) control is a slow process; they have to stay motivated for years and not give up because diagnosis is hindered by low-sensitivity tests, and 2) effective control implies strong commitment and constant application of the chosen measures.

3.2.4.2. Test and cull strategy

Test and cull is one of the most frequently used tools for achieving control of paratuberculosis. This type of measure is applied in control programs in order to remove infected animals from the herd, especially those excreting high loads of bacteria since they are supposed to be the most likely source of transmission (Dorshorst *et al.*, 2006). This identification and elimination measure aims to eradicate the disease from the entire herd (Kalis *et al.*, 2001). The most widely diagnostic test used has been the faecal culture. However, due to its long incubation period and high economic cost, serological- (Kalis *et al.*, 2002) and molecular techniques (Sevilla *et al.*, 2014) have been introduced to identify infected animals.

Control of the disease following a test and cull strategy is slow and often quite disappointing since positive animals continue to appear over the years even after periods of negative results and absence of clinical cases (Bastida and Juste, 2011).

Nevertheless extensive test and cull strategies used alone have resulted to be ineffective and costly for producers (Groenendaal and Wolf, 2008). In addition eradication of the disease has been pointed out as very unlikely to be gained by just following this strategy (Smith *et al.*, 2015). In order to achieve optimal results rapid and high sensitive and specific diagnostic techniques (Whitlock *et al.*, 2000; Sevilla *et al.*, 2014) as well as a combination of hygienic measures are required.

3.2.4.3. Vaccination

Vaccination has been used since 1926 to achieve control of the disease (Vallee and Rinjard, 1926). The types of vaccines used have included live attenuated strains of *Map* and whole-cell inactivated preparations. The first were replaced by the latter due to biosecurity reasons (possible reactivation of the antigen) and a poor stability over time (Aduriz *et al.*, 2000). However whole-cell inactivated vaccines are not allowed in

cattle in many countries because of the interference produced by the vaccine with the official diagnostic techniques used for detecting *M. bovis*.

On the other hand vaccination in small ruminants not subjected to tuberculosis eradication programs has been carried out for years (Sigurdarson and Gunnarsson, 1983). It is estimated that millions of vaccine doses are currently used annually. However not many studies have been carried out except for some performed in Spain, Cyprus, United Kingdom, New Zealand, Norway and Australia (Juste and Perez, 2011). In sheep, clinical expression of the disease and level of shedding by infected animals have been greatly reduced after vaccination in countries like Australia (Windsor, 2012, 2015), Iceland (Fridriksdottir *et al.*, 2000) or Spain (Aduriz *et al.*, 1991; Juste and Perez, 2011). In addition, in Iceland vaccination of sheep in infected area is compulsory since 1966 which has contributed to reduce significantly the economic impact of the disease (Sigurdarson and Gunnarsson, 1983). In goats, although vaccination has been carried out for years in different countries (Sigurdarson and Gunnarsson, 1983; Saxegaard and Fodstad, 1985; Corpa *et al.*, 1994; Juste and Perez, 2011), relevant publications are even scarcer. However in recent years there has been an increase in the use of the vaccine against *Map* in Spain as a control measure in goats (Lozano de Arcenegui *et al.*, 2012).

Nowadays there are three vaccines approved for sale: Silirum® (CZ Veterinaria, Porriño, Spain), Mycopar® (Boehringer Ingelheim Vetmedica) and Gudair® (CZ Veterinaria, Porriño, Spain). However their use is not approved worldwide. Bovines are the target animals for Silirum® and Mycopar® whereas Gudair® use is approved for sheep and goats. All three belong to the whole-cell inactivated type vaccines which have proven to enhance both cellular and humoral immune response in cattle (Stabel *et al.*, 2011; Muñoz, 2014), goats (Hines *et al.*, 2014) and sheep (Begg and Griffin, 2005).

Although it is well-known that vaccines do not fully prevent new infections in experimental (Juste *et al.*, 1994; Muñoz *et al.*, 2005; Muñoz, 2014) and field assays (Körmendy, 1994; Windsor, 2006), vaccination has proven to be a very useful tool for achieving control of the disease. Several studies have concluded that vaccines reduce the infectious level of the disease by diminishing the shedding load (Körmendy, 1994; Harris and Barletta, 2001; Reddacliff *et al.*, 2006; Juste *et al.*, 2009; Alonso-Hearn *et al.*, 2012; Tewari *et al.*, 2014; Dhand *et al.*, 2016) and it may also reduce the cumulative incidence of clinical cases over time. In addition vaccination limits the extent of the typical diffuse paratuberculosis lesions (Juste *et al.*, 1994; Sweeney *et al.*, 2009; Muñoz, 2014) as well as the colonization of intestinal tissues by *Map* (Juste *et al.*, 1994; Uzonna *et al.*, 2003; Sweeney *et al.*, 2009; Alonso-Hearn *et al.*, 2012; Muñoz, 2014; Arrazuria, Molina, *et al.*, 2016) .

The main drawbacks of vaccination against *Map* are: 1) prevention of new infections is not fully accomplished, 2) risk of nodule formation at the injection point, 3) accidental self-inoculation of the vaccine by operators can cause painful lesions and 4) there is some interference with the immune diagnostic techniques used for tuberculosis and paratuberculosis. *Map* vaccination leads to a strong cellular and humoral immune response impossible to be differentiated from the one triggered after a *Map* infection using the current immune diagnostic techniques (Tewari *et al.*, 2014). On the other hand vaccination against *Map* is not allowed in cattle because of the interference it may cause with the bovine tuberculosis diagnostic tests, identifying healthy animals as reactors (Garrido *et al.*, 2013).

Almost all *Map* vaccines are suspended in a mineral oil adjuvant (Bastida and Juste, 2011) to cause a higher and more persistent immune response. Oil acts as an irritant and, because it is non-absorbable, increases the antigen persistence eliciting a longer immune response (Hope, 1995). However the subcutaneous nodule formation

seems to be related with these oil-based adjuvants (Halgaard, 1984; Köhler *et al.*, 2001).

The economic impact of paratuberculosis, the limited effectiveness of all the vaccines used so far, both live and inactivated, the adverse effects produced by them as well as the interference in the diagnosis of paratuberculosis and tuberculosis, are aspects that have favored scientific research to find new vaccine products. One alternative could be the subunit vaccines which are still under study and evaluation. This type of vaccine aims to avoid interference in tuberculosis and paratuberculosis diagnostic tests with animals vaccinated against *Map* since they use *Map* specific proteins (Rosseels and Huygen, 2008). Different proteins have been identified as possible candidates for its use as subunit vaccines: Hsp70 (Koets *et al.*, 1999), lipoproteins (J. F. J. Huntley *et al.*, 2005), proteins of the 85 antigen complex (Shin *et al.*, 2005), proteins of the PPE family (Nagata *et al.*, 2005), the superoxide dismutase enzyme (Shin *et al.*, 2005) and the alkyl hydroperoxide reductase (Olsen *et al.*, 2000) However these types of vaccines have shown a much lower degree of protection (Koets *et al.*, 2006; Kathaperumal *et al.*, 2009).

DNA vaccines, consisting of the inoculation of mammalian expression vectors containing *Map* genes have been used in different species including mice, humans and sheep (Velaz-faircloth *et al.*, 1999; J. F. Huntley *et al.*, 2005; Sechi *et al.*, 2006; Park *et al.*, 2008; Roupie *et al.*, 2008). After its inoculation, an increase in both cellular and humoral responses has been observed (Bull *et al.*, 2007), however no real protection studies have been carried out and therefore more experimental studies are needed in order to become a practical alternative to the classic vaccines.

Despite all the drawbacks noted above, according to Bastida and Juste conclusions vaccination is one of the best paratuberculosis control strategies currently available (Bastida and Juste, 2011).

3.2.4.4. Control programs

Paratuberculosis is categorized as a notifiable disease by the OIE, therefore all countries members of the organization (almost all countries worldwide) have the obligation of reporting their incidence data. However, unlike for other notifiable diseases, such as tuberculosis (OIE, 2018a), there are no guidance measures designed by the OIE to address paratuberculosis (OIE, 2018b). This lack of guidance seems to entitle each country to decide individually which measures to apply regarding the control of the disease.

Although an increasing number of control programs have being initiated since the 1990's, success has been limited (Kennedy and Benedictus, 2001; Barkema *et al.*, 2018). Implementation remains mostly voluntary and because infected animals can take several years to present clinical signs, most farmers take action only when the disease becomes obvious and economically relevant for the cow productivity and could imply a significant impact on their farms economic viability. In addition programs vary from small, independent plans targeting limited production systems within a defined region to national programs taking into account all cattle bred in a whole country (Nielsen, 2009). Therefore control measures are not homogeneously applied at an international or national level, and hence results do not reflect the actual magnitude of the disease.

As already mentioned above, control programs for paratuberculosis have been implemented in the last decades, particularly in developed countries. However, reviews of their activities and results are scarce and focus mostly on cattle (Nielsen, 2009; Bakker, 2010; Whitlock, 2010; Geraghty *et al.*, 2014) . Nevertheless there is a more recent study which has focused on gathering information from 48 different countries (Figure 2) in order to assess the existence and nature of *Map* control programs during a six-year period (2012-2018) (Whittington *et al.*, 2019). Although the disease was notifiable in most countries, not only for cattle but also for the seven groups of ruminants assessed in the survey (Table 2), formal control programs were present in

only 22 countries (Figure 3). In most countries *Map* control programs continue to be voluntary (60%) and they are generally supported by incentives for joining or financial compensations (Whittington *et al.*, 2019).

Table 2. Notifiability of paratuberculosis in each type of ruminant in 48 countries. Source: (Whittington *et al.*, 2019).

Species/type	Number of countries			% countries in which species is applicable and paratuberculosis is notifiable
	Notifiable	Not notifiable	Not applicable	
Dairy cattle	35	13		72.9
Beef cattle	33	12	3	73.3
Sheep	28	16	4	63.6
Goats	28	16	4	63.6
Camelis	12	16	20	42.9
Deer – farmed	15	15	18	50.0
Other	10	11	27	47.6

Figure 2. The 48 countries represented in the study. *Source:* (Whittington *et al.*, 2019).

Figure 3. The 22 countries represented in the study that had a control program for paratuberculosis between 2012 and 2018. Source (Whittington *et al.*, 2019).

Map control programs can have different aims. According to Whittington and co-workers, prevalence reduction was the major objective in most of the 22 countries with control programs (n=17 → 77.3%), followed by reduction of the incidence of clinical cases (n=10 → 45.5%) and improvement of the consumer safety by reducing *Map* contamination in human food (n=7 →31.8%). Only two countries, Norway and Sweden, aimed to eradicate the disease. To achieve their main goals 19 of the 22 countries with control programs culled the clinical cases and 16 used the test and cull strategy in subclinical cases. Hygienic measures for rearing the neonates and juveniles were implemented in their control programs by 17 countries and biosecurity practices at farm level were also used by 17 countries to prevent introduction of the infection (Whittington *et al.*, 2019). Vaccination as a tool to control the disease has been used by 7 countries during this six-year survey (2012-2018) (Whittington *et al.*, 2019). In addition vaccination of sheep in Iceland is mandatory since 1966 (Sigurdarson and Gunnarsson, 1983), where it is believed that without it paratuberulosis would be more widespread.

Control programs from 16 (73%) of the 22 countries taken part in the survey of Whittington and co-workers were reported to be successful (Whittington *et al.*, 2019). However, recommendations for future control programs included the primary goal of

establishing an international code for paratuberculosis, which would lead to universal acknowledgment of the principles and methods of control in relation to endemic and transboundary disease. An holistic approach across all ruminant livestock industries and long-term commitment will be also required for achieving control over paratuberculosis (Whittington *et al.*, 2019).

3.3. TUBERCULOSIS

3.3.1. Journey from the past to the present

Although the etiological agent of the human tuberculosis was not successfully isolated until 1882 by Robert Koch (Koch, 1882), mycobacteria are supposed to have been around for millions of years (Hayman, 1984; Bates and Stead, 1993). This extremely vast period of time has allowed them to adapt themselves to almost any environment on Earth (ground, water, faeces, air…) and survive in all of them for long periods. Their worldwide distribution as well as their omnipresence during the history of humankind is sustained by paleomicrobiology (Donoghue *et al.*, 2004; Drancourt and Raoult, 2005). The first weak evidence of tuberculosis in humans appears on the clinical descriptions of lesions compatible with bone tuberculosis found in a 500,000-year-old *Homo erectus* skull in Turkey (Kappelman *et al.*, 2008). Nevertheless, to this day, the oldest irrefutable proof of *M. tuberculosis* in humans goes back to the Neolithic period, 9,000 years ago. The remains belong to an infant and a woman from one of the first villages with evidence of agriculture and animal domestication located in the Eastern Mediterranean. In this case, human tuberculosis was confirmed by morphological and molecular methods (Hershkovitz *et al.*, 2008). However, the oldest evidence to date of MTC has been found in an animal, a 17,000-year-old bison in Wyoming (USA) (Rothschild *et al.*, 2001).

As man began to settle down, agriculture evolved (about 7,000 BC) and domestication of cattle followed. These conditions would have been favorable for direct

transmission between humans and animals but very unlikely between families isolated from each other. Indeed tuberculosis must have been of little importance until then, since it received almost no attention in written records or artifacts. The establishment of larger and crowded cities led to an increased contact between inhabitants favoring the transmission of the disease.

The existence of tuberculosis has been documented by different civilizations throughout the human history. In Egypt tuberculosis can be traced back over 5,000 years. Vertebral lesions characteristic of Pott's disease (spinal tuberculosis) were found in Egyptian mummies and were clearly depicted in paintings and statues (Cave, 1939; Zimmerman, 1979). More recently, the cause of the disease could be confirmed thanks to the amplification of *M. tuberculosis* DNA extracted from the Egyptian human remains (Nerlich *et al.*, 1997; Crubézy *et al.*, 1998).

The first bibliographical reference of a clinical tuberculosis sign was included in the Code of Hammurabi from the Babylonian Empyre (about 2,000 BC). Other written documents connected to tuberculosis are related to the Hebraism. The ancient Hebrew word schachepheth was used in the Old Testament in order to describe tuberculosis (Daniel and Daniel, 1999).

At the other edge of the world, Peruvian mummies provided archeological evidence of early tuberculosis, including Pott's deformities, suggesting that the disease was already present in South America before the first European explorers arrived. And, as in Egypt, *M.tuberculosis* DNA was recovered from the remains (Salo *et al.*, 1994; Arriaza *et al.*, 1995).

The symptoms of the disease as well as the characteristic tubercular lung lesions were not accurately defined until the Ancient Greek period (800 - 500BC) by Hippocrates. Tuberculosis was called then Phitsis and it was described as a fatal disease especially for young adults (Coar, 1982).

During the 18th and 19th centuries tuberculosis in Europe was also known as the "white plague". Favored by overcrowded cities, *M. tuberculosis* is believed to have been responsible for causing 25% of all adults' deaths during this period. Until the 19th century tuberculosis was considered to be congenital, inherited or spontaneous. The transmission of the disease to a healthy person due to a patient's cough was unbelievable until 1865 when Jean Antoine Villemin, a French surgeon, proved that the disease could be transmissible between humans and animals. The rabbits used for his experiments developed extensive tuberculosis after being inoculated with purulent liquid from a tuberculous lesion from an individual who died of tuberculosis (Villemin, 1868). In 1882, Robert Koch published "Die Ätiologie der Tuberkulöse". There he described the bacillus of tuberculosis and the infectious nature of the disease, but always denied the zoonotic capability of the bacillus (Koch, 1882). Subsequently, the acid-alcohol resistance characteristic of the bacterium was described (Ziehl, 1882; Neelsen, 1883) followed by the Pirquet and Mantoux tuberculin skin test discoveries (Huebner *et al.*, 1993; Lee and Holzman, 2002). In addition, Albert Calmette and Camille Guérin (BCG) vaccine was developed (Calmette *et al.*, 1926) and Selman Waksman streptomycin and other anti-tuberculous drugs were described (Schatz *et al.*, 1942).

From the moment Lehmann and Neumann introduced the genus *Mycobacterium* into the scientific literature in 1896 a very interesting and decisive fact took place. The study of the different mycobacteria species was shaped by the fact that only a small number from the nearly 170 currently recognized species are source of human disease, being *M. tuberculosis* on top of all of them. As a result, studies of microbial physiology, structure, genetics and diagnostic tools have been mainly focused on *M. tuberculosis* and secondarily on *M. leprae* (van Ingen, 2017). In addition the medical community was reluctant to accept tuberculosis as a zoonosis. That would have meant that Koch was mistaken denying the zoonotic nature of the disease.

It is true that *M. bovis* is not the most relevant pathogen responsible for human tuberculosis but the non-recognition of the disease as a zoonosis led to insufficient measures at the farm level and in the slaughterhouses. *M. bovis* affected carcasses were confiscated but there was no restrictive policy for the animal products such as milk or cheese or for the farm of origin (Agenjo Cecilia, 1942). Furthermore, although the World Organization for Animal Health (OIE) was created in 1924 and information about bovine tuberculosis (including both bovine and avian tuberculosis) had been published in 1927 in the *Bulletin of the Office International des Epizooties*, it was not until 1964 that the bovine tuberculosis was included among the OIE-listed diseases. Moreover in spite of the general acceptance of *M. bovis* as different from *M. tuberculosis*, it was not until 1970 that was officially recognized as a new species and *M. bovis* was proposed as the name for the bovine tubercle bacillus (Karlson and Lessel, 1970).

3.3.2. Etiology: *Mycobacterium bovis*

As already mentioned *M. bovis* belongs to the MTC which includes the causative agents of tuberculosis in mammals. Species within the MTC are the following: *M. tuberculosis, M. bovis*, *Mycobacterium bovis BCG*, *Mycobacterium microti* (*M. microti*), *Mycobacterium pinnipedii*, *Mycobacterium canettii*, *Mycobacterium africanum* (*M. africanum*) *and Mycobacterium caprae* (*M. caprae*). However in this dissertation only *M. bovis* and its relationship with *Map* are going to be assessed.

Over the years, different hypotheses about the taxonomic development of the mycobacteria species have been formulated. *M. bovis* has a much broader host range than *M. tuberculosis* and initially it was suggested that *M. tuberculosis* evolved from *M. bovis* by specific adaptation of an animal pathogen to the human host (Stead *et al.*, 1995). It was thought that being *M. tuberculosis* almost an exclusive human pathogen it should have evolved from *M. bovis*, able of inducing tuberculosis in a much wider

range of hosts. However this hypothesis was turned down after the sequencing of the entire *M. tuberculosis* genome (Cole *et al.*, 1998) and the discovery of 20 variable genomic regions in MTC members (Brosch *et al.*, 1999; Gordon *et al.*, 1999). In 2002 Brosh *et al.* analyzed the distribution of these 20 variable regions in 100 strains from the MTC and confirmed that the *M. bovis* genome has suffered several deletions in relation to the *M. tuberculosis* (Brosch *et al.*, 2002). These results match other publications where the outcomes indicate that the *M. bovis* genome is smaller than that of the *M. tuberculosis* (Gordon *et al.*, 2001; Garnier *et al.*, 2003).

In a study conducted in 2001, DNA extraction and identification were carried out on the oldest animal remains with compatible tuberculosis lesions (Rothschild *et al.*, 2001). Remains belonged to a 17.000 year old bison and the obtained results demonstrated that the DNA belonged to the MTC but at the same time eliminated the possibility of matching with modern *M. bovis*. Indeed the outcomes showed higher spoligotype similarity patterns with *M. africanum* followed by *M. tuberculosis* (Rothschild *et al.*, 2001). Nowadays, the hypothesis of the pathogen transmission to animals from a previously adapted human strain is the most supported, considering *M. africanum* as the origin of the current animal lineages instead of *M. tuberculosis* (Smith *et al.*, 2009).

3.3.3. Epidemiology *M. bovis*

3.3.3.1. Host range

Bovine tuberculosis can be triggered by different mycobacteria (Domingo *et al.*, 2014). However the pathogen affecting the highest number of hosts is *M. bovis*. Disease caused by this microorganism has been described most frequently in cattle (Aagaard *et al.*, 2010) due to its greatest economic relevance throughout the world. Nevertheless it is also well known that *M. bovis* affects other domestic members from the Bovidae family like goats (Crawshaw *et al.*, 2008) and sheep (Muñoz-Mendoza *et*

al., 2015). In addition it has been demonstrated that other non-ruminant domestic species such as cats (Aranaz *et al.*, 1996), dogs (Rocha *et al.*, 2017), pigs (Parra *et al.*, 2003) and horses (Sarradell *et al.*, 2015) might be susceptible to *M. bovis* and to clinical disease development. A large list of wildlife hosts for *M. bovis* does exist too. Infection has been confirmed in different feral animals: deers (Schmitt *et al.*, 1997), badgers (Anderson and Trewhella, 1985), wild boars (Serraino *et al.*, 1999), hares (Coleman and Cooke, 2001), possums (Buddle *et al.*, 1994)… *M. bovis* infection cases in humans have also been reported (Robinson *et al.*, 1988; Cosivi *et al.*, 1998). Person-to-person transmission of *M. bovis* has been proven too and not only among immune-deficient patients (Evans *et al.*, 2007), but also among immune-competent persons (Sunder *et al.*, 2009). However intra-specific transmission in humans is considered rather unlikely (Berg and Smith, 2014).

3.3.3.2. Transmission

Different transmission routes for *M. bovis* have been described. However there is a general acceptance that the airborne route is the most effective transmission pathway because of the low number of organisms required as an infective dose (J Francis, 1947). When sneezing, an infected host generates aerosols containing *M. bovis* which can be inhaled by uninfected animals resulting in infection. *M. bovis* transmission via inhalation is effective in herding animals like cattle (Pollock and Neill, 2002) or wild ruminants such as cervid species kept confined (De Lisle *et al.*, 2001). Inhalation of the pathogen can end up in infection too in free-ranging feral species maintaining social or familial interactions in underground dens, such as badgers (Delahay *et al.*, 2002) or brushtail possums (Jackson *et al.*, 1995).

Although respiratory transmission is the most important route of infection of groups of animals in close contact, oral transmission through ingestion of *M. bovis* is also considered of great importance. Transmission can occur by direct or indirect

ingestion of the pathogen. In calves direct consumption of contaminated food takes place. Intraspecific transmission of *M. bovis* occurs when contaminated milk from infected dams is ingested by their offspring (S. D. Neill *et al.*, 1994). Because *M. bovis* is shed through faeces, urine or nasal secretions and due to its high resistance in the environment, the alimentary route by indirect consumption of contaminated water or pastures is of great importance too (Domingo *et al.*, 2014). In humans the most common route of infection of *M. bovis* is through the oral route by consumption of contaminated milk or other dairy products (Acha and Szyfres, 1987). Thus, milk pasteurization plays a crucial role in preventing human infection (Collins, 2006). Another less common form of infection is through transcutaneous transmission. This has been proven in humans in contact with infected carcasses where infection was spread through cuts or abrasions (Vayr *et al.*, 2018). In animals, transcutaneous transmission is primarily caused by bites from infected animals. This has been reported in domestic cats (Ragg and Moller, 2000), ferrets (Ragg and Moller, 2000), and badgers (Gavier-Widen *et al.*, 2001).

Most likely routes for *M. bovis* transmission have been already determined. However the minimum infective dose necessary to trigger the disease in experimental assays as well as the dose for wild animals and cattle naturally infected is difficult to quantify. An experiment carried out in 2004 concluded that ingestion of as few as $5x10^3$ colony forming units (CFUs) of *M. bovis* resulted in infected calves and lesion development (Palmer *et al.*, 2004), whereas $1.3x10^6$ and $1x10^7$ CFUs of *M. bovis* administered orally was needed to establish infections in cattle and sheep in others studies (Sigurdsson, 1945; J Francis, 1947). In Eurasian badger the minimum infective dose was estimated as <10 CFUs through the endobronchial route for latent infection and $1x10^{3-4}$ CFUs for generalized infection (Corner *et al.*, 2007, 2012).

3.3.3.3. Bovine tuberculosis in humans: a One Health issue

M. bovis is a recognized Public Health hazard in developing and industrial nations (Grange and Yates, 1994). The advent of milk pasteurization and eradication programs in developed countries reduced greatly the prevalence of tuberculosis due to *M. bovis*. Still few new infections occur when individuals get in contact with infected cattle (Pfeiffer and Corner, 2014), captive wildlife (Stetter *et al.*, 1995), or contaminated animal carcasses (Pfeiffer and Corner, 2014). Meanwhile the risk for zoonotic tuberculosis increases in rural areas of developing countries where *M. bovis* pasteurization is not an established control measure and people live in conditions that favour direct contact with infected animals.

Since it is categorized as a zoonotic organism, cooperation between Animal and Human Health is required in order to achieve control over the disease. This movement is known as One Health (OH). The World Health Organization (WHO) and OIE support the one world OH concept, which constitutes a collaborative and holistic approach between both organisms on a global scale.

Different authors concluded that disease control programs for *M. bovis* in humans should be considered a priority and called for evaluation of the level of the zoonotic problem, especially in rural areas and in workplaces like slaughterhouses (Cosivi *et al.*, 1998; Müller *et al.*, 2013). Obstacles against the acceptance of the benefits from an OH approach are mostly economic together with a lack of accurate and representative data especially from developing regions. Historically, *M. bovis* cases in humans have been often reported as a relative proportion of the total number of tuberculosis cases in persons, diminishing its impact on society. Lack of accurate and representative *M. bovis* data in developing regions, incorrect extrapolation of data from high-income countries and low *M. bovis* tuberculosis burden countries have led to

the misconception that globally only a small number of humans suffer from tuberculosis caused by *M. bovis* (Olea-Popelka *et al.*, 2017).

However, according to recent data published by the WHO, it has been estimated that 142,000 new human cases of zoonotic tuberculosis occurred worldwide in 2017 and 12,500 persons died due to the disease (Table 3) (World Health Organization, 2018). This burden of disease cannot be reduced without improving standards of food safety and controlling *M. bovis* in animals and humans.

Table 3. Estimated incidence and mortality due to *M. bovis* in 2017. Best estimates (absolute numbers) are followed by the lower and upper bounds of the 95% uncertainty interval. Source: (World Health Organization, 2018).

Region	Incident cases		Deaths	
	Best estimate	**Uncertainty interval**	**Best estimate**	**Uncertainty interval**
Africa	70,000	18,800 - 154,000	9,270	2,450 - 20,500
The Americas	821	222- 1,800	45	12 - 98
Eastern Mediterranean	7,660	1,980 - 17,100	733	194 - 1620
Europe	1,150	308 – 2,550	87	24 - 191
South-East Asia	44,900	11,500 - 100,000	2,090	568 - 4,590
Western Pacific	18,000	4,740 - 40,000	309	84 - 678
GLOBAL	142,000	70,600 - 239,000	12,500	4,910 - 23,700

3.3.3.4. Bovine tuberculosis in cattle, prevalence.

Despite the efforts to eradicate *M. bovis,* the presence of bovine tuberculosis in cattle still has a worldwide distribution and remains as a major livestock problem.

In Europe, according to the Zoonoses Directive 2003/99/EC, monitoring data about the bovine tuberculosis herd prevalence have to be gathered by the member states and reported to the European Food and Safety Authority (EFSA) annually. In order for a member state to be declared officially tuberculosis free (OTF) the following requirements described in the Council Directive 64/432/EEC have to be met: 1) the percentage of infected bovine tuberculosis cattle herds should not be higher than 0.1%

for 6 years in a row and 2) at least 99.9% of the OTB bovine herds must maintain that status for at least 6 years in a row.

According to the latest annual report from the EFSA the current situation in Europe on bovine tuberculosis infection, detection and control is heterogeneous (EFSA and ECDC, 2018). In 2017 the following 22 countries and regions were categorized as OTF: Austria, Belgium, the Czech Republic, Denmark, Estonia, Finland, France, Germany, Hungary, seven regions and 14 provinces in Italy, Latvia, Lithuania, Luxembourg, Malta, the Netherlands, all administrative regions within the superior administrative unit of the Algarve in Portugal, Poland, Slovakia, Slovenia, the Canary Islands and Pontevedra in Spain, Sweden, Scotland and the Isle of Man in the United Kingdom (EFSA and ECDC, 2018). On the other hand 10 member states (Bulgaria, Croatia, Cyprus, Greece, Ireland, Italy, Portugal, Romania, Spain and the United Kingdom) had not yet achieved the country-level OTF status in 2017 (EFSA and ECDC, 2018).

According to the EFSA, bovine tuberculosis was reported by 16 member states in 2017. In spite of the heterogeneous distribution the overall proportion of positive cattle herds remained very low: only 0.9% herds in all OTF and non-OTF regions from the European Union were positive for bovine tuberculosis (EFSA and ECDC, 2018). The prevalence varies from the absence of infected animals in most OTF regions to 13.5% within the United Kingdom in the non-OTF region England-Wales. The total number of positive cattle herds reported in non-OTF regions increased by 8% compared to 2016, and the prevalence of bovine tuberculosis-positive cattle herds also increased from 1.6% in 2016 to 1.8% in 2017 (Figure 4) (EFSA and ECDC, 2018).

As for the OTF regions, during 2017 detection of bovine tuberculosis-infected herds remained a rare event, as in the previous years, but compared to 2016, two more

member states, Malta and Portugal detected bovine tuberculosis infection in their OTF regions (Figure 4).

Figure 4. Proportion of cattle herds positive for bovine tuberculosis according to regional boundaries of official status. Source: (EFSA and ECDC, 2018).

According to the European Union policy on the eradication of bovine tuberculosis, eradication of the disease must be the final target and the member states are primarily responsible for achieving this purpose (Reviriego Gordejo and Vermeersch, 2006) .

In Spain, the fight against bovine tuberculosis was initiated at the beginning of the 50's. However it was not until 1987, after becoming part of the European Union, that Spain presented an Accelerated Eradication Program according to the following Directives 77/391/CEE y 78/52/CEE and 87/58/CEE. This program, based on a test and cull strategy, yielded highly satisfactory results during its early years. The annual National Bovine Tuberculosis Eradication Programs were established in 2006. One of the objectives of these programs was to gradually increase the diagnostic sensitivity

not only at herd but also at individual level. Other additional measures such as management of possible wild reservoirs and the establishment of monitoring systems in slaughterhouses were introduced gradually. As a result of these new measures prevalence at herd level got reduced, but over the last decade it has remained at around 1.5-2% (MAGRAMA, 2019).

Nevertheless, as already mentioned, in Spain there are great tuberculosis prevalence differences among geographical areas. The highest prevalence rates can be found in the southeast of the country (Andalusia= 12.34%, Castilla la Mancha= 10.35%) (MAGRAMA, 2019). Meanwhile the Canary Islands (MAGRAMA, 2019) and Pontevedra (according to the Executive Directive (UE) 2019/64 of the Commission of January 14th 2019) achieved the OTF status. In the Balearic Islands the prevalence dropped till 0.00% in 2016. In the peninsula the lowest herd prevalence is found in the northern regions. Data obtained during the latest eradication campaign showed that in Galicia, Asturias and the Basque Country only 0.08%, 0.02% and 0.09% of the herds were positive for tuberculosis respectively (MAGRAMA, 2019).

Over the last 15 years the trend of the herd level prevalence has showed a moderate but continuous decrease in Spain until 2013, after which an increase was detected, especially in 2015 and 2016 (MAGRAMA, 2019).

3.3.4. Pathogenesis

3.3.4.1. Entry and survival of *M.bovis* in the host

In accordance with an EFSA evaluation, nowadays *M. bovis* risk of transmission through milk or dairy products is considered insignificant due to pasteurization at least in developed countries. The same for tuberculosis transmitted by meat to humans (EFSA BIOHAZ Panel (EFSA Panel on Biologicial Hazards), 2013).

The location of the lesions largely depends on the route of infection. Inhalation is the most commonly accepted pathway of infection. After an airborne transmission,

lesions are more likely to appear in the nasopharynx and lower respiratory tract, including the lungs and associated lymph nodes (S. D. Neill *et al.*, 1994; Neill *et al.*, 2001). Adult cattle typically show lesions associated to the respiratory tract. In contrast, if the pathogen enters the organism via ingestion of contaminated pasture, water or feed, development of a gastrointestinal form of the disease is more likely. In calves bovine tuberculosis is usually transmitted by ingestion; and lesions tend to affect the mesenteric lymph nodes (Menzies and Neill, 2000) and the intestinal wall.

Once *M. bovis* goes through mucous membranes or into alveolar spaces, recognition of the bacterial cell wall components and activation of inflammatory pathways in phagocytes begins. Afterwards the pathogen is phagocytized by macrophages and neutrophils are attracted to the site of the initial infection. These cells interact with other cells responsible for the innate and adaptive immune responses (Arentz, 2008). Unlike in humans (O'Garra *et al.*, 2013), in cattle it is unknown if latent infections or even the elimination of mycobacteria after a primary infection occur. It has been hypothesized that some animals reactors to the skin test may be latently infected even if no tuberculous lesion is found or no *M. bovis* positive culture result is obtained (Pollock and Neill, 2002) but no definitive conclusion on latency has been established.

3.3.4.2. Macroscopic pathology of bovine tuberculosis

The primary lesion is often found within the dorsal area of one of the lung lobes. After the initial infection has been established, phagocytic cells transport the viable mycobacteria to the draining lymph node through lymphatic capillary vessels. A new infection point is then developed in the lymph nodes. The presence of both: primary pulmonary lesion and lymph node lesion, is known as the primary or Ghon complex, which was already described many years ago (Ghon, 1912). This primary complex can be subclassified as complete or incomplete depending on whether both lesions are

present or the lesion at the infection establishment point is missing (Domingo *et al.*, 2014).

The bovine tuberculosis typical macroscopic lesion is described as a tubercle: a delimited yellowish granulomatous inflammatory nodule of variable size more or less encapsulated by connective tissue which often contains a central core of necrotic tissue with different degrees of mineralization (Aranday-Cortes *et al.*, 2013; Domingo *et al.*, 2014).

The primary lesion generally progresses towards an encapsulated and mineralized lesion. The existence of a potent cell-mediated immune (CMI) response may prevent the growth of lesions and extension to other organs. However, if the infected animal is immunocompromised, or the immune response is unable to stop the spread of the infection, the primary infection may generalize during the initial stages, in a process known as 'early generalization'. Generalization via haematogenous or lymphatic dissemination can also occur after reinfection or in a post-primary phase, therefore called 'late generalization' (Domingo *et al.*, 2014).

The primary lesion can progress and generalize, inducing a 'miliary' form with abundant nodules of small size throughout the lung and pleura (early generalization). The lesion can grow, showing different forms depending on the development and involvement of adjacent tissues, including: (1) an 'acinar' form showing numerous small yellowish nodules affecting primary pulmonary lobules; (2) a 'cavernous' form when the bronchial lumen is dilated due to the accumulation of caseum coming from the lesion or when the caseum breaks out into a bronchus; and (3) an 'ulcerative' form in the trachea and bronchi when bacilli infect small erosions within the airway epithelium (Salguero, 2018).

Tubercle appearance is not only limited to the respiratory tract and adjacent lymph nodes they can also be found in the head and neck area (Aranday-Cortes *et al.*,

2013; Salguero *et al.*, 2017; Ameni *et al.*, 2018) as well as in extra-thoracic or gastrointestinal lymph nodes and other abdominal organs.

3.3.4.3. Microscopic pathology of bovine tuberculosis

The granuloma is the microscopic lesion observed in tuberculosis affected individuals. It is a characteristic morphological lesion present in chronic inflammatory reactions with abundant epithelial-like macrophages (Palmer *et al.*, 2015). Granulomas have been described as a physical barrier to impede the mycobacterial growth and spread (Aranday-Cortes *et al.*, 2013). Apart from the macrophages different kind of cells can be observed within the granuloma: lymphocytes, plasma cells, neutrophils and Langhan's multinucleated giant cells which can be found surrounding the caseous necrotic core.

After a mycobacterial infection, cytokines and chemokines are in charge for recruiting monocytes, lymphocytes, neutrophils and tissue-resident macrophages (Mattila *et al.*, 2013) in an attempt to control the infection, forming cellular aggregates (Aranday-Cortes *et al.*, 2013).

Microscopic features of tuberculous granulomas in lymph nodes have been described exhaustively and are used to classify these injuries according to morphological criteria such as the presence or absence of necrosis, mineralisation, and fibrous capsules (Wangoo *et al.*, 2005). This classification system can likely be applied to lung granulomas (Domingo *et al.*, 2014). Four types of granulomas are described. Early lesions, categorized as stage I ('initial'): small granulomas are formed by an accumulation of neutrophils, epithelioid macrophages, a small number of lymphocytes and a few Langhan's multinucleated giant cells. Necrosis is absent in stage I granulomas (Wangoo *et al.*, 2005). The lesion will progress to stage II ('solid'). Granulomas at this stage are primarily composed of epithelioid macrophages and are confined partly or completely by a thin capsule. Hemorrhages can be found, with

infiltration of lymphocytes, neutrophils and often Langhan's multinucleated giant cells. Sometimes minimal necrotic areas can be present, generally composed of necrotic inflammatory cells (Wangoo *et al.*, 2005). The lesion will start to form a central area of caseous necrosis and progress to stage III ('necrotic'). Stage III granulomas are fully encapsulated, with a central caseous necrotic area and occasionally minimal mineralization. Epithelioid macrophages, Langhan's multinucleated giant cells and a peripheral zone of macrophages with clusters of lymphocytes and scattered neutrophils surround the necrotic area (Wangoo *et al.*, 2005). In stage IV ('necrotic and mineralized') large, irregular, multicentric granulomas are completely surrounded by a relatively thick capsule of fibrous tissue. Areas of caseous necrosis with extensive islands of mineralization are seen. Epithelioid macrophages and multinucleated giant cells surround the necrosis with dense clusters of lymphocytes near the peripheral fibrotic capsule (Wangoo *et al.*, 2005). Stage IV granulomas can be multicentric, with several granulomas coalescing to form one very large granuloma, displaying multiple necrotic cores. Large stage IV granulomas are often surrounded by a small amount of 'satellite' stage I and stage II granulomas (Aranday-Cortes *et al.*, 2013).

3.3.4.4. Immune response

Immunity against mycobacteria is a multifactorial process which depends on the balance between an inflammatory (Th1) and an anti-inflammatory (Th2) response. The first one allows the host to develop a granuloma, which contains the microorganism and hinders its spread. And the later, the anti-inflammatory response, restricts the extent of the granuloma and allows the contact between the effectors T-cells and the infected cells resulting in the killing of the infecting pathogen (Villareal-Ramos *et al.*, 2003).

The pathological tissue alteration after a MTBC infection is the result of the host's immune response against a tuberculous infection (Ulrichs and Kaufmann, 2006)

in an attempt to isolate the pathogen and impede the development of the disease. The interactions between the mycobacteria and the different cellular targets present in the host's respiratory mucosa determine the outcome of pulmonary tuberculosis. Both the innate and the adaptive immune response play a crucial role. Experimental infection assays with *M. bovis* in cattle has provided insight into important aspects of the consequent immune response development. Being *M. bovis* an intracellular pathogen, the most effective type of immune response to fight the microorganism is the CMI response, which shows its greatest intensity in the early stages of the disease (Figure 5) (Pollock and Neill, 2002; Schiller *et al.*, 2010). In fact protection against *M. bovis* is directly related to the Th1 type CMI response (Villareal-Ramos *et al.*, 2003) and the response intensity is measured by the quantity of interferon-gamma (IFN-γ) produced by the lymphocytes.

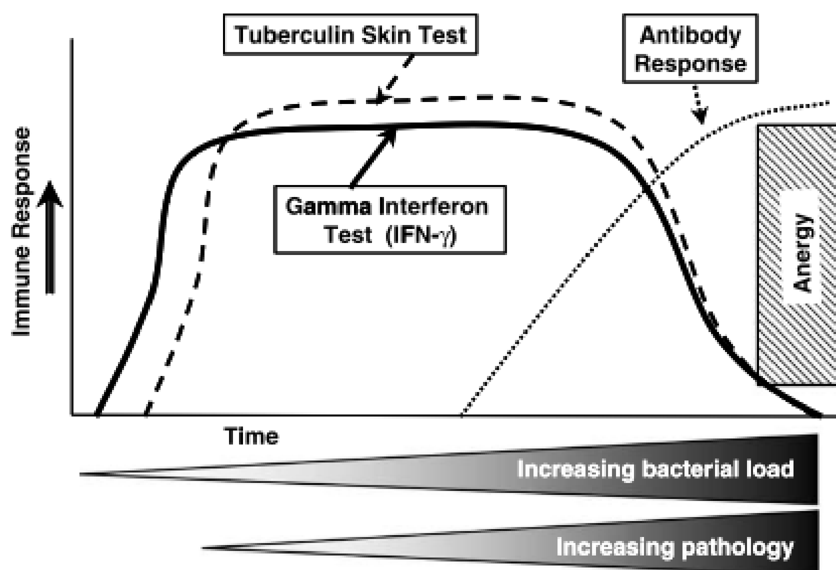

Figure 5. Evolution of the immune response through time. *Source:* (adapted from (Vordermeier *et al.*, 2004)

Antibodies are also produced after an *M. bovis* infection. However the humoral response begins to acquire greater intensity and relevance in advanced stages of infection, when the CMI response is decreasing (Figure 5). The relative importance of the humoral response in terms of protection against infection is explained by the fact

that *M. bovis* is an intracellular pathogen and therefore it is considered to be safe from the action of antibodies since the serum components are thought not to get access to the pathogen. Until recently B lymphocytes have largely been considered to be supportive, rather than required in the protective response (Maglione and Cahn, 2009). Currently this concept is changing and the importance of B cells and antibodies in the development of a protective immune response against tuberculosis has become more appealing. Although CMI remains the predominant correlate of protection, evidence suggests that antibodies may contribute, at least in part, to immunity. Studies have shown that monoclonal antibodies against surface antigens of *M. tuberculosis* give rise to protective immunity in mice and prolong their survival after infection with lethal doses of *M. tuberculosis* or *M. bovis* (Chambers *et al.*, 2004). In addition, recent evidence suggests that B cells may be more important than first thought. A large number of studies have assessed the humoral response after an infection by measuring antibodies for diagnostic purposes, particularly in humans, to differentiate latent from active tuberculosis (Chan *et al.*, 2014). However due to its greater relevance, all vaccine models tested as an alternative for controlling the disease in cattle are mainly designed to stimulate the CMI response.

3.3.4.4.1. Innate Immune Response

The host innate immune response provides the initial resistance to infections produced by intracellular pathogens before the adaptive Th1 CMI response fully develops.

The following major cellular components are included in the innate immune response: macrophages, dendritic cells (DCs) and natural killer (NK) cells. When mycobacteria trigger an infection, the innate immune system is able to recognize the bacilli thanks to the pathogen-recognition receptors (PRRs). These PRRs are responsible for recognizing conserved structures in the pathogens known as pathogen-

associated molecular patterns (PAMPs). This identification allows the activation of intracellular signaling and the production of pro-inflammatory molecules such as IFN-γ or interleukins (IL) 1β, IL-12 or IL-18. There are different types of PRRs for *M. bovis* recognition: Toll-like receptors (TLRs), mannose-binding receptor 3, complement receptor, nucleotide-binding oligomerization domain among others. However the TLRs are the PRRs most thoroughly studied in the innate immune response against tuberculosis. The TLRs allow the macrophage activation as well as the production of pro-inflammatory mediators and oxygen and nitrogen–reactive intermediates that hinder the growth of the bacteria (Carrisoza-Urbina *et al.*, 2018).

- **Macrophages:**

Macrophages are antigen presenting cells. They are considered as the first line of defense against various intracellular pathogens. Infected macrophages produce IL-12, a pro-inflammatory cytokine which plays a crucial role in host-defense against intracellular pathogens (D'Andrea *et al.*, 1992). This is one of the first host responses against infection and it induces IFN-γ production by NK cells (D'Andrea *et al.*, 1992).

There are different known factors responsible for the macrophage activation such as tumor necrosis factor-alpha (TNF-α) and IFN-γ produced by CD8+ and CD4+ lymphocytes as well as NK cells (Nathan *et al.*, 1983). This early activation mediated by IFN-γ induces a strong innate effect that converts the macrophages into potent killing cells towards intracellular pathogens and initiates differentiation of Th1 cells (Manetti *et al.*, 1993). As a result macrophages produce high amounts of reactive oxygen intermediates, reactive nitrogen intermediates and pro-inflammatory cytokines that induce cytoxicity against the invading pathogens and generate innate immune responses. In addition activated macrophages increase their antigen presentation capabilities,

initiating the adaptive immune response (Martinez *et al.*, 2008). Very interestingly, these cells seem to significantly increase their lytic activity efficiency after systemic initial contact of an individual with *M. bovis* antigens (Juste *et al.*, 2016)

- **Dendritic cells:**

Dendritic cells are the most potent antigen presenting cells and they are able to activate different cells of the immune system such as NK cells, T γδ cells and naive T lymphocytes (Pearce and Everts, 2015). Like macrophages, dendritic cells use different PRRs for bacterial recognition. After the intake of the pathogen, antigens are processed and expressed by the major histocompatibility complex (MHC) type II. This way the DCs present the intruder microorganism to the T lymphocytes in order for them to recognize and destroy it.

Mycobacteria interactions with DCs increase the expression of surface molecules from the latter, such as MHC type II and the co-stimulatory molecules CD40 and CD80 (Hope *et al.*, 2004; Pearce and Everts, 2015). This action leads to T cell activation in an attempt to eliminate the bacterial invader. In addition, the DCs cytokine profile gets altered after a mycobacterial infection too. An infection produced by *M. tuberculosis* or *M. bovis* BCG has been associated with a higher expression of pro-inflammatory molecules such as: IL-12, TNF-α, IL-1 and IL-6, which are essential in the control of tuberculosis. These cytokines play major roles in protective anti-mycobacterial immune responses. IL-12 secreted by DCs is able to trigger a reciprocal action. It can stimulate IFN-γ and TNF-α secretion by T cells, which, at the same time, may serve to boost up the macrophage anti-microbial activity to destroy invading

bacilli. Taken this together it suggests that infected DCs have an augmented capacity to stimulate mycobacteria reactive T cells.

On the other hand mycobacterial interaction with DCs may lead to the production of IL-10, an anti-inflammatory cytokine. This molecule may inhibit the cellular immune response by donwregulating the IL-12 secretion (Giacomini *et al.*, 2001; Hickman *et al.*, 2002) since it affects the antigen presentation process by inhibiting the migration and maturation of DCs (Hope *et al.*, 2004; Fabrik *et al.*, 2013). This kind of response may serve to limit the activation of DCs and macrophages and therefore control the potentially harmful immune response that occurs in tissues *in vivo*.

- **Natural Killer cells**

NK cells are large granular lymphocytes with diverse functions that include cytotoxicity and cytokine production and interact with antigen presenting cells to eliminate damaged and infected cells (Bastos *et al.*, 2008; Boysen and Storset, 2009). NK cells together with macrophages and DCs are responsible for restricting infections in early stages and then influence adaptive immune responses against pathogen invasion, so that B and T lymphocytes eliminate the stranger microorganisms. Studies have proven that particularly DCs are crucial for efficient NK cell responses (Lucas *et al.*, 2007) in order to acquire full capacity for cytolysis or cytokine secretion. Mature DCs migrate or are resident in secondary lymphoid tissues, a major site of NK-cell activation during innate immune responses (Ferlazzo *et al.*, 2004; Lucas *et al.*, 2007).

As innate effectors NK cells contain preformed granules of lytic proteins including perforin and granulysin which are released upon recognition of target cells (Davis and Dustin, 2004; Chauveau *et al.*, 2010). These granule components can directly kill extracellular bacilli and are able to substantially

reduce the viability of intracellular mycobacteria (Stenger *et al.*, 1998). Once activated, NK cells contribute to the development of the immune response mainly via cytotoxicity and cytokine production (Cooper *et al.*, 2001; Boysen *et al.*, 2006).

In addition to the interactions between NK cells and antigen presenting cells described above, NK cells with memory functions have been described recently (Sun *et al.*, 2011; Peng and Tian, 2017). Therefore NK cells may be a bridge between the innate and adaptive immune systems with bidirectional interactions influencing not only the innate response but also the adaptive immune response (Siddiqui *et al.*, 2012).

Evidences suggest that NK cells play a main role in the immune response against mycobacterial infection. Olsen et al. observed that after stimulation with mycobacterial antigens, NK cells from cattle were able to significantly increase their IFN-γ production. In addition they figured out that the IFN-γ increase was dependent upon IL-12 released by antigen presenting cells (Olsen *et al.*, 2005). Another experiment carried out in dairy cattle demonstrated that bovine NK cells could induce significant reductions of the *M. bovis* replication in macrophages and that this was dependent of two factors: the action of IL-12 and cognate signals delivered through cell contact (Denis *et al.*, 2007). In the same study significant increases of IL-12 were detected which may boost additional NK cell activation and facilitate the amplification of Th1 mediated immune responses. This would lead to an increased capacity of bovine NK cells to control *M. bovis* growth.

3.3.4.4.2. Adaptive Immunity

Unlike the innate mechanisms, the adaptive immune response requires the specific recognition of antigens. Both are very important and interact in different ways.

Being the first to react the innate immune system has a great impact on the development of adaptive immune mechanisms. In addition, the adaptive immunity is able to activate components of the innate immune system after executing several of its functions.

Adaptive immunity can be divided into CMI and humoral responses. The first one includes T-lymphocytes activation and effector mechanisms, and the latter involves B-lymphocytes maturation and antibody production. Both responses are not independent: their mechanisms are related to each other. T lymphocytes are required for antibody maturation, isotype switching and memory. B lymphocytes also work as antigen presenting cells by activating T lymphocytes.

As for other intracellular infections, the primary protective immune response against MTC infections is cell mediated rather than antibody mediated. *M. bovis* resides inside the macrophage and is relatively resistant to mechanisms that efficiently eliminate other phagocytosed bacteria. This is due in part to the ability of the bacilli to hinder macrophage activation by IFN-γ and IL-12. In addition, deficiencies in IL-12 or IFN-γ, or their receptors, make the individual more susceptible to mycobacterial infections (Jouanguy *et al.*, 1999; Alcaïs *et al.*, 2005).

- **Cellular immune response**

The adaptive immunity, fundamentally the CMI response, plays a main role in MTC infections. Once the pathogen agent reaches the lymph nodes, the naive T lymphocytes are activated by the antigen presenting cells, in most of the cases by DCs, that migrate from the alveolar interstitium to the lymph nodes (Demangel *et al.*, 2002; Bhatt *et al.*, 2004). Antigen presentation to the CD4+ T lymphocytes is performed by the antigen presenting cells using the MHC type II receptors, while the presentation to the CD8+ T cells is carried out by the MHC type I.

Activated CD4+ effectors T lymphocytes migrate from the lymph node through the circulatory torrent and are recruited into the primary focus of infection to participate in the inflammatory response. According to a study conducted in mice this can take up

from 15 to 18 days after infection (Reiley *et al.*, 2008). Both CD4+ and CD8+ T lymphocytes populations play an essential role against MTC infections.

After activation the CD4+ T lymphocytes will be differentiated into different subpopulations: Th1 or Th2 cells (pro-inflammatory or anti-inflammatory cells respectively). DCs exposed to MTC produce IL-12 which is responsible for the CD4 + T cells differentiation into Th1 cells (Flynn and Chan, 2001; Flynn, 2004).

The main function of Th1 cells is the production of cytokines such as IL-2, which participates in the activation and proliferation of T lymphocytes. They also produce IFN-γ and TNFα that activate macrophages (Foulds *et al.*, 2006). It has been assumed that the induction of a Th1-type immune response provides the host the greatest protective capacity. That is why the Th1 stimulation is a crucial point for vaccine candidates (Kaufmann, 2005).

The participation of CD8+ T cells in the control of the infection is well known. In the lungs of infected mice, CD8+ T cells showed to be able to secrete IFN-γ through activation of the T-cell receptor or by interaction with MTC infected DCs (Serbina and Flynn, 1999). Once again, the function performed by this IFN-γ is the activation of the macrophage and promotion of bacterial destruction. In addition, CD8+ T cells proved to be efficient in lysing infected cells and in reducing the number of intracellular bacteria (Stenger *et al.*, 1997).

- **Humoral immune response**

The role of antibodies in intracellular bacterial infections has gained renewed attention. Their participation in the control of chronic infections produced by *M. tuberculosis* (Williams *et al.*, 2004; Reljic *et al.*, 2006), has been lately revised. Because the organism lives within cells, usually macrophages, it is frequently assumed that tubercle bacilli are not exposed to antibodies and therefore this type of immune response is considered to be non-protective. However, during the initial steps of infection, antibodies alone or together with the proper cytokines may provide important functions, such as prevention of entry of bacteria at mucosal surfaces.

Antibodies can be used in two different ways: in the clinical management and in the control of tuberculosis, either as active participants in protection against the disease or as convenient serologic diagnostic tools. Serological methods have been considered as attractive techniques for the rapid diagnosis of tuberculosis because of the simplicity, quick turn back of results and low cost.

As for their use in protection against tuberculosis, antibodies could enhance immunity through many mechanisms including neutralization of toxins, opsonization, complement activation, promotion of cytokine release, antibody-dependent cytotoxicity and enhanced antigen presentation. In this sense it has been repeatedly observed that anti-mycobacterial antibodies play an important role in various stages of the host response to tuberculosis infection (Costello *et al.*, 1992; Hoft *et al.*, 2002; Williams *et al.*, 2004; Valliere *et al.*, 2005). In particular, De Vallière *et al.* showed that specific antibodies increased the internalization and killing of BCG by neutrophils and macrophages. Moreover, antibody-coated BCG bacilli were more effectively processed and presented by dendritic cells for stimulation of CD4+ and CD8+ T-cell responses (Valliere *et al.*, 2005).

These findings suggest the need to reconsider the role of antibody responses in MTC infections. In particular, the mechanism involved in antibody mediated enhancement of innate and cell-mediated immunity should be addressed, in order to analyze whether these mechanisms could be used to develop better vaccines against tuberculosis or to design alternative immunotherapeutic tools (Hernández-Pando *et al.*, 2007).

3.3.5. Diagnosis

The course of an *M. bovis* infection is slow and signs may be lacking, even in advanced phases when many organs are affected. Furthermore, if present, clinical signs may vary largely depending on the lesions location. Lung involvement may be manifested by a cough, dyspnoea or signs of low-grade pneumonia. In advanced

cases, lymph nodes can become so enlarged that they may hinder the passage of air, food or blood by blocking the respiratory pathway, the alimentary track or blood vessels. Involvement of the digestive tract is manifested by intermittent diarrhoea or constipation in some instances. Extreme emaciation and acute respiratory distress may occur during the terminal stages of the disease. However, since the introduction of eradication programs this type of scenarios has become very infrequent.

In order to eradicate the bovine tuberculosis it is of critical importance to be able to identify all infected animals regardless of their infective stage as quickly as possible. Currently, the official diagnosis of bovine tuberculosis is based primarily on the detection of the CMI response triggered by the infection and on the direct detection of the etiological agent by using microbiological, histopathological or molecular techniques.

The diagnostic tests used to confirm a positive animal can be divided into two groups: ante-mortem tests detecting the host's immune response or post-mortem tests which directly identify the pathogenic agent.

3.3.5.1. Diagnostic tests based on the immune response

The intradermal tuberculin test is the standard method for detection of bovine tuberculosis in cattle (Office International des Epizooties, 2015). It has been used for ante-mortem diagnosis in humans and animals for over 100 years. This test involves the intradermal injection of tuberculin purified protein derivatives (PPDs) and their capability to elicit a delayed hypersensitivity in animals currently infected. Two approaches for this technique are currently in use in Europe: the SIT measures the response against bovine PPD whereas the comparative intradermal test (CIT) compares the response against bovine and avian PPDs. According to the OIE the CIT is used with the aim of increasing the specificity by differentiating between animals infected with *M. bovis* and those responding to bovine tuberculin as a result of

exposure to other mycobacteria (Office International des Epizooties, 2015). The procedure is readily available in the legislation related to eradication programs and in the case of the EU can be found in Directive 64/432/EEC that is briefly described below.

Before injection the skin should be clipped, cleansed and measured with a caliper. According to Council Directive 64/432/ECC, the ideal injection site should be situated at the border of the anterior and middle third of the neck since it has been demonstrated that maximizes the test sensitivity (Casal *et al.*, 2015). The tuberculin can be injected using different syringes. When both avian and bovine PPDs are injected in the same animal, the site for injection of the avian PPD should be about 10 cm apart from the bovine PPD or on different sides of the neck. After 72 hours (±4 hours), the skin fold thickness at each injection site should be measured a second time and checked out for the existence of possible negative side effects (pain, edema, lymph node infarction, exudation, blisters…) by the same veterinary. Interpretation should be performed according to the Council Directive 64/432/ECC.

Since the intradermal tuberculin test yields a numeric value, different positivity thresholds can be established that will lead to different levels of sensitivity and specificity. False negative results may be caused by immunological factors (early infection, anergy or concurrent immunosuppression), factors related to the PPDs (expired product, product stored under inappropriate conditions, manufacturing errors, low potency) or to the methodology (doses, site of injection, inexperience) (de la Rua-Domenech *et al.*, 2006; Humblet *et al.*, 2011) or by co-infection with *Map* (Seva *et al.*, 2014). On the other hand false positive results might be triggered by co-infection or pre-exposure to other related non-tuberculous or environmental mycobacteria due to their similar antigenic composition (Humblet *et al.*, 2011; Jenkins *et al.*, 2018).

The subjective nature of the intradermal tuberculin test has led to a very wide range of sensitivity and specificity reported values. Reported estimates of sensitivity for the cervical SIT ranged from 80.2% (González-Llamazares *et al.*, 1999) to 100% (Lesslie and Herbert, 1975). The sensitivity achieved by the CIT is lower than for the SIT and has been ranged from 52% (Quirin *et al.*, 2001) to 100% (Lesslie and Herbert, 1975). The SIT shows a specificity ranging between 75.5% (Francis *et al.*, 1978) and 99.0% (Wood *et al.*, 1991) (median value of 96.8%) whereas the CIT specificity lies between 78.8% (Francis *et al.*, 1978) and 100% (Lesslie and Herbert, 1975; Norby *et al.*, 2004) (median value of 99.5%).

Over the last years efforts have been made to find out alternative more specific antigens. The lack of specificity of the technique has been associated with the fact that some components of the bovine PPDs are shared with other environmental mycobacteria (Hope *et al.*, 2005a). These specific antigens might be especially useful in situations where co-infections with *Map* exist, in order to overcome any potential interference (Aagaard *et al.*, 2010; Flores-Villalva *et al.*, 2012).

Different studies have been carried out searching for new more specific antigens. Identification of specific antigens present only in *M. bovis* and absent from environmental mycobacteria can help to increase the specificity of the different diagnostic techniques.

The most relevant alternative antigens tested in the tuberculin skin test are the early secretory antigenic target-6kDa (ESAT-6), the culture filtrate protein 10 (CFP-10) and the Rv3615c (Mb3645c) applied alone or combined with each other or with other antigens. The specificity obtained in different studies under different epidemiological conditions was always higher when the alternative more specific antigens were used in comparison with the official tuberculins (Pollock *et al.*, 2003; Aagaard *et al.*, 2010; Xin *et al.*, 2013). On the other hand it appears that the sensitivity gets compromised when

the specific antigens are used instead of the official tuberculins (Pollock *et al.*, 2003; Aagaard *et al.*, 2010). However Jones and co-workers proved that addition of the Rv3020c antigen improves the sensitivity without compromising the specificity of the technique (Jones *et al.*, 2012).

The IFN-γ release test, developed in the late 1980s, is recommended by the OIE as an ancillary laboratory-based test to the tuberculin intradermal test. Most of the bovine tuberculosis control programs are based on the use of the IFN-γ test as a parallel test to the intradermal test to maximise the detection of infected animals. The assay is accepted for use as ancillary test to the intradermal test in the European Union since 2002 [Council Directive 64/432/EEC, amended by (EC) 1226/2002].

This in vitro assay is a laboratory- based test detecting specific CMI responses by circulating lymphocytes. This technique can be divided into two stages. First, heparinized whole blood is incubated with antigens (PPDs or specific antigens) for 18–24 h. Antigenic stimulation induces production and release of IFN-γ by predominantly T lymphocytes. Second, plasma supernatants are collected and the IFN-γ present in them is quantified by carrying out a sandwich ELISA. Optical density is measured and the difference value between tuberculins is determined by subtracting the optical density value achieved with the avian PPD stimulation from that of the bovine PPD. In general, most laboratories also include a no stimulation negative control (PBS or media) and a mitogen or superantigen positive control.

Sensitivity and specificity values for the IFN-γ release test have been estimated by different studies as shown in a meta-analysis conducted by de la Rua-Domenech and co-workers (de la Rua-Domenech *et al.*, 2006). IFN-γ test is considered to show a similar or slightly lower specificity than the obtained for the SIT, and definitely lower than for the CIT skin test version. However, the sensitivity obtained with the IFN-γ release test is considered equal or slightly higher than for the skin test. The most likely

explanation might be that the IFN-γ release test is able to identify reactor animals at an earlier stage of infection than the intradermal tuberculin skin test (Pollock *et al.*, 2005).

Reported IFN-γ estimates of sensitivity range between 73% (Whipple *et al.*, 1995) and 100% (Lilenbaum *et al.*, 1999) with a median value of 87.6%. Specificity for this technique ranges from 85% (Buddle *et al.*, 2001) and 99.6% (S D Neill *et al.*, 1994) with a median value of 96.6%.

As for the tuberculin skin test, different alternative antigens have also been assayed for the IFN-γ release test. Similar to what happens in the skin test, the use of alternative antigens seems to increase the specificity of the IFN-γ assay compared to the avian and bovine PPDs (Bezos *et al.*, 2014). In relation to the sensitivity values, in a review carried out by Bezos and co-workers, it was pointed out that no antigen ,singularly tested, gave equivalent sensitivity compared to the PPD-based INF-γ assay (Bezos *et al.*, 2014). These results agreed with another study which concluded that the combined use of the Rv3615c antigen with ESAT-6 and CFP-10 seems to increase the diagnostic sensitivity without decreasing the specificity (Sidders *et al.*, 2008).

Currently, the most commonly diagnostic tools used for bovine tuberculosis are based on the measurement of the delayed hypersensitivity reaction and the IFN-γ production. However **antibody-based bovine tuberculosis assays** show an improvement over the methods traditionally used. Many samples can be tested in a short time, it is a simple and quick as well as inexpensive test which also allows the standardisation of the technique in the different laboratories (Cho *et al.*, 2007). However, the development of an accurate antibody-based *M. bovis* test has been very difficult. The titer of antibodies changes significantly during the infection and they are mainly produced in advanced stages of the *M. bovis* infection (Pollock and Neill, 2002). Furthermore *M. bovis* infection in cattle leads to an early and strong CMI response

while the antibody response remains discourangingly weak (Pollock *et al.*, 2001; Welsh *et al.*, 2005).

Animals presenting an advanced and generalised infection status not responding to the intradermal tuberculin skin due to an impaired CMI response are described as anergic. Although ELISA serological tests are able to identify a proportion of these anergic non-CMI reactor animals, when applied in the early stages of infection the sensitivity levels are especially low (Figure 5). These kind of tests have been characterized by a low sensitivity when compared with skin and IFN-γ release tests (Plackett *et al.*, 1989; Fifis *et al.*, 1992). Schiller and co-workers pointed out that the combination of methods based on the CMI response against *M. bovis* together with serological tests could be of help in the control of the disease by increasing the level of detection of the pathogen (Schiller *et al.*, 2010).

Several more specific antigens have been described as potential diagnostic targets (ESAT-6, CFP-10 and MPB70). However the antibody response is mainly triggered against the MPB83 antigen in domestic livestock and also in wild animals (Mcnair *et al.*, 2001; Amadori *et al.*, 2002; Wiker, 2009). Responses against MPB83 is detected earlier in the course of experimental infections, observing an increase in the antibody response at 3–4 weeks post infection (O'Loan *et al.*, 1994; W.R. *et al.*, 2006). In addition a recent study evaluated a new multiprotein complex (P22) for the detection of specific antibodies against the MTC. Not only in cattle but also in sheep did this ELISA show high specificity values (≥98%) after using a stringent cut-off. However the optimal specificity was obtained when serum belonging to pigs was used (specificity=100%). This P22 ELISA could be a cost effective, quick and reliable tool for the screening of bovine tuberculosis at herd level.

3.3.5.2. Diagnostic tests based on agent detection

The identification of characteristic **histological lesions**, together with the observation of resistant alcoholic acid bacilli, may lead to a presumptive diagnosis of tuberculosis. The presence of mycobacteria in a sample can be uncovered by a Ziehl-Neelsen stainning However bacterial burden of tuberculous lesion can be so low that the presence of bacilli may not be detectable in histological samples from infected animals (Office International des Epizooties, 2015). Also other organisms belonging to the *Mycobacterium* genus may also grow in the same locations, be Ziehl-Neelsen positive and develop histopathologically similar lesions.

Microbiological culture is considered the gold standard to confirm whether or not an animal is infected with bovine tuberculosis despite some limitations: difficulty in obtaining valid samples from live animals, need for samples pre-treatment, slow growth and extra time for identification by additional methods (Medeiros *et al.*, 2010). MTC microorganisms are slow-growing and therefore their culture has a long duration and, due to its complexity, requires specialization of the personnel in charge. The presence of mycobacterial growth in a culture confirms the positivity of the sample. However a negative result does not rule out infection in the sampled animal. The sensitivity of this technique is relatively low. It depends on several factors: quality of the tissue to be cultured, stage of the infection (animals in early stages of the disease cannot be detected by microbiological culture) or bacterial load. The major limitation for systematic cultivation of *M. bovis* in animals has been pointed out to be the collection of poor quality samples during necropsies or at slaughterhouses.

Once in the laboratory, samples are thinly sliced searching for lesions and afterwards they are processed. Tissue samples have to be homogenized and decontaminated in order to prevent growth of other bacteria in the culture medium.

Different solid media (mainly Coletsos and Löwenstein-Jensen) are used for primary isolation. Cultures are incubated at 37ºC for a minimum of 10-12 weeks.

Nowadays **automated liquid culture** systems are available such as the BACTEC MGIT 960 (Mycobacterial Growth IndicatorTube). The liquid culture shows important advantages over the solid one: it reduces the culture outcome time and increases the sensitivity of the technique (Robbe-Austerman *et al.*, 2013). Studies have compared the sensitivity of different media (Hines *et al.*, 2006). The sensitivity for the BACTEC MGIT 960 was estimated at 94.6% and 74.4% for the solid media (Hines *et al.*, 2006). An average detection time of 15.8 days was estimated for the BACTEC MGIT 960 whereas 43.4 days were estimated as the average detection time needed for solid media (Hines *et al.*, 2006). The principal disadvantage for this liquid culture system is the higher economic cost.

In addition, the combined used of liquid and solid culture media has been suggested to improve the culture sensitivity (Hines *et al.*, 2006).

Once growth has been detected in culture media, the presence of the MTC members is usually confirmed by the identification of specific DNA fragments through PCR (Wilton and Cousins, 1992). Afterwards different molecular techniques are used to identify the specific strain.

PCR-based tests are being increasingly used for the identification of *M. bovis* in suspect tuberculous lesions. The main advantage is that since it is not necessary to wait for the bacteria to grow in specific media, the infection confirmation period gets shortened and therefore it is possible to reduce the exposition of susceptible individuals to the infection sources by culling the shedders earlier (Courcoul *et al.*, 2014).

Until recently the sensitivity of PCR tests has been lower than culture for detecting *M. bovis* in tissues. However the design of a tetraplex real-time PCR by Sevilla and co-workers for simultaneous detection of *Mycobacterium* genus, *M. avium*

subspecies and MTC represents a highly specific and sensitive tool for the detection and identification of mycobacteria in routine laboratory diagnosis (Sevilla *et al.*, 2015). Different sequences were used: 1) the mycobacterial ITS located between 16S and 23S rRNA genes for the *Mycobacterium* genus, 2) insertion sequence IS1311 for the *M. avium* subspecies and 3) the devR gene for the MTC (Sevilla *et al.*, 2015). The sensitivity and specificity values achieved were 97.5% and 100% respectively using a verified culture-based method as the reference method (Sevilla *et al.*, 2015). In addition a more recent study describes the development, optimization and validation of a Real-Time PCR based on the mpb70 gene to detect MTC members in clinical tissue samples from cattle. Results obtained from 200 bovine tissue samples showed sensitivity and specificity values of 94.59% and 96.03% respectively in relation to bacteriological culture (Lorente-Leal *et al.*, 2019).

Currently, **DVR-spoligotyping** is the technique of choice for the identification of strains of the MTC complex. DVR-spoligotyping is aimed to detect the DR-locus which consists of a variable number of direct repeat sequences interspersed with non-repetitive single sequences, called spacers, and reveals the presence or absence of these spacers. The result is a spoligum profile that can be presented as a binary numerical code and can also be assigned a name according to international nomenclature (https://www.visavet.es/mycodb/index-en.php).

3.3.6. Bovine tuberculosis programs

Eradication of bovine tuberculosis has been a major objective for Animal Health and Public Authorities for almost a century. As for the European Economic Community this disease has always been considered of great concern. Current European policies on the eradication of bovine tuberculosis are better understood after considering the progressive development of relevant Community legislation.

During the last decade of the 19th century and the beginning of the 20th the infectious nature of the tubercle bacillus as well as its zoonotic character seemed to be not well appreciated. Motivation for control of the disease during this time seemed to be predominantly economic (John Francis, 1947).

Afterwards the main objectives changed and measures were introduced to enable international trade of live cattle and their products. Historically the first legal measures regarding bovine tuberculosis at community level were established with the aim of favouring the intra-community trade among the European Economic Community Member States by establishing generalised animal health requirements. In the Council Directive 64/432/EEC of 26 June 1964 on animal health problems affecting intra-community trade of cattle and swine not only specific requirements for the trade of cattle in relation with bovine tuberculosis were specified but also the OTF herd status was defined. Since 1964 this legislative act has been amended frequently. In 1997 the requirements for achieving the OTF status were modified by the Council Directive 97/12/EC. Most recently in 2002 the diagnosis of bovine tuberculosis was thoroughly reviewed and the INF-γ was presented as a supplementary test to enable detection of the maximum number of infected and diseased animals in a herd or in a region by the Commission Regulation (EC) No. 1226/2002.

The second important step was Council Directive 77/391/EEC of 17 May 1977, which laid down community measures for the eradication of brucellosis, tuberculosis and leucosis in cattle. The Member States were, and still are, obliged to design eradication programs in order to accelerate, intensify or carry through the eradication of bovine tuberculosis. Financial support to these programs from the community budget was also foreseen. This legislation, which has been amended frequently, provides the legal framework for the bovine tuberculosis eradication programs.

Bovine tuberculosis programs have been successful in several Member States or regions that have reached the OTF status. Denmark achieved the OTF status in 1980, the Netherlands in 1995, Germany and Luxembourg in 1997, Austria and some regions of Italy in 1999, France in 2001 and Belgium in 2003. Nowadays 22 countries and regions were categorized as OTF (see the “Bovine tuberculosis in cattle, prevalence” section) (EFSA and ECDC, 2018).

The European Union policy on the eradication of bovine tuberculosis is based on two fundamental principles: (1) Member States are primarily responsible for the eradication of bovine tuberculosis and may receive community financial support for the eradication program and (2) eradication of bovine tuberculosis in the European Union must be the final target and the Member States must consider eradication as the defined aim.

In Spain, as already mentioned in previous sections, the first actions to fight back bovine tuberculosis are dated from the early 1950s. However it was not until 1965 when a National Plan to Combat Tuberculosis and Bovine Brucellosis was established according to the Ministry of Agriculture Order of 24 May. Once Spain entered the European Economic Community, an Accelerated Eradication Program was designed in accordance with Directives 77/391/EEC and 78/52/EEC and Decision 87/58/EEC. The National Programs for the Eradication of Bovine Tuberculosis 2006-2010 represented a qualitative change in the approach of the objectives. The principal goal of these programs was to gradually increase the diagnostic sensitivity, both at herd and individual level in order to eliminate as many infection foci as possible. Additional measures such as management of possible wild reservoirs (according to the Plan of Action on Tuberculosis in Wild Species PATUBES) or the integration of the surveillance system in slaughterhouses have been gradually introduced to help gaining control over the disease.

It has already been pointed out in this section that the Member States are primarily responsible for the eradication of bovine tuberculosis. However, before achieving this ultimate objective, in Spain it is necessary to fulfil previous goals: reduction of prevalence and incidence rates at herd level and individual incidence has to be achieved alongside with an increase in the number of herds categorized as T3 (OTF herds within the meaning of Directive 64/432 /EEC) in the different regions. The diagnostic tests used for that purpose are the intradermal tuberculin test and the IFN-γ. The intradermal tuberculin test is performed on all older than six-week animals and the IFN-γ is performed on older than six-month individuals. Depending on the herd category the SIT or CIT are used as the routine technique which can be strategically complemented by the use of the IFN-γ test in positive herds considered as infected. Sacrifice of reactive animals is mandatory. In addition, hygienic measures in farms where positive animals have been detected should be applied. The Official Veterinary Services are in charge of verifying that good management practices such as the correct management of manure and the cleaning and disinfection procedures are carried out appropriately. In addition, where appropriate, a minimum period of 60 days of sanitary emptying has to be respected for the reuse of pastures. Finally an exhaustive control of the movements and of the replacement of these farms, as well as intensification of the diagnostic tests should be carried out in order to raise their sanitary qualification as soon as possible.

3.3.7. Vaccination

Nowadays there is no tuberculosis vaccine approved for its use in cattle or wildlife animals with the exception of Great Britain where the BCG vaccine was licensed for intramuscular administration to badgers in 2010 (Perrett *et al.*, 2018). The major reasons for this banning are that vaccination may interfere in the traditional bovine tuberculosis diagnostic tests by sensitizing the vaccinated animals and turning

them into false-positive, and that the degree of protection conferred after vaccination is not complete.

BCG is the only vaccine licensed for use against tuberculosis in humans. It is a live attenuated vaccine obtained from a *M. bovis* strain after 230 successive passages in the laboratory between 1908 and 1921. It is an inexpensive vaccine which has been inoculated to more than 2.5 billion people. Moreover it has a long-established safety profile and an outstanding adjuvant activity, eliciting both humoral and CMI responses. It can be delivered at birth or at any time thereafter, and a single dose can produce long-lasting immunity (Martín *et al.*, 2007). Recent long-term follow-up studies demonstrated that a single dose in childhood preserves immunization for up to 50–60 years after vaccination (Aronson *et al.*, 2004). Nevertheless, different studies have concluded that protection conferred by BCG decreases over time. Efficacy of BCG in adolescent and adult populations is reported to be highly variable among different geographical regions (Andersen and Doherty, 2005).

Nowadays human preferred BCG route of administration is the intradermal route. However oral BCG administration was used in multiple controlled studies performed in the 1920s and 30s and showed protection against tuberculosis (Kereszturi, 1929; Aronson and Dannenberg, 1935). More recent studies demonstrated that oral BCG administration induces mucosal immunity compared to intradermal vaccination (Lai *et al.*, 2015; Hoft *et al.*, 2018). Enhanced protection after a pulmonary mucosal BCG vaccination was described in studies carried out on rhesus macaques compared to the standard intradermal use of the BCG (Verreck *et al.*, 2017). Matching the route of vaccination and natural infection seems convenient (Manjaly Thomas and McShane, 2015).

In cattle, the route of transmission is primarily linked to the airborne pathway, although calves are commonly infected through ingestion of infected milk. Transmission of wildlife species can occur through different routes involving aerosol

inhalation or oral ingestion. Thus, induction of mucosal immunity could be helpful and oral vaccination with BCG might be beneficial. Results obtained after BCG being orally delivered to protect cattle against bovine tuberculosis have been very promising (Nugent *et al.*, 2017; Buddle *et al.*, 2018). In additioin its use in wildlife reservoirs, such as free-living badgers (Gormley *et al.*, 2017) or possums (Tompkins *et al.*, 2009) and under experimental conditions in wild boars (Garrido *et al.*, 2011) has been very encouraging too. Test and cull strategies or treatments are not feasible control options for wildlife species. Vaccination seems to be the only reasonable approach and oral delivery might be the most cost-effective option. Furthermore in developing countries, the test and cull strategy for diminishing the prevalence of bovine tuberculosis in cattle rapidly is too expensive and alternative control methods such as vaccination could be a very appealing approach.

The BCG efficacy is very variable. A review of different experimental trials and studies conducted by different researchers in different countries between 1959 and 2002 estimated that the BCG efficacy ranges between 0% and 75% (Hewinson *et al.*, 2003). To optimise the use of the BCG vaccine it is crucial to determine factors influencing its efficacy. Results obtained from different studies suggest that BCG doses of 10^4-10^6 CFU administered parenterally induced equivalent protection (Buddle *et al.*, 1995, 2013), while higher doses (10^8 CFU) were required to induce protection when BCG was administered orally (Buddle *et al.*, 2011)..

Over the past decades, a large number of vaccination and challenge experiments have been carried out in cattle using standardized models. This makes possible the comparisons between different studies with BCG tested alone or compared to other vaccines. Challenge models have focused on using relatively low *M. bovis* challenge doses (10^3-10^4 CFU) administered via endobronchial/intratracheal inoculation or by aerosol (Buddle *et al.*, 1995; Palmer *et al.*, 2002). These low-dosed challenges turned out into the development of tuberculous lesions mimicking those observed in the lower respiratory tract of naturally infected animals. Similar BCG strains have been used

(initially Pasteur, then BCG Danish 1331) and vaccine protection has been assessed by comparing quantitative gross, histopathological, and microbiological findings. In a recent study carried out by Ameni and co-workers BCG was administered subcutaneously against bovine tuberculosis under field conditiond. The vaccine efficacy for protection against disease was low (30%), whereas its effect on reducing the extent of gross pathology, microscopic lesion, or isolation of *M. bovis* was significant (Ameni *et al.*, 2018). These results show a lower BCG efficacy than the obtained in previous studies under the same circumstances in Mexico (Lopez-Valencia *et al.*, 2010) and Ethiopia (Ameni *et al.*, 2010). This difference in full protection could be explained by differences in the severity of the infected reactor animals that served as sources of infection.

Outcomes of one study carried out by Buddle and co-workers suggested that natural pre-sensitisation to environmental mycobacteria appeared to have an adverse effect on subsequent immunity induced by BCG vaccine. Lower degree of protection was induced compared to that developed by two other attenuated *M. bovis* vaccines (Buddle *et al.*, 2002). However in another study conducted by Hope *et al.*, it was observed that *M. avium* exposure induced partial protection against *M. bovis* infection, which could possibly mask subsequent immunity induced by BCG (Hope *et al.*, 2005a).

Regarding the optimal age for vaccination it has been demonstrated that BCG inoculation of young calves induced a PPD-B specific IFN-γ secretion of longer duration than that observed following BCG vaccination of adult cattle (>1 year old) (Hope *et al.*, 2005b). Neonatal vaccination with BCG induces significant protection from experimental *M. bovis* infection (Hope *et al.*, 2005b, 2011) and it has been corroborated by field studies too (Ameni *et al.*, 2010). Another advantage of neonatal vaccination of calves observed in different studies involves significant reductions of the tuberculous lesions as well as pathology scores and bacterial burden which were not observed in calves aged 5–6 months (Buddle *et al.*, 2002, 2003).

It is well-known that BCG vaccination may interfere with the tuberculin skin test interpretation. Using the SIT at the cervical region, 80% of BCG-vaccinated calves were categorized as reactors to the tuberculin skin test six months after vaccination. However this figure dropped to 10–20% after nine months since vaccination (Whelan *et al.*, 2011). In another study the highest maximum skin test reactivity was observed only five weeks after vaccination, but disappeared completely 18 months after vaccination (Moodie, 1977).

To avoid this interference DIVA tests will be needed in countries willing to implement BCG vaccination into the test and cull based programs. For the development of DIVA tests, antigens present in the MTC but not expressed or secreted by BCG have been studied so that they can be used instead of bovine PPD in the whole blood IFN-γ or skin test. A recent study assessed the incorporation of ESAT-6, CFP10, and Rv3615c to a whole blood IFN-γ test and results were similar to that obtained using the traditional avian and bovine PPDs. When tested in non-infected animals, both the DIVA and tuberculin readouts gave specificities between 97 and 99%. The relative specificity of the DIVA blood test was also high (95%) in BCG-vaccinated cattle and was significantly greater than that observed for the tuberculin readout (71%) (Vordermeier *et al.*, 2016). Same DIVA antigens have been used for the skin test in cattle showing a high sensitivity for *M. bovis* infected cattle, similar to the level reached by the CIT in non-vaccinated cattle while not compromised by vaccination with BCG or with vaccines against PTB (Whelan *et al.*, 2010; Jones *et al.*, 2012).

The alternative of using heterologous vaccines to overcome this diagnostic problem has been never reported.

During the last 25 years research for the development of new human vaccines against tuberculosis has been very intense and efforts to create and evaluate vaccines against *M. bovis* in cattle have benefited from this investigation. Different types of vaccines have been recently tested in cattle: live attenuated mycobacteria and subunit

vaccines such as DNA, protein, and virus-vectored vaccines, which could be used together with the BCG to boost its immunity (Table 4) (Parlane and Buddle, 2015).

Table 4. Types of new vaccines tested in cattle. Source: (Parlane and Buddle, 2015)

Type of Vaccine	Vaccine	Protection against tuberculosis compared to BCG	Reference
Modified BCG	· BCG over-expressing Ag85B	+	(Rizzi *et al.*, 2012)
	· BCG Δ zmp1	+	(Khatri *et al.*, 2014)
	· BCG mutants (BCGDleuCD, BCGDfdr8, BCGDmmA4, BCGDpks16)	=	(Waters *et al.*, 2016)
Attenuated *M. tuberculosis* strain	· M. tuberculosis ΔRD1 ΔpanCD	-	(Waters *et al.*, 2007)
Attenuated *M. bovis* strain	· UV-irradiated *M. bovis*	+	(Buddle *et al.*, 2002)
	· *M. bovis* Δ leuD	NT[b]	(Khare *et al.*, 2007)
	· *M. bovis* Δ RD1	=	(Waters *et al.*, 2009)
	· *M. bovis* Δ mce2	+	(Blanco *et al.*, 2013)
DNA vaccine	· Mycobacterial DNA	=	(Maue *et al.*, 2004; Cai *et al.*, 2005)
	· Heterologous prime boost: mycobacterial DNA + BCG	+	(Maue *et al.*, 2007)
Adjuvanted protein vaccine	· Simultaneous protein + BCG	+	(Wedlock *et al.*, 2008)
Virus-vectored vaccine	· Heterologous prime boost: BCG + Ad85A	+	(Vordermeier *et al.*, 2009)

NT Not tested against TB challenge or compared with BCG
[b] Significant protection against TB, but not tested against BCG
+ Significantly better than BCG
= Equivalent protection to BCG
– No protection against TB

In addition vaccination of wildlife species with a heat-inactivated vaccine has been carried out recently in different assays. Results obtained from wild boars suggest that oral and parenteral vaccination with the heat-inactivated *M. bovis* strain triggered out an immune response that conferred a similar degree of protection as BCC (Garrido *et al.*, 2011). Same effects were observed in a study carried out in red deer. The orally vaccinated group with the heat-inactivated *M. bovis* strain and subsequently infected with *M. bovis* presented a reduced lesion score level. No interference with the *in vivo* bovine tuberculosis diagnosis techniques were reported (Thomas *et al.*, 2017). Results regarding protection conferred by this vaccine in cattle have not been published yet. However a study carried out by Jones and co-workers stated that oral vaccination of

cattle with the *M. bovis* heat inactivated vaccine did not compromise the bovine tuberculosis diagnostic tests when using regular or specific antigens (Jones *et al.*, 2016).

Furthermore a new vaccine has been designed and already tested in humans showing promising protection results. M72/AS01$_E$ is a subunit vaccine composed by an immunogenic fusion protein (M72) derived from two *M. tuberculosis* antigens (MTB32A and MTB39A). After administrating two doses of the M72/AS01$_E$ vaccine, 54% of the *M. tuberculosis*-latent-infected and HIV-negative adults were protected from turning into active pulmonary tuberculosis patients (Van Der Meeren *et al.*, 2018).

4. METHODOLOGY

In all three studies we refer to in this dissertation calves were involved. All the experimental procedures involving animal housing and care as well as the clinical practices they were submitted to were carried out in agreement with the European, Spanish and Regional Law and Ethics Comitee Regulations. The experimental design underwent ethical review and approval by NEIKER's Animal Care and Use Committee and by the Agriculture Department of Diputación Foral de Bizkaia.

Calves taking part in the three studies were selected from the same feedlot that had previously bought them from different commercial farms with no known history of bovine tuberculosis. Thirty calves (approximately 2 months old) were preselected from the feedlot farm for every study. Prior to being included in each study three blood samplings were carried out on week 0, 4 and 12. In order to confirm the absence of previous contact with *M. bovis* an IFN-γ release test on whole blood was carried out. Samples for the IFN-γ tests were stimulated with avian and bovine PPD and other more specifc antigens as ESAT-6/CFP10 and Rv3615c.

Vaccination against *Map* with the whole-cell heat-inactivated Silirum® vaccine was carried out in the feedlot at week 0 just before blood extraction. Twenty out of the 30 calves were randomly vaccinated subcutaneously in the dewlap. Finally, after the third sampling, 10 vaccinated and 5 non-vaccinated calves showing the lowest optical densities as a result for the INF-γ test were selected. The 15 remaining calves were kept at the feedlot and no longer followed. During the 16th week post-vaccination animals were shipped to the biosafety level 3 facilities in NEIKER where they were divided into three experimental groups of five calves each according to their vaccination status.

After a two-week adaptation period, at week 18 post-vaccination, five vaccinated and five non-vaccinated calves taking part in the first and second study were challenged through the endotracheal route with 10^6 CFUs of *M. bovis* suspended in 2

ml of phosphate-buffered saline. However for the third study five vaccinated and five non-vaccinated calves were challenge orally with the same *M. bovis* isolate and at the same concentration. Heads of the animals were fastened upwards until deglutition was observed to avoid accidental spills. The *M. bovis* field isolate (SB0339 spoligotype profile) used was originally obtained from a naturally infected wild boar. After challenge the groups were recalled as: 1) *Map* vaccinated and *M. bovis* infected, 2) *Map* vaccinated and *M. bovis* not infected and 3) *Map* non-vaccinated and *M. bovis* infected.

Five more blood samplings were carried out during each experiment at the biosafety level 3 facilities at weeks 18, 20, 22, 26 and 30 post-vaccination.

The tuberculin skin test was carried out one week before the necropsies of the animals according to the Spanish and EU regulations. The skin test was performed by inoculating 0.1ml (2500 UI) of avian and bovine-PPD in the cervical neck region. In addition to the standard PPD antigens, 0.1 ml of a peptide cocktail (100 µg/ml/peptide) and a protein cocktail (100 µg/ml/protein) representing ESAT-6/CFP10 and Rv3615c were also used in the first study. The skin fold thickness was measured right before and 72 hours after the inoculation with a caliper. The skin thickness increase results for the standard antigens were interpreted according to the standards of official criteria (EU Council Directive 64/432/CEE and Spanish RD 2611/1996) for both SIT and CIT. In relation to the specific antigen cocktails, a result was considered positive if the skin thickness increase was equal to or bigger than 2 mm 72 hours post-inoculation.

Before the necropsies all individuals were sedated by an intramuscular injection of XILAGESIC® 2% and euthanized by intravenous injection of T-61 (Intervet S.A., Salamanca, Spain). A systematic necropsy of every animal was carried out with the main objective of assessing the presence and extension of tuberculous lesions. Collected samples were distributed within five body areas as follows: head (nasal turbinate, palatine tonsils and mandibular, parotid and retropharyngeal lymph nodes),

thorax (tracheal, prescapular, tracheobronchial and mediastinal lymph nodes), lung (right and left cranial and caudal lobes and medium and accessory lobes), abdomen (hepatic, jejunal and ileocecal lymph nodes as well as liver and spleen) and others (prefemoral and popliteal lymph nodes).

All collected tissues were visually inspected for the presence of macroscopic lesions and sliced as thin as possible in search of deeper lesions. The severity of the gross pathological changes compatible with tuberculosis lesions were scored according to a semiquantitative system (Palmer *et al.*, 2007). Briefly, two scoring system were established to measure the severity of the lesions in lung and the lymph nodes. Lung lobes were subjected to the following scoring system: (0) no visible lesions; (1) no external gross lesions, but lesions seen upon slicing; (2) less than 5 gross lesions of <10mm in diameter; (3) more than 5 gross lesions of <10mm in diameter; (4) more than one distinct gross lesion of >10mm in diameter and (5) coalescing gross lesions. Scoring of lymph node gross lesions was based on the following scoring system: (0) no visible lesions; (1) small focal lesion (1–2mm in diameter); (2) several small foci and (3) extensive lesions.

For bacteriology analysis samples were maintained refrigerated until bacteriological processing (after bacteriological processing the remaining samples were stored at -20°C in case further analysis or retesting was needed). The BACTEC system and classical solid media were used for mycobacterial culture. According to the protocol described previously by Garrido and coworkers (Garrido *et al.*, 2011) samples were thoroughly homogenized in sterile distilled water (2 g in 10 ml or equivalently). Five ml were decontaminated in hexadecyl-pyridinium chloride at a final concentration of 0.75% (w/v) for 12–18 h. Afterwards samples were centrifuged at 2.500 x g for 5 min and pellets were cultured in Coletsos and Löwenstein Jensen tubes (bioMèrieux, maisons-Alf) at 37°C for 4 months. The remaining 5 ml of the homogenized suspension were decontaminated and processed following the instructions of the

manufacturer to inoculate BBL MGIT tubes supplemented with BBL MGIT PANTA and BACTEC MGIT growth supplement (Becton Dickinson). BBL tubes were incubated for 42 days in a BACTEC MGIT 960 System.

Colonies in solid media were visualized under a stereomicroscope and infection level of samples were categorized according to the number of colonies per tube as follows: (0) no growth, (1) less than 10 colonies, (2) between 10 and 50 colonies and (3) more than 50 colonies (Garrido *et al.*, 2011).

DNA was extracted from all positive cultures and a MTC specific PCR (Sevilla *et al.*, 2015) was carried out to confirm that *M. bovis* was responsible for the colonies growth. Finally, all isolates were spoligotyped in order to confirm that the isolated strain was the same than the used for the challenge (Kamerbeek *et al.*, 1997).

In relation to the statistical work Table 5 shows a summary of the tests carried out in the different studies.

Table 5. Summary of the statistical tests used.

Objective	Test
Calculate the reduction by vaccination of: -Solid culture score -Nº of positive tissues by culture -Gross lesion score -Nº of affected areas by gross pathology	(1-VI/NVI) * 100
Differences in the degree of pathology and bacterial burden (lesions scores and culture scores).	Mann-Whitney U-test
Differences in the number of affected tissues by gross pathology and culture.	Student's paired two-sample t-test
Proportions of animals with lesions.	Fisher's exact test
Association between culture and gross pathology results.	Spearman's correlation
Differences in the distribution of lesions and positive culture results between groups	Chi square test
Correlation between skin test results and lesion scores and skin test results and Nº of tissues with lesions.	Spearman

5. RESULTS

5.1. FIRST STUDY

Tuberculosis Detection in Paratuberculosis Vaccinated Calves: New Alternatives against Interference. Serrano, M., Elguezabal, N., Sevilla, I.A., Geijo, M. V., Molina, E., Arrazuria, R., Urkitza, A., Jones, G.J., Vordermeier, M., Garrido, J.M., Juste, R.A., 2017. Tuberculosis Detection in Paratuberculosis Vaccinated Calves: New Alternatives against Interference. *PLOS ONE*, 12(1), p. e0169735. doi: 10.1371/journal.pone.0169735.

Tuberculosis Detection in Paratuberculosis Vaccinated Calves: New Alternatives against Interference

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ABSTRACT

Paratuberculosis vaccination in cattle has been restricted due to its possible interference with the official diagnostic methods used in tuberculosis eradication programs. To overcome this drawback, new possibilities to detect *Mycobacterium bovis* infected cattle in paratuberculosis vaccinated animals were studied under experimental conditions. Three groups of 5 calves each were included in the experiment: one paratuberculosis vaccinated group, one paratuberculosis vaccinated and *M. bovis* infected group and one *M. bovis* infected group. The performance of the IFN-gamma release assay (IGRA) and the skin test using conventional avian and bovine tuberculins (A- and B-PPD) but also other more specific antigens (ESAT-6/CFP10 and Rv3615c) was studied under official and new diagnostic criteria. Regarding the IGRA of vaccinated groups, when A- and B-PPD were used the sensitivity reached 100% at the first post-challenge sampling, dropping down to 40–80% in subsequent samplings. The

sensitivity for the specific antigens was 80–100% and the specificity was also improved. After adapting the diagnostic criteria for the conventional antigens in the skin test, the ability to differentiate between *M. bovis* infected and non-infected animals included in paratuberculosis vaccinated groups was enhanced. Taking for positive a relative skin thickness increase of at least 100%, the single intradermal test specificity and sensitivity yielded 100%. The comparative intradermal test was equally accurate considering a B- PPD relative skin increase of at least 100% and greater than or equal to that produced by A- PPD. Using the specific antigens as a proteic cocktail, the specificity and sensitivity reached 100% considering the new relative and absolute cut-offs in all experimental groups ($\Delta \geq 30\%$ and $\Delta mm \geq 2$, respectively). Results suggest that the interference caused by paratuberculosis vaccination in cattle could be completely overcome by applying new approaches to the official tuberculosis diagnostic tests.

INTRODUCTION

Bovine tuberculosis (bTB) and paratuberculosis (PTB) are widespread infectious diseases that affect many domestic (Aagaard *et al.,* 2010; Muñoz-Mendoza *et al.,* 2015) and wild (Aranaz *et al.,* 2004; Carta *et al.,* 2013) species. The impact of these diseases derives from losses to the livestock industry, especially dairy cattle (Harris and Barletta, 2001; Waters *et al.,* 2012), from hunting and wildlife conservation as well as from their recognized (bTB) or suspected (PTB) zoonotic character. The relevance of bTB as a zoonosis has been substantially reduced in the more developed countries, but it is still a frequent cause of morbidity and mortality in countries that cannot afford strong control measures like milk thermal treatment and compulsory bTB eradication schedules (Waters *et al.,* 2012). It has been estimated that about 10% of the total human tuberculosis cases around the world are caused by *Mycobacterium bovis* (*M. bovis*) (Müller *et al.,* 2013; Rodwell *et al.,* 2010). On the other hand *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) is considered potentially zoonotic since it was

first isolated from human patients in 1984 (Chiodini *et al.,* 1984) and has also been firmly associated with some forms of chronic regional intestinal inflammatory disease (Elguezabal *et al.,* 2012; Ramón A. Juste *et al.,* 2009; Naser *et al.,* 2004), although an etiological role has not been widely accepted by the medical community.

The live *M. bovis* Bacillus Calmette-Guerin (BCG) has been used as a vaccine in humans (Waters *et al.,* 2012) as well as in cattle (Buddle *et al.,* 1995), showing different levels of protection against *M. bovis* infection. One of the major disadvantages of the use of this attenuated vaccine in cattle is the interference with bTB diagnostic tools due to cross reactivity of the tuberculins with antigens of the vaccine itself.

PTB is considered one of the most important diseases in dairy cattle, decreasing the milk production by up to 10% (Harris and Barletta, 2001; Hasonova and Pavlik, 2006). It has been proven that PTB vaccination in sheep and goats with *Map* whole heat-inactivated vaccines efficiently prevents the disease and significantly diminishes the bacterial burden reducing the chance for other animals of becoming infected (Juste and Perez, 2011). As a consequence PTB vaccination should be taken into account in countries where bTB prevalence is really low and the Animal Health System works efficiently (Ramon A Juste *et al.,* 2009). In Spain, PTB vaccination is not allowed in cattle due to the possible interference in the official immunological bTB diagnostic tests (Garrido *et al.,* 2013). bTB herd prevalence in Spain is 1.2% (European Food Safety Authority, 2015) but the Basque Country is one of the regions with lowest frequencies (0.25%) (Ministerio de agricultura alimentación y medio ambiente, 2014) and it can be very closely monitored thanks to its small size and well developed veterinarian services. For these reasons a field clinical trial for an inactivated vaccine registration was authorized by the competent authorities (local Animal Health and Animal Experimentation Authority, the Spanish Drug Registration Authority and the Central Animal Health Authority) whose results have been partially published (Alonso-Hearn *et al.,* 2012; Garrido *et al.,* 2013; Ramon A Juste *et al.,* 2009).

Moreover, simultaneous infection of herds with *Map* and *M. bovis* may occur (Seva *et al.,* 2014) and it may be responsible for a reduced sensitivity (Se) of the cell-mediated immune (CMI) response based tests to detect bTB (Álvarez *et al.,* 2009). Over the last few years, antigens that are present in *M. bovis* but absent in both: tuberculosis (BCG) and PTB vaccines, such as ESAT-6/CFP-10 or Rv3615c, have been assayed as an alternative to avian and bovine purified protein derivatives (A-PPD and B-PPD), traditionally used in the current Comparative Intradermal Test (CIT) and interferon (IFN)-gamma assays (Flores-Villalva *et al.,* 2012).

Another issue that may affect bTB diagnosis is exposure to environmental mycobacteria, especially *Mycobacterium avium avium* (*M. a. avium*). Previous studies have concluded that exposure to *M. a. avium* may impart a degree of immunity to *M. bovis* infection that can compromise currently used diagnostic tests, making improvement of test Se dependent on the use of specific antigens (Hope *et al.,* 2005). Regarding vaccine protection, some authors have concluded that sensitization with environmental mycobacteria may adversely affect the efficacy of the BCG vaccination (Buddle *et al.,* 2002) whereas others suggest that there is no evidence that natural pre-exposure to M. avium reduces the effectiveness of BCG vaccination (Howard *et al.,* 2002) and that it rather causes an overall protection that cannot be further increased by vaccination.

The goal of this work was to assess different strategies to avoid PTB vaccination interference with CMI response-based bTB detection tests in cattle experimentally challenged by the use of both: alternative interpretation criteria for the standard comparative intradermal test and new more specific antigens (Hope *et al.,* 2005).

MATERIALS AND METHODS

Calves, inclusion criteria and vaccination

The experimental scheme is detailed on Fig 1. Thirty Fresian calves from a feedlot were preselected to carry out this experiment. These animals were born in 13 different farms located in Northern Spain. Two months after birth, at week 0 (W0), animals were submitted to a IFN-gamma release assay with standard (A-PPD, B-PPD) or more specific (ESAT-6/CFP10 and Rv3615c) antigens for the diagnosis of bTB. Immediately afterwards, 20 of them were subcutaneously vaccinated against *Map* with 1ml of a heat inactivated vaccine (Silirum1 CZV, Porriño, Pontevedra, Spain) in the dewlap (Garrido *et al.,* 2013). To confirm absence of contact with *M. bovis*, the samplings were repeated twice (W4, W12). Only 15 animals: 10 vaccinated and 5 non-vaccinated were kept and transferred to the biosafety level 3 (BSL-3) facilities in NEIKER. After arrival, calves were split into three separate groups of five animals each. The 15 remaining calves were kept in the feedlot and not further followed.

M. bovis challenge

All the experimental procedures were carried out according to the European, National and Regional Law and Ethics Committee regulations. The experimental design underwent ethical review and approval by NEIKER's Animal Care and Use Committee and by the Agriculture Department (PARAPATO-1264-BFA). Once animals were transferred to the BSL-3 facilities, they went through a two-week adaptation period. At W18 animals were sampled and five vaccinated and five non-vaccinated animals were challenged with 10^6 colony forming units (CFU) of an *M. bovis* field isolate suspended in 2 ml of phosphate-buffered saline (PBS) by the endotracheal route. The isolate used for challenge was originally obtained from a naturally infected cow and identified as *M. bovis* spoligotype profile SB0339 according to the M.bovis Spoligotype Database website (www.mbovis.org). Prior to challenge, animals

underwent intramuscular sedation with Xylazine (10 mg/50 kg). The final experimental groups were: PTB vaccinated and *M. bovis* infected (VAC/INFEC), PTB vaccinated and *M. bovis* non-infected (VAC/NIN), and PTB non vaccinated and *M. bovis* infected (NVAC/INFEC).

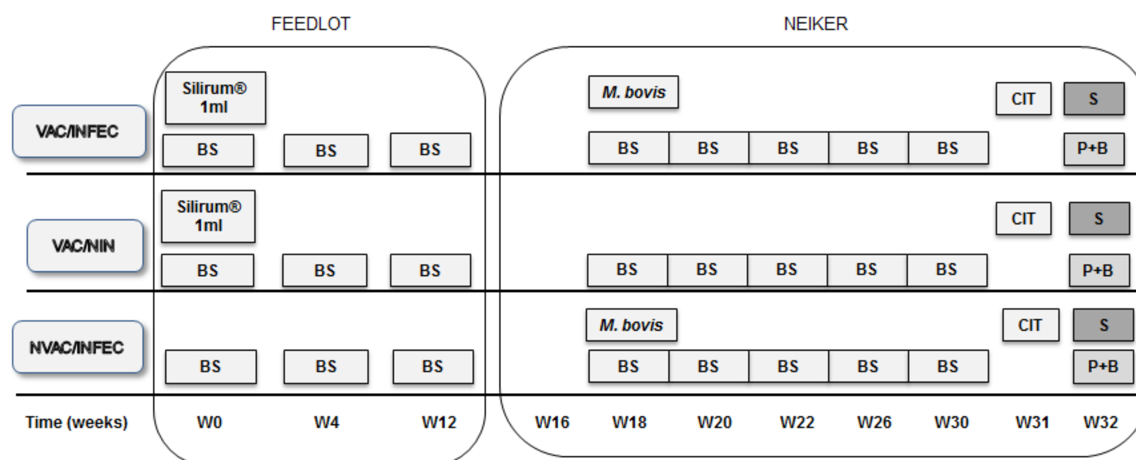

Fig 1. Experimental scheme. VAC/INFEC: vaccinated infected group. VAC/NIN: vaccinated non-infected group. NVAC/INFEC: non-vaccinated infected group BS: Blood Sampling. S: Slaughter. P+B: Pathology and Bacteriology. CIT: Comparative Intradermal Test. W: Week.

Blood sampling

Blood was collected from the jugular or caudal vein at W0, W4, W12, W18, W20, W22, W26 and W30 in tubes with lithium heparin and immediately aliquoted and processed in the laboratory.

DIAGNOSTIC TESTS

IFN-gamma release assay.

Stimulation of whole blood with B-PPD, A-PPD and defined antigens, ESAT-6/CFP-10 and Rv3615, was carried out within 8 hours after sample collection. Five 1.5 ml aliquots of whole blood from each animal were stimulated with: 100 µl of PBS, 100 µl of A- and B-PPD (20µg/ml as assay concentration) (CZV, Porriño, Pontevedra, Spain) respectively, and with 150 µl (to achieve an assay concentration of 5 µg/ml each peptide) of ESAT-6/CFP10 and Rv3615c antigens that were tested as sets of

overlapping peptides representing either ESAT-6/CFP-10 in one peptide cocktail or, alternatively, a peptide cocktail representing Rv3615c (Vordermeier *et al.,* 1999). After incubating the plates for 16-24h at 37°C 5 - 7% CO2 the stimulated samples were centrifuged and subsequently the plasma was separated.

Quantification of IFN-gamma concentration in the plasma's supernatant was performed by ELISA using the Bovigam® commercial kit according to the manufacturer's instructions (Prionics, Schlieren, Switzerland).

For result analysis, our first approach was to represent mean optical density (OD) values for each antigen to compare the treatment groups. The OD of the non-stimulated samples was subtracted from the OD of those stimulated with the different antigens. Afterwards, OD cutoffs were established for each defined *M. bovis* antigen, and frequencies of positive results were calculated and compared among groups.

Cut-offs alternative to the currently established ones for the standard antigens were studied for this experiment. Also, considering that our group of interest was the VAC/NIN, a new cutoff for the specific antigen (ESAT-6/CFP10) different from the previously defined cut-off by Vordermeier et al. (Vordermeier *et al.,* 2001), was selected. The goal was to find cut-offs that would allow us to improve and maximize both: Se and Sp of the diagnostic techniques. This approach will be thoroughly explained in the results section.

Comparative intradermal test (CIT).

The CIT was carried out at W3, this is, one week before slaughter. The test was performed according to the European Communities Commission Regulations (regulation1226/2002, amending annexes A and B of the consolidated Council Directive 64/432/EEC) and the Royal Decree RD2611/1996 by the Official Veterinary Services inoculating 0.1 ml of B-PPD and A-PPD. In addition to the standard PPD antigens, 0.1 ml of a peptide cocktail (100 µg/ml/peptide) and a protein cocktail (100

µg/ml/protein) representing ESAT-6/CFP10 and Rv3615c were also used. Four sites on the necks of the animals were selected and the skin thickness of every injection site was measured before and 72 h after the inoculation. The interpretation was carried out according to official criteria (EU Council Directive 64/432/CEE and RD 2611/1996).

Since standard criteria showed lower Se or Sp in vaccinated animals, alternative criteria were studied for skin test interpretation in the different tests regarding not only the absolute but also the relative skin thickness increase threshold and antigen comparison. The obtained outcomes are shown in the result section.

Post mortem studies

The animals were slaughtered at W32 in three consecutive days, five calves per day. The animals underwent sedation with XILAGESIC® 2% (10 mg/ 50 kg l.w) (Laboratorios Calier, S.A., Barcelona, Spain) and then euthanized by an intravenous injection of T61 (4-6ml/50kg). Complete necropsies were carried out and samples were collected from several organs (lymph nodes, lung, tonsils, liver and kidney) for histopathological and microbiological analysis.

Data analysis.

Frequency of positive results in each group/technique/antigen and time was used as a qualitative variable for diagnostic time dynamics description and group Se and Sp estimates comparison.

RESULTS

Success of challenge procedure

Infection was achieved in all challenged animals as all were confirmed infected by bacteriological and pathological analysis. VAC/NIN animals did not present lesions compatible with bTB, whereas all the animals belonging to the challenged groups did.

All isolates displayed the same *M. bovis* spoligotype as the challenge strain. Post-mortem findings will be reported elsewhere.

IFN-gamma

The mean value for every IFN-gamma result obtained was calculated. If the outcome for the B-PPD was ≥ 0.1 and B-PPD>A-PPD or Rv3615c was ≥ 0.1 or ≥ 0.3 for ESAT-6/CFP10 the results were considered *M. bovis* positive, respectively.

Evolution of average IFN-gamma release over time per group and antigen is shown on Fig 2. The IFN-gamma levels upon stimulation with standard antigens A-PPD and B-PPD (Fig 2A, 2B and 2C) showed slight response increase in both vaccinated groups (VAC/NIN and VAC/INFEC) at W12, whereas the NVAC/INFEC group did not respond to standard antigens during the pre-infection period. However, upon challenge, reactions against A-PPD and B-PPD rose in all three groups of animals. The VAC/NIN (Fig 2A) group showed a heterogeneous response with peaks and troughs from W18 to W26, always higher to the A-PPD than to the B-PPD and declining sharply in both cases after W26. On the contrary, the NVAC/INFEC group (Fig 2C) showed an increasing response following B-PPD stimulation throughout the post-infection period.

The response to the defined antigens showed higher definition than the standard ones as seen in Fig 2D, 2E and 2F. During the pre-challenge period none of the animals in any of the groups showed any kind of response against ESAT-6/CFP10 or Rv3615c. However, infection immediately triggered a response in both infected groups (VAC/INFEC and NVAC/INFEC) (Fig 2E and 2F) although the NVAC/INFEC animals (Fig 2F) showed slightly higher IFN-gamma levels compared to the VAC/INFEC animals (Fig 2E). Surprisingly, the VAC/NIN group (Fig 2D) showed a minimum response at W22. ESAT-6/CFP10 showed higher discrimination power since differences between infected and non-infected groups were greater.

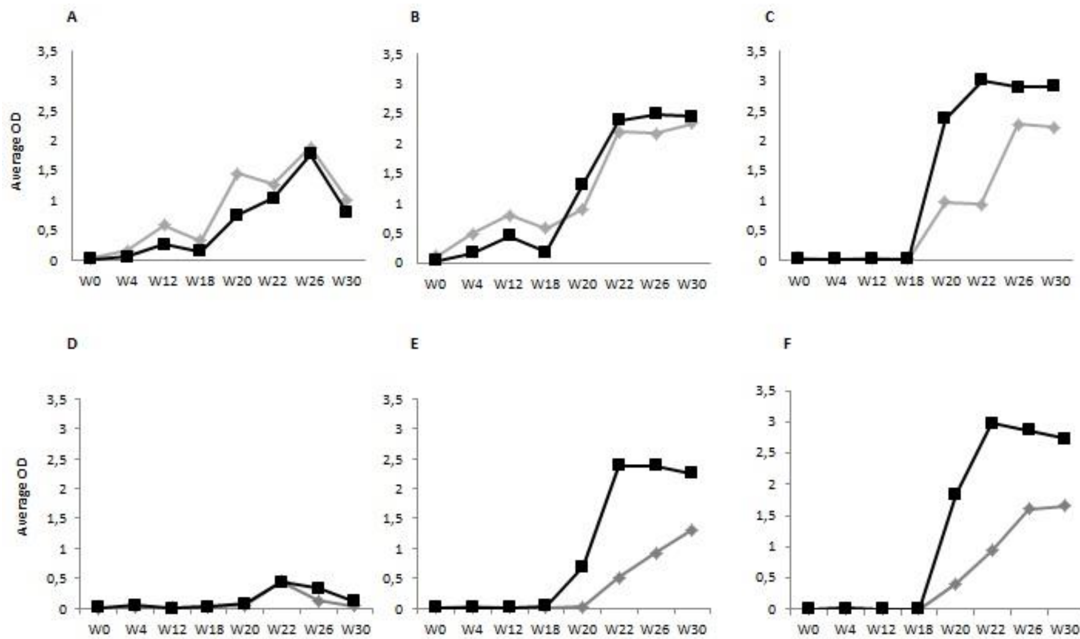

Fig 2. Cellular immune response measured as IFN-gamma release. Avian-PPD (gray rhombus), Bovine-PPD (black square) (A, B, C), ESAT-6/CFP10 (black square) and Rv3615c (gray rhombus) (D, E, F). Groups: vaccinated non-infected (VAC/NIN) (A and D), vaccinated infected (VAC/INFEC) (B and E) and nonvaccinated infected (NVAC/INFEC) (C and F).

The qualitative results for the IFN-gamma release test are shown on Table 1. The B-PPD detected all infected animals in the NVAC/INFEC during the post-infection period, showing different degrees of detection in the VAC/INFEC group and slight cross reactivity in some of the VAC/NIN animals. Applying the calculated cut-offs for the defined *M. bovis* antigens, in the VAC/NIN group a slight non-specific reaction could be observed for both during the postinfection weeks although infection was discarded according to histopathological and microbiological results. This behavior tended to disappear in the last sampling at W30. These defined specific antigens exhibited a mild delay in the detection of infected animals in the VAC/INFEC group. Regarding Rv3615c, the reactivity seemed to decrease at W30. Both, Rv3615c and ESAT-6/CFP10 peptide cocktails were equally efficient at classifying animals from the NVAC/INFEC group from W22 on

Table 1. Percentage of positive animals for INF-gamma testing.

STATE	ANTIGEN	W0	W4	W12	W18	W20	W22	W26	W30
VAC/INFEC	Bovine – Avian PPD	0%	0%	0%	0%	0%	20%	40%	20%
	Rv3615c	0%	0%	0%	0%	20%	100%	20%	0%
	ESAT-6/CFP-10	0%	0%	0%	0%	0%	60%	20%	0%
VAC/NIN	Bovine – Avian PPD	0%	0%	0%	0%	100%	40%	40%	80%
	Rv3615c	0%	0%	0%	0%	0%	80%	100%	80%
	ESAT-6/CFP-10	0%	0%	0%	0%	80%	100%	100%	100%
NVAC/INFEC	Bovine – Avian PPD	0%	0%	0%	0%	100%	100%	100%	100%
	Rv3615c	0%	0%	0%	0%	80%	100%	100%	100%
	ESAT-6/CFP-10	0%	0%	0%	0%	100%	100%	100%	100%

VAC/NIN: vaccinated non-infected group. VAC/INFEC: vaccinated infected group. NVAC/INFEC: non-vaccinated infected group. W: week. The Optical Density (OD) cut-off for Rv3615 - PBS is >0.1 and > 0.3 for ESAT-6/CFP10 - PBS. For the standard antigens the met requirements are B-PPD > A-PPD and OD for B-PPD - PBS is > 0.1. Vaccination was performed at W0 and experimental challenge at W18.

Comparative Intradermal Test (CIT).

Skin test raw data and interpretation are detailed on Table 2. All calves included in the VAC/NIN group (5/5) showed a PPD-A response bias and were therefore classified as CIT-negative. However, substantial responses were recorded to PPD-B which meant that 3/5 calves were classified as SIT-positive (Table 2).

The skin test was performed using two more antigenic preparations: ESAT-6/CFP10-Rv3615c as a peptide or protein cocktail. We considered a skin increase of 2 mm or greater as a positive result. Using this cut-off value, 1/5 VAC/NIN animals tested positive to the ESAT-6/CFP10-Rv3615c peptide cocktail while none responded to the protein cocktail (0/5, Table 2). Taken together, while the Sp of the SIT is compromised by PTB vaccination, this limitation can be overcome by using the CIT or defined protein antigens as skin test reagent.

Table 2. Intradermal Response: raw data and interpretation.

State	Calf	A-PPD (Δmm)	B-PPD (Δmm)	Interpretation		ESAT-6/CFP10/Rv3615c cocktail (Δmm interpretation)			
				SIT	CIT	Peptide		Protein	
VAC/NIN	1	12	5	+	N	0	N	0	N
	2	14	5	+	N	3	+	0	N
	3	9	3	N	N	1	N	0	N
	4	6	4	+	N	0	N	0	N
	5	8	3	N	N	0	N	0	N
VAC/INFEC	6	9	9	+	N	3	+	5	+
	7	7	6	+	N	6	+	4	+
	8	12	23	+	+	11	+	15	+
	9	11	10	+	N	6	+	3	+
	10	8	18	+	+	7	+	7	+
NVAC/INFEC	11	2	9	+	+	5	+	6	+
	12	1	8	+	+	8	+	6	+
	13	1	12	+	+	9	+	7	+
	14	0	9	+	+	3	+	3	+
	15	1	5	+	N	4	+	6	+

VAC/NIN: vaccinated non infected group. NVAC/INFEC: non vaccinated infected group. VAC/INFEC: vaccinated infected group. PPD: protein purified derivative. SIT: single intradermal test, CIT: comparative intradermal test. N: negative. +: positive. Δmm: skin thickness increase in mm. The SIT is considered positive when an increase equal or bigger than 4mm is observed in the PPD-B injection site after 72h. The CIT is considered positive when the skin thickness increase at the PPD-B injection site is 4 mm greater than the increase induced at the PPD-A injection site after 72h. A ESAT 6/CFP10/Rv3615c peptide or protein cocktail result will be considered positive if the skin thickness increase is equal to or bigger than 2mm.

Following *M. bovis* infection, the detection (Se) of infection in the PTB vaccinated group (VAC/INFEC) was severely compromised as 3/5 animals escaped CIT detection due to the PPD-A biased responses (Table 2) as well as in the non-vaccinated/infected group (NVAC/INFEC) were an animal out of 5 was considered as a negative one (4/5 Table 2). As discussed, the SIT is severely compromised in respect of Sp following PTB vaccination, and therefore the observation that it detected all infected animals regardless of their vaccination status is irrelevant (Table 2, VAC/INFEC and NVAC/INFEC groups, 10/10, Table 2). In marked contrast, the ability of the defined

antigen reagents to detect *M. bovis* infected calves was not affected by their vaccination status, as 10/10 animals in the VAC/INFEC and NVAC/INF groups were detected by protein and peptide cocktails (Table 2).

Table 3 shows the relative increase of the skin thickness 72h after the intradermal inoculation. The VAC/INFEC group showed the greatest (Mean = 230%) relative skin increase to the B-PPD followed by the NVAC/INFEC while the VAC/NIN group exhibited the smallest (Mean = 63.8%) skin relative increase with the B-PPD. The A-PPD showed the greatest skin increase in the vaccinated groups (VAC/INFEC (Mean = 127.4%) and VAC/NIN (Mean = 128.6%)). Both, peptide and protein cocktails showed highest skin increase percentages in the infected groups, (VAC/INFEC: Mean = 87.6% and Mean = 101%; NVAC/INFEC: Mean = 92.6%; and Mean = 86.4% respectively) (Table 3).

Table 3. Individual skin test results: skin thickness increase relative to the initial reading.

State	Calf	Avian-PPD Δ%	Bovine-PPD Δ%	ESAT-6/CFP10/Rv3615c Peptide cocktail Δ%	ESAT-6/CFP10/Rv3615c Protein cocktail Δ%
VAC/NIN	1	120	63	0	0
	2	140	56	30	0
	3	150	60	17	0
	4	100	80	0	0
	5	133	60	0	0
	MV=	128.6	63.8	9.4	0
VAC/INFEC	6	150	150	43	71
	7	140	150	120	100
	8	109	383	100	167
	9	138	167	75	50
	10	100	300	100	117
	MV=	127.4	230	87.6	101
NVAC/INFEC	11	33	180	83	120
	12	13	133	133	100
	13	13	171	180	88
	14	0	113	27	38
	15	17	62	40	86
	MV=	15.2	131.8	92.6	86.4

VAC/NIN: vaccinated non infected group. NVAC/INFEC: non vaccinated infected group. VAC/INFEC: vaccinated infected group. Δ%: skin thickness increase at 72 h after inoculation expressed as a percentage. MV: mean value.

Table 4 shows the performance of the different alternative diagnostic criteria. A SIT interpretation with an absolute skin thickness increase of 6 mm or more detected all the VAC/INFEC animals but none of the VAC/NIN, accounting for 100% Se and Sp.

With a comparative interpretation where an increase of 6 mm in the B-PPD that was equal to or greater than the A-PPD, all VAC/NIN were correctly classified as negative, but only 3/5 of the VAC/INFEC were scored as positive. Thus the Se was 60% and the Sp 100%.

Table 4. Skin thickness interpretation criteria after application of the different antigens in the skin test.

Antigen	Test type and interpretation	Cut-off positive criteria	Sensitivity %	Specificity %
Official diagnostic criteria: vaccinated and non-vaccinated animals				
PPD	Single official	Standard: Δmm B-PPD≥4	100	40
		Strict: : Δmm B-PPD>2	100	0
	Comparative official	Standard: Δmm B-PPD≥4 and >A-PPD	70	100
		Strict: Δmm B-PPD>2 and >A-PPD	70	100
Alternative diagnostic criteria (vaccinated animals only)				
PPD	Simple absolute	Δmm ≥ 6	100	100
	Comparative absolute	Δmm ≥ 6 and B-PPD ≥ A-PPD	60	100
	Single relative	≥ 100%	100	100
	Comparative relative	≥ 100% and B-PPD ≥ A-PPD	100	100
Peptide cocktail	Simple absolute	Δmm ≥ 3	100	80
	Single relative	≥ 40%	100	100
Protein cocktail	Simple absolute	Δmm ≥ 3	100	100
	Single relative	≥ 30%	100	100
Alternative diagnostic criteria (non-vaccinated animals only)				
Peptide cocktail	Simple absolute	Δmm ≥ 3	100	100
Peptide cocktail	Simple absolute	Δmm ≥ 3	100	100

A-PPD: avian purified protein derivative. B-PPD: bovine purified protein derivative.

In order to establish criteria, focus was set on the vaccinated groups because the aim of this work was to set up the parameters to be able to differentiate VAC/INFEC animals from those which were only PTB-vaccinated. Regarding relative readings, for the SIT, a cut-off at a 100% skin increase classified all the animals in the VAC/INFEC as bTB positive, while all in the VAC/NIN group were classified as non-bTB reactors (Table 3). This yielded 100% Se and 100% Sp (Table 4). A comparative reading, scoring as positive B-PPD relative increase greater than the A-PPD increase, also yielded 100% Se and Sp. Using the protein cocktail two different cut-offs were established. The first one takes into account the absolute skin increase and the second the relative. Despite the low number of animals belonging to each group, these results are very interesting since all the animals were correctly classified.

DISCUSSION

The main aim of this study was to assess the interference of an inactivated PTB vaccine with the official bTB diagnostic tests. In order to improve the diagnostic efficacy of the CMI-based test, the immunological response of the animals was determined comparing the standard antigens PPD-A and PPD-B with two different formulations of defined antigens (ESAT-6/CFP10 and Rv3615c) (Vordermeier *et al.,* 2011). In addition, new criteria for result interpretation according to the vaccination status of animals were explored.

During the last decade great efforts have been dedicated to the study and development of new specific antigens (Meng *et al.,* 2015; Vordermeier *et al.,* 2011) that have been thoroughly assessed by in vitro IFN-gamma tests (Casal *et al.,* 2012). The use of these antigens has received attention for the skin test (Whelan *et al.,* 2010) in PTB vaccinated animals, and it has been proven that protein (Flores-Villalva *et al.,* 2012; Whelan *et al.,* 2010) and peptidic cocktails (Casal *et al.,* 2012) can be suitable under field conditions.

Furthermore, to the best of our knowledge, this is the first time that the skin test has been performed with these specific cocktails in PTB vaccinated animals and subsequently *M. bovis* infected cattle, although these antigens had been previously shown not to be recognized following PTB vaccination in goats (Pérez de Val *et al.,* 2012).

The *M. bovis* challenge process was carried out successfully; all the calves belonging to infected groups became infected regardless of their vaccination status. However, the severity and extension of the lesions was lower in the VAC/INFEC animals (data not shown). These results are in agreement with the findings from Pérez del Val et al (Pérez de Val *et al.,* 2012) who also reported that *Map* vaccinated and *M. bovis* infected goats showed minor severity and extension of the lesions in an experimental setting, suggesting that PTB vaccination could provide a certain degree of containment of bTB dissemination (Pérez de Val *et al.,* 2012).

The dynamics of the IFN-gamma test results show that PTB vaccination induced a response against standard PPD mycobacterial antigens that was predominantly of the avian type before to *M. bovis* challenge. Although a noticeable degree of cross reactivity to B-PPD was observed it did not affect the Sp of the CIT. After infection, all groups switched to a predominantly bovine PPD-biased response that was slightly lower in the vaccinated group compared to the non-vaccinated group. All the animals belonging to the VAC/INFEC group were categorized as infected only in the first sampling post-challenge at W20, while all NVAC/INFEC animals were positive throughout the complete post-infection period. This indicates that IFN-gamma test did not work well in vaccinated animals in terms of Se and also Sp.

The more specific antigens worked better in terms of Se, in particular ESAT-6/CFP10 that only failed to detect one animal on W20, two weeks post challenge. It was also the most specific since it only yielded false positive results in the non-infected

group for two animals four weeks post-infection (W22) and for one calf eight weeks post-infection (W26). The movement of the animals to the P3 facilities might have changed the microbial environment and induced an unspecific cellular immune response that reached a certain development in the VAC/NIN group, but that was quickly replaced by a more specific one in the animals that were challenged with *M. bovis*.

Results have demonstrated that the response pattern of the traditional antigens versus the more specific ones can be very different. The ESAT-6/CFP10 and Rv3615 antigens did not show any kind of response during the post-vaccination period and they just underwent a raise in their response after *M. bovis* challenge. A-PPD and B-PPD responses based on the IFN-gamma release made differentiation difficult especially among the VAC/INFEC animals. Similar results have been observed with the same antigens in goats (Pérez de Val *et al.,* 2012).

In this experiment we decided to apply 2 interpretation criteria for the qualitative IFN-gamma results: one for the B-PPD and a second one for the specific antigens responses. The most extended OD cut-off point (0.1) was selected for the B-PPD. For the ESAT-6/CFP10 the OD cut-off was set at 0.3 and at 0.1 for Rv3615c. Selecting these cut-offs, the Se of the technique in the most problematic group (VAC/INFEC) reached 100% when the ESAT-6/CFP10 antigen was used. This Se rate was maintained from the second post-infection sampling throughout the experiment. Our data showed that the Se obtained with this specific antigen was higher than the one obtained with B-PPD as previous researchers demonstrated (Aagaard *et al.,* 2006; Flores-Villalva *et al.,* 2012).

Regarding the conventional CIT results, all the animals belonging to the VAC/NIN and NVAC/INFEC groups were correctly classified in relation to the infectious status according to treatment and necropsy results. The interference problem arose

when the VAC/INFEC group has to be diagnosed using the same criteria. Applying the traditional comparative skin test 60% of the VAC/INFEC animals were misclassified as *M. bovis* non-infected.

If new cut-off points were set up, this drawback could be overcome. Regarding the vaccinated and non-vaccinated groups alternative cut-offs were established for every antigen studied (Table 4). As it can be observed, from all the assessed possibilities, those which measured the relative increase of the skin thickness were the most reliable in both vaccinated groups, in terms of Se and Sp.

When the non-vaccinated animals were taken into account, whether the chosen diagnostic interpretation is the official criterion or the new criterion, 100% of the animals were diagnosed correctly in relation to our gold standard: the necropsy results. These outcomes reinforce the idea that our efforts should focus on validating a technique able to identify all the animals from the problematic group, VAC/INFEC animals.

In spite of the low number of animals and that they were kept under experimental conditions, the Se and Sp obtained (100% respectively) when using the protein cocktail in the skin test were very encouraging. All the animals belonging to the three different groups were correctly classified. That is why in view of the outstanding results it would be of great interest to test the protein cocktail under field conditions.

In this experimental scheme, we have demonstrated that it is possible to differentiate *M. bovis* infected animals even after PTB vaccination by including the specific antigens into the skin test, or by adopting new interpretation criteria for the conventional ones. These findings lend support to the PTB vaccination strategies showing that vaccination in bTB affected environments should not be a problem.

CONCLUSIONS

Our results prove that the ESAT-6/CFP10 and Rv3615c proteinic and peptidic cocktails can be used as skin test reagents in the face of PTB vaccination and *M. bovis* infection without compromising either Se or Sp. In respect to IFN-gamma results, use of these defined antigens improved the Se and Sp compared to the conventional antigens A-PPD and B-PPD. In conclusion PTB vaccination produces interference in cattle experimentally infected with *M. bovis*, but this could be overcome if new testing strategies such as new specific antigens were applied alternative to or complementing the official bTB diagnostic tests or if new diagnostic criteria with traditional antigens were established. Although the results presented are promising, this study has been performed in experimental bTB infection conditions and they should be further validated in field conditions.

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5.2. SECOND STUDY

Preliminary results indicate That inactivated Vaccine against Paratuberculosis could Modify the course of experimental *Mycobacterium bovis* infection in calves. Serrano, M., Elguezabal, N., Sevilla, I.A., Geijo, M. V., Molina, E., Juste, R.A., Garrido, J.M., 2017. Preliminary Results Indicate That Inactivated Vaccine against Paratuberculosis Could Modify the Course of Experimental *Mycobacterium bovis* Infection in Calves. *Frontiers in Veterinary Science*, 4, p. 175. doi: 10.3389/fvets.2017.00175.

Preliminary results indicate that inactivated vaccine against paratuberculosis could modify the course of experimental *Mycobacterium bovis* infection in calves

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Abstract

Although paratuberculosis (PTB) vaccination has been recognized as an effective tool to control the disease, its use has been limited in countries undergoing bovine tuberculosis (bTB) eradication programs because of its interference with the diagnostic techniques. Due to this restraint, little is known about the effect of vaccinating against PTB on the progression of bTB infection. To assess this topic, an experimental infection was carried out including the following three groups of five calves each: non-vaccinated infected with *Mycobacterium bovis* (NVI), vaccinated against PTB infected with *M. bovis* (VI), and vaccinated against PTB non-infected (VNI). The level of infection attending to pathological and bacteriological parameters was evaluated at necropsy in collected tissue samples. Infection was confirmed in all challenged animals being the lung and thoracic regions most affected for all studied parameters. The VI group presented 15.62% less gross lesions in the thoracic region than the NVI, although no significant differences were found. Only one vaccinated animal presented gross lesions in the lung, compared to three non-vaccinated calves. NVI animals showed an average of 1.8 lung lobes with gross lesions whereas in the vaccinated

group the average number of affected lobes was 0.2, representing an 89% reduction. Significant differences were not found, although a tendency was observed ($p = 0.126$). Pathological and culture scores showed the same tendency. Vaccination induced a 71.42 and 60% reduction in lesion and culture scores in the lung as well as a 23.75 and 26.66% decline, respectively, in the thoracic region. The VI group showed lower positivity in the rest of the areas for all measured criteria except for the head. In order to reinforce our results, further research on a larger sample size is needed, but the results from this study suggest that PTB vaccination could confer certain degree of protection against bTB infection, supporting the view that PTB vaccination could increase resistance to the main mycobacterioses that affect animals.

Keywords: paratuberculosis, bovine tuberculosis, vaccine, interference, crossed-protection.

INTRODUCTION

Bovine tuberculosis (bTB) and paratuberculosis (PTB) are mycobacterial diseases that have a huge economic impact on cattle, especially on dairy herds (Hasonova and Pavlik, 2006; Waters *et al.,* 2012). Both present a widespread distribution, affecting domestic hosts (Aagaard *et al.,* 2010; Muñoz-Mendoza *et al.,* 2016) and wildlife species (Aranaz *et al.,* 2004; Carta *et al.,* 2013), promoting the successful dissemination of their etiological agents.

Paratuberculosis or Johne's disease is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Progression of the disease causes a chronic gastrointestinal granulomatous inflammation. Different factors, such as the long incubation period of the disease, fecal oral route transmission, intermittent excretion periods added to high resistance of mycobacteria in the environment, and limited performance of diagnostic methods, make control of the infection difficult to achieve. PTB vaccination has been proven to restrain the disease in cattle (Juste *et al.,* 2009), sheep (Reddacliff *et al.,* 2006), and goats (Singh *et al.,* 2007). Excretion of bacterial burden is considerably reduced, containing the spread of the infection and therefore diminishing the number of

clinical cases (Alonso-Hearn *et al.,* 2012; Bastida and Juste, 2011). Nevertheless, the interference induced by PTB vaccination with the current interpretation criteria (Serrano *et al.,* 2017) of the bTB official diagnostic tests (Garrido *et al.,* 2013) results in the restricted use of PTB vaccination. MAP-based vaccination in cattle is not allowed by the Animal Health Authorities of most countries. Despite the diagnostic interference, a certain degree of containment of the lesion dissemination from the target organs after a bTB infection has been previously reported in PTB-vaccinated goats (Pérez de Val *et al.,* 2012). This suggests that if interference issues are avoided, PTB vaccination can be used for PTB control and might also be beneficial against bTB conferring some degree of protection.

The degree of interference of PTB vaccination with official bTB diagnostic tests has been evaluated previously in cattle (Garrido *et al.,* 2013; Köhler *et al.,* 2001) and goats (Bezos *et al.,* 2012; Chartier *et al.,* 2012). Cross reactivity in the skin test has proven to be limited if the comparative intradermal test is used in bTB-free bovine herds (Garrido *et al.,* 2013). These findings have been based on an exhaustive analysis of results from a vaccine clinical trial under field conditions. To evaluate the effect of PTB vaccination on bTB infection and the interference with bTB diagnosis, an experimental infection with *Mycobacterium bovis (M. bovis*) in bovines previously vaccinated against PTB was performed. Results on interference using alternative diagnostic criteria and specific antigens have been reported in a separate paper (Serrano *et al.,* 2017) while pathological and bacteriological changes associated with vaccination in a bTB experimental infection are the goal of this report.

MATERIALS AND METHODS

Ethics Statement

Animals used in this study had their origin in commercial farms. With the purpose of obtaining data for this trial, calves were submitted to clinical practices standardized and regulated by the European, Spanish and Regional Law and Ethics Committee. The

experimental design underwent ethical review and approval by NEIKER's Animal Care and Use Committee and by the Agriculture Department (PARAPATO-1264-BFA).

Animal Selection

Thirty Friesian calves from 13 different farms in northern Spain were selected in a feedlot. For final selection of animals, absence of previous contact with mycobacteria was considered. IFN-γ release assay (IGRA) with standard tuberculins (A-PPD, B PPD) as well as with more specific antigens (ESAT-6/CFP10 and Rv3615c) (Juste and Perez, 2011) already tested for the diagnosis of bTB was the assay used for this purpose. The first blood sampling was carried out at week 0, at the age of 2 months. After the blood samples were collected, 20 randomly selected animals were vaccinated subcutaneously in the dewlap with 1 ml of a heat-inactivated vaccine (Silirum® CZV, Pontevedra, Spain) and 10 remained unvaccinated. In order to reassure the absence of previous contact with *M. bovis* or other mycobacteria, blood samplings at the feedlot were repeated twice, at weeks 4 and 12. Finally 10 vaccinated and five non-vaccinated animals with negative results for IGRA and without evident pathologies were selected and transported to the biosafety level 3 (BSL-3) facilities in NEIKER where three groups of five animals each were formed.

M. bovis Challenge

A 2-week adaptation period was established for the calves after their arrival at the BSL-3 facilities. At week 18 post-vaccination, five vaccinated and five non-vaccinated animals were challenged by the endotracheal route with 10^6 colony-forming units (CFUs) of a *M. bovis* field isolate suspended in 2 ml of phosphate-buffered saline after intramuscular sedation with Xylazine (10 mg/50 kg). The *M. bovis* challenging isolate was spoligotype SB0339 according to the *M. bovis* Spoligotype Database website (http://www.mbovis.org). The final experimental groups were as follows: PTB vaccinated and *M. bovis* infected (VI), PTB vaccinated and *M. bovis* non-infected (VNI),

and PTB non-vaccinated and *M. bovis* infected (NVI).

Post mortem Studies

The animals were slaughtered at week 14 post-infection in three consecutive days, five calves per day. Upon sedation with XILAGESIC® 2% (10 mg/50 kg lw) (Laboratorios Calier, S.A., Barcelona, Spain), animals were euthanized by an intravenous injection of T61 (4–6 ml/50 kg) (Intervet International GMBH, Unterschleissheim, Germany). Complete necropsy was performed although special focus was set on the respiratory system. All tissue specimens were individually collected and processed for pathological and microbiological analysis. Collected tissues per region included were: head [tonsils, nasal turbinate, and parotid and retropharyngeal and mandibular lymph nodes (LNs)], thorax (tracheal, tracheobronchial, mediastinal, pulmonary, and prescapular LNs), lung (right and left cranial and caudal lobes and medium and accessory lobes), abdomen (liver, spleen, and hepatic LNs), and others (prefemoral LNs).

Gross Pathology

All tissues were visually inspected for lesions compatible with TB infection. Scoring of lesions according to Palmer et al. (Palmer *et al.,* 2007) was performed independently by two researchers, and the mean value of both scores was used. Briefly, the scoring system for lung was as follows: 0: no visible gross lesion, 1: no visible external gross lesion but internal detected after splicing, 2: less than five lesions smaller than 10 mm, 3: over five lesions smaller than 10 mm, 4: more than one lesion bigger than 10 mm, and 5: gross confluent lesions. In the case of LNs, scoring was as follows: 0: no visible gross lesions, 1: focal lesions of 1–2 mm, 2: a lot of small foci, and 3: extended lesions. Once scores were assigned to each lesion, total and regional scores per animal were calculated by adding them.

Bacterial Culture

Bacterial tissue culture was performed as described previously (Garrido *et al.,* 2011). Briefly, 2 g of tissue samples were homogenized in 10 ml of sterile distilled water. Five millilitres of the suspension was decontaminated and processed for liquid culture in BBL MGIT tubes supplemented with BBL MGIT PANTA and BACTEC MGIT growth supplement (Becton Dickinson, Franklin Lakes, NJ, USA) following manufacturer's instructions. BBL MGIT tubes were incubated for 42 days in a BACTEC MGIT 960 System. The remaining 5 ml were decontaminated in hexadecyl-pyridinium chloride 0.75% (w/v) for 12–18 h for solid culture. After a 5 min centrifugation step at 2,500 × g, pellets were cultured in Coletsos tubes (bioMérieux SAF-69280 Marcy l'Etoile France) at 37°C during 4 months.

Once the MGIT incubation period finished, DNA extraction was performed on culture from all positive tubes and some negative tubes. PCR was carried out subsequently to confirm that growth was due to *M. bovis* (Sevilla *et al.,* 2015).

After the incubation period for solid culture was completed, colonies were visualized under a stereoscope and scraped for DNA extraction and M. tuberculosis complex PCR confirmation (Sevilla *et al.,* 2015). All isolates were confirmed as *M. bovis* SB0339 by spoligotyping (Kamerbeek *et al.,* 1997). A culture score was defined in order to categorize the infection level of each tissue. In this case, scores were as follows: 0: no growth, 1: less than 10 colonies, 2: between 10 and 50 colonies, and 3: over 50 colonies (Garrido *et al.,* 2011).

A tissue was considered positive for culture when it gave a positive result by solid culture, liquid culture, or both.

Statistical Analysis

Reduction by vaccination was calculated by the formula (1 − VI/NVI) × 100 for each of the following parameters: solid culture score, number of positive tissues by culture, gross lesion score, and number of affected areas by gross pathology.

For the analysis, the number of tissues with positive cultures, with bTB compatible gross lesion and the solid culture and lesion scores, was calculated per area and animal. Differences in the degree of pathology and bacterial burden (lesion scores and culture scores) were compared using the Mann–Whitney *U*-test. Differences in the number of affected tissues by gross pathology and culture were assessed using Student's paired two-sample t-test, whereas Fisher's exact test was used for proportions of animals with lesions. Spearman's correlation test was applied to assess the association between culture and gross pathology results. In all cases, significance of the differences among groups for all variables was accepted at $p < 0.05$.

RESULTS

Clinical Signs

All animals included in the study went through the whole experiment,and no adverse reactions were reported after vaccination or challenge. No clinical signs of bTB such as wasting and coughing were observed in any of the animals after challenge. Infection of all challenged animals was confirmed by bacteriological and pathological analysis. The VNI group did not present gross lesions compatible with bTB or culture positive tissues.

Post mortem Analysis

Detailed pathology results from the infected groups of the study are shown in Table 1. The thoracic region and lung were the areas presenting a higher number of affected tissues and the NVI presented slightly higher scores as expected. Culture

results from the infected groups of the study are shown in Table 2. In this case, again thorax and lung were the most affected areas, although the VI group only presented one animal with one positive tissue. The analysis has been focused separately on head, thorax, and lung as well as on the total where all areas of the animal have been considered. The analysis of number of tissues presenting pathology and culture positive results is shown in Table 3.

Table 1. Tissues with tuberculosis compatible lesions in the infected groups of the study.

		Head					Thorax					Lung			Abdomen			Others	
Group	Animal ID	Nasal turbinate	Palatine tonsil	Parotid LN	Retropharingeal LN	Mandibular LN	Pulmonary LN	Tracheobronchial LN	Prescapular LN	Tracheal LN	Mediastinal LN	Right lobe	Left lobe	Accesory lobe	Hepatic LN	Liver	Spleen	Prefemoral LN	Score
VI	1								3										3
VI	2			1			4	4	3	2	6				2	1	1		24
VI	3							2	3	5									10
VI	4			2				5	3	9	5	4							28
VI	5								3	4									7
NVI	6							2		7	4	4	4	2					23
NVI	7								3	8	2	1	1					1	16
NVI	8									2	2	1		1					6
NVI	9							6	3	10	9								28
NVI	10			1	1			4	3	9	6				2		1		27

VI, vaccinated infected; NVI, non-vaccinated infected; LN, lymph node. One LN affected (right or left): dotted; two LNs affected (both right and left or two of three, cranial, caudal, or medial): light gray; three LNs affected (cranial, caudal, and medial): dark gray. Lesion score is the sum of the scores of all tissues.

Table 2. Tissues with positive culture results in the infected groups of the study.

		Head					Thorax							Lung					Abdomen		Others	
Group	Animal ID	Nasal turbinate	Right Palatine tonsil	Right Parotid LN	Right Retrophar LN	Right Mandibular LN	Caudal Tracheal LN	Middle Tracheal LN	Craneal Tracheal LN	Right Prescapular LN	Right Tracheobronquial LN	Left Tracheobronquial LN	Craneal Mediastinal LN	Right craneal lobe	Right caudal lobe	Right middle lobe	Left craneal lobe	Left caudal lobe	Hepatic LN	Spleen	Left Prefemoral LN	Score
VI	1							1		2												3
VI	2						2				2		1									5
VI	3												1									1
VI	4								2		2		2		2							8
VI	5					1			3	3	1											8
NVI	6								3	3			1	1			2	1				11
NVI	7								3	1												4
NVI	8								1						1							2
NVI	9								3	3	2		2									10
NVI	10				1				2	3	2		1						1			10

VI, vaccinated infected; NVI, non-vaccinated infected; LN, lymph node. MGIT culture positive: dotted; solid culture positive: light gray; both MGIT and solid culture positive: dark gray. Culture score is the sum of the scores of all tissues.

In the thoracic region, the NVI group presented a mean of 6.4 LNs with gross lesions with a minimum of two and a maximum of 10 affected LNs in each animal compared to the VI group with an average of 5.4 LNs affected with a minimum of two and a maximum of nine affected LNs. Significant differences among groups were not observed (p = 0.643). However, the reduction due to vaccination was of 15.62%.

Of the six lung lobes evaluated, the NVI group presented an average of 1.8 lobes with gross lesions with a minimum of zero and a maximum of five affected lobes per animal, much higher than the mean of 0.2 affected lobes found in the VI group, which had a minimum of zero and a maximum of one affected lobes. Although significant differences were not observed between the number of affected lobes, the tendency should be considered (t-test, p = 0.126). This represents an 89% reduction due to vaccination. Only one animal (1/5, 20%) from the VI group presented lesions in the lung compared to three animals (3/5, 60%) from the NVI group (Fisher's test; p = 0.189).

Table 3. Gross pathology and bacteriology analysis considering number of affected tissues per area.

	Pathology				**Bacteriology**			
	Mean (SEM)		**t-test**		**Mean (SEM)**		**t-test**	
	NVI	**VI**	***p***	**% R**	**NVI**	**VI**	***p***	**% R**
Head	0.4 (0.40)	0.6 (0.4)	0.733	0.00	1.0 (0.55)	1.4 (0.4)	0.572	0.00
Thorax	6.4 (1.43)	5.4 (1.50)	0.643	15.62	3.4 (0.60)	3.4 (0.40)	1.000	0.00
Lung	1.8 (0.92)	0.2 (0.20)	0.126	89.00	1.0 (0.55)	0.2 (0.20)	0.207	80.00
Total	9.2 (1.74)	6.8 (2.35)	0.436	26.10	6.6 (1.40)	5.4 (0.93)	0.495	18.18

NVI, non-vaccinated infected; VI, vaccinated infected; R, reduction due to vaccination.

Table 4. Gross pathology and bacteriology analysis considering lesion and culture score per area.

	Pathology				**Bacteriology**			
	Mean (SEM)		***U*-test**		**Mean (SEM)**		***U*-test**	
	NVI	**VI**	***p***	**% R**	**NVI**	**VI**	***p***	**% R**
Head	0.4 (0.40)	0.6 (0.40)	0.606	0.00	0.2 (0.20)	0.2 (0.20)	1.000	0.00
Thorax	16.0 (4.13)	12.2 (3.60)	0.401	23.75	6.0 (1.58)	4.4 (1.07)	0.344	26.66
Lung	2.8 (1.85)	0.8 (0.80)	0.288	71.42	1.0 (0.77)	0.4 (0.40)	0.521	60.00
Total	20.0 (4.10)	14.4 (4.90)	0.530	28.00	7.4 (1.83)	5.0 (1.37)	0.248	32.43

NVI, non-vaccinated infected; VI, vaccinated infected; R, reduction due to vaccination.

The number of affected tissues was always lower in the VI group than in the NVI groups except for the area that compromised the head. In this case, two animals presented gross lesions, one in one tissue and another in two in the VI group, whereas the NVI group presented one animal with gross lesions in two tissues. Tissue culture positivity was also slightly higher in the head in the VI group where an average of 1.4 ± 0.4 tissues with detectable bacteria was observed compared to the mean of 1±0.55 tissues in the NVI group. In the VI group, four animals presented positive tissue culture (4/5, 80%) compared to three in the NVI group (3/5, 60%).

Pathology and culture scores were always lower in the VI group than in the NVI group (Table 4 and Figure 1) except for the head where lesion scores were slightly higher in the VI group (0.6 ± 0.4 vs .0.4 ± 0.4). In any case, significant differences were not observed among analyzed areas. However, reductions in lung of 71.42 and 60% in lesion and culture scores, respectively, and of 23.75 and 26.66% in the vaccinated group for the same parameters in thorax should be noted. Correlation between both diagnostic methods considering number of affected tissues was best fit in lung ($r = 0.988$, $p < 0.0001$), followed by head ($r = 0.746$, $p = 0.013$), total ($r = 0.667$, $p = 0.035$), and thorax ($r = 0.655$, $p = 0.04$).

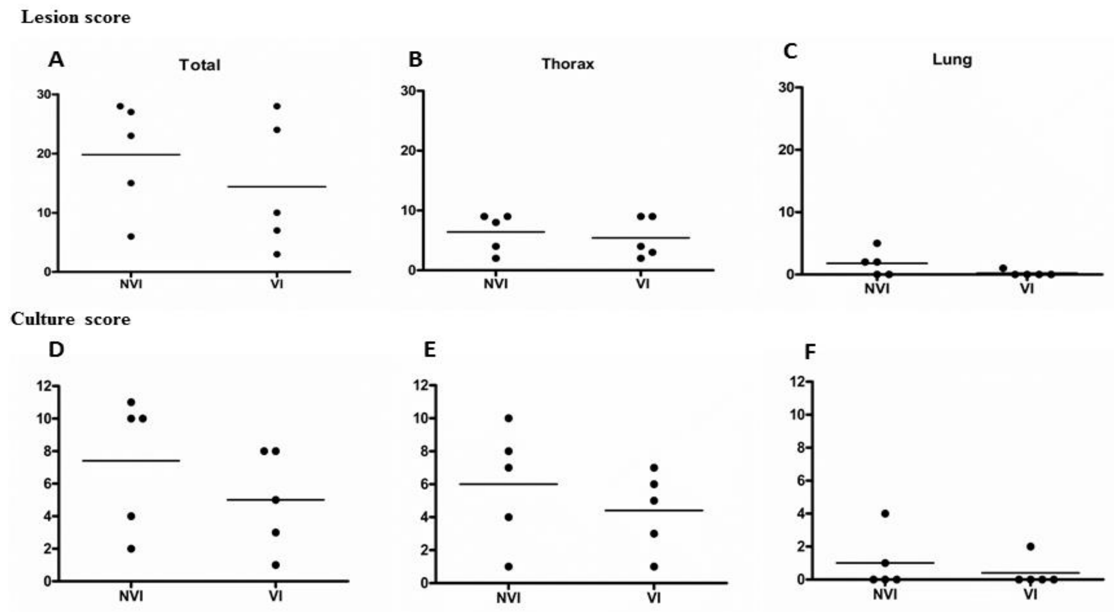

Figure 1. Bovine tuberculosis lesion and culture scores for total (A,D), thorax (B,E), and lung (C,F). The solid lines present median values

DISCUSSION

Although the efficacy of PTB vaccination has been repeatedly demonstrated (Alonso-Hearn *et al.,* 2012; Bannantine *et al.,* 2014; Juste *et al.,* 2009; Juste and Perez, 2011), its use has been restricted due to the cross-reactivity with current bTB diagnostic methods. Interference issues have probably led to the lack of knowledge on the effect of PTB vaccination in the development of bTB infection. This is the first study examining this topic in bovines under experimental conditions.

In this particular experimental setting, all animals became infected regardless of their vaccination status showing positive culture results and macroscopic lesions compatible with bTB. In both groups, thorax and lung were the most affected areas. The majority of tissue sites affected by infection in terms of lesion development or bacterial colonization, as well as the highest lesion and culture scores appeared in these two areas. However, PTB vaccination seemed to induce a moderate protective response against *M. bovis* challenge, which led to a reduction of the pathological and bacteriological results in both areas of the VI group. The effect of vaccination was most

evident in the lung as seen by the fact that only one animal (1/5, 20%) of the VI group presented lesions and detectable bacteria in this area compared to the NVI group where three animals were affected (3/5, 60%). Same tendency was observed in the remaining studied areas except for the head, where the protective effect was not evident. Although differences among groups were not statistically significant and only tendencies have been observed, the degree of protection observed can be considered biologically relevant. To reinforce the appealing results obtained from this first trial, it would be necessary to carry out further studies with a larger sample size.

These results suggest that a certain degree of heterologous protection against *M. bovis* infection takes place after PTB vaccination, and although the protection conferred is probably not enough to impede the establishment of the disease or prevent horizontal transmission within a herd, it may contain the infection to some extent. Results from this experiment indicate that this containment would clearly benefit the lung (60–89%) reduction in lesions and bacterial burden) since reduction due to vaccination in the thoracic region was less notorious and absent in the head area. This may be important considering that the main excretion route of *M. bovis* is through the respiratory system, and therefore, the reduction of the bacterial load in the lung may lead to a reduction of this figure in the environment.

These findings partially agree with the results obtained by Pérez de Val et al. (Pérez de Val *et al.,* 2012) in goats after PTB vaccination and subsequent challenge with *M. bovis* where, lesions in vaccinated goats appeared only in the lung and corresponding LNs whereas non vaccinated animals showed an increased dissemination frequency of the lesions. In that case (Pérez de Val *et al.,* 2012), goats were challenged through the endobronchial route and, therefore, infection progressed mainly affecting the lower respiratory tract. In another study in goats in which the transthoracic route was used, lesions were mainly located in lung and mediastinic and mesenteric LNs (Bezos *et al.,* 2010). In our study, thorax was the primary focus and

extrathoracic and extrapulmonary dissemination of bacteria to the upper respiratory tract or head area (retropharyngeal, mandibular, parotid LNs, and nasal turbinate) occurred in four animals of the VI group and three animals of the NVI group. This could be due to a pulmonary dissemination to the head by mycobacterial shedding in the tracheobronchial secretions and subsequent ingestion as hypothesized in previous reports (Pérez De Val *et al.,* 2011). Bacteria and gross lesions were detected in spleen of one animal of the NVI group, whereas only gross lesions were observed in spleen and liver of one animal of the VI group indicating that systemic circulation of mycobacteria had occurred.

Added to the route, the challenging dose can be crucial for the pathological outcome of the infection. In previous studies in goats (Bezos *et al.,* 2010; Pérez de Val *et al.,* 2012; Pérez De Val *et al.,* 2011), animals were challenged with lower doses of *Mycobacterium caprae* (10^2–1.5×10^3 CFUs), but as stated before in those studies, the inoculum was directly deposited into the lung. The selected dose (10^6 CFU) and infection route (endotracheal) may be responsible for the wider spreading of the lesions in our study. This high dose was applied to guarantee infection for vaccine evaluation. In any case, considering the fact that in experimental conditions the bacterial load administered for challenge is most probably many logs higher than the amount of *M. bovis* that an animal will be in contact with in field conditions it could be expected that higher protection levels would be observed in these cases.

Positive correlations were found between pathological and bacteriological techniques as expected. These were best fit in the lung, area that poses the most noticeable partial protective effect by the vaccine.

The results presented here suggest that vaccination against PTB modifies the course of experimental bTB infection by decreasing the severity of the lesions and the bacterial burden. Although our results are not conclusive, they support the view that

mycobacterial vaccines could potentially be useful tools for disease control in specific settings where vaccination does not interfere with eradication programs.

ETHICS STATEMENT

With the purpose of obtaining data for this trial, calves were submitted to clinical practices standardized and regulated by the European, Spanish and Regional Law and Ethics Committee. The experimental design underwent ethical review and approval by NEIKER's Animal Care and Use Committee and by the Agriculture Department (PARAPATO-1264-BFA).

AUTHOR CONTRIBUTIONS

RJ, JG, and IS conceived the study. MS, EM, MG, IS, and NE carried out the laboratory work. RJ, NE, and MS compiled and analyzed the data. MS, NE, and IS collated the results. MS, NE, IS, and JG drafted the preliminary manuscript. All authors participated in the review and the editing of the final draft and also read and approved its final version.

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5.3. THIRD STUDY

Different lesion distribution in calves orally or intratracheally challenged with *Mycobacterium bovis*: implications for diagnosis. Serrano, M., Sevilla, I.A., Fuertes, M., Geijo, M., Risalde, M.Á., Ruiz-Fons, J.F., Gortazar, C., Juste, R.A., Domínguez, L., Elguezabal, N., Garrido, J.M., 2018. Different lesion distribution in calves orally or intratracheally challenged with Mycobacterium bovis: Implications for diagnosis. *Veterinary Research*, 49, p. 74. doi: 10.1186/s13567-018-0566-2.

Different lesion distribution in calves orally or intratracheally challenged with *Mycobacterium bovis*: implications for diagnosis

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ABSTRACT

Animal tuberculosis (TB) remains a major problem in some countries despite the existence of control programmes focused mainly on cattle. In this species, aerogenous transmission is accepted as the most frequent infection route, affecting mainly the respiratory system. Under the hypothesis that the oral route could be playing a more relevant role in transmission, diagnosis and disease persistence than previously thought, this study was performed to assess the course of TB infection in cattle and its effects on diagnosis depending on the route of entry of *Mycobacterium bovis*. Two groups of five calves each were either endotracheally (EC) or orally (OC) challenged.

Necropsies were carried out 12 weeks after challenge except for three OC calves slaughtered 8 weeks later. All animals reacted to the tuberculin skin test and the entire EC group was positive to the interferon-gamma release assay (IGRA) 2 weeks after challenge and thereafter. The first positive IGRA results for OC calves (3/5) were recorded 4 weeks after challenge. Group comparison revealed significant differences in lesion and positive culture location and scoring. TB-compatible gross lesions and positive cultures were more frequently found in the thorax ($p < 0.001$) and lung ($p < 0.05$) of EC animals, whereas OC animals presented lesions ($p = 0.23$) and positive cultures ($p < 0.05$) mainly located in the abdomen. These results indicate that the infection route seems to be a determining factor for both the distribution and the time needed for the development of visible lesions. Our study suggests that confirmation of TB infection in some skin reactor animals can be problematic if current post-mortem examination and diagnostics are not improved.

INTRODUCTION

Mycobacterium bovis (*M. bovis*) is the main etiological agent of animal tuberculosis (TB), a mycobacterial infectious disease with a worldwide distribution (Humblet *et al.,* 2009) that affects cattle (Pollock and Neill, 2002), other domestic hosts (Pesciaroli *et al.,* 2014), wildlife (Gortázar *et al.,* 2015) and humans (Olea-Popelka *et al.,* 2017). The huge economic losses caused by bovine TB added to the impact of its zoonotic nature led to implement control strategies for over a century in many countries (Caminiti *et al.,* 2016; Good *et al.,* 2018). Although eradication of TB has been accomplished in some countries, the presence of *M. bovis* in herds continues to pose serious problems for animal and human health in many others (De Kantor and Ritacco, 2006; Essey and Koller, 1994; Good *et al.,* 2018; Radunz, 2006; Rivière *et al.,* 2014). This discrepancy has been observed despite the similarity of the eradication programs used in the different countries (Collins, 2006; Reviriego Gordejo and Vermeersch, 2006). There are several reasons for the persistence of the disease in cattle, but it is usually attributed to the existence of wild reservoirs (Gortázar *et al.,* 2015). Domestic

reservoirs include goats (Napp *et al.,* 2013), sheep (Muñoz-Mendoza *et al.,* 2015) and pigs (Di Marco *et al.,* 2012), depending on the characteristics of the local host community.

Different transmission pathways do exist for cattle. These include direct or indirect inhalation, oropharyngeal exposure and/or ingestion of *M. bovis* and, more unlikely because of the active eradication programs, transplacental or mammary transmission (Domingo *et al.,* 2014). Lesion distribution and progression seem to be shaped by the route of introduction of the bacterium (Liebana *et al.,* 2008; Neill *et al.,* 2005). There is a general acceptance that the aerogenous transmission is the most frequent one in cattle and lesions are usually found in the respiratory system and associated lymph nodes (LN) (Domingo *et al.,* 2014; Liebana *et al.,* 2008). This also seems to be the case for natural intra-species transmission in the badger, the principal wild animal reservoir in Ireland and UK (Gormley and Corner, 2018). Lesions can also reach these LN and other tissues or LN of the head region (Domingo *et al.,* 2014; Fitzgerald *et al.,* 2016; Liebana *et al.,* 2008) after oral exposure to *M. bovis*. However, ingestion of bacilli is usually associated with affected LN and tissues of the digestive system with or without visible lesions (Domingo *et al.,* 2014; Liebana *et al.,* 2008). Oral exposure to *M. bovis* could represent a more relevant route of infection than previously thought. In the wildlife-livestock interface inter-species transmission is of an indirect nature, for instance through shared water or food (Barasona *et al.,* 2017; Cowie *et al.,* 2016). In these cases, infection will most likely enter the host by the oral route. Widespread contamination of environmental samples in the Iberian Peninsula suggests that indirect transmission contributes to the maintenance of tuberculosis in multi-host–pathogen systems (Santos *et al.,* 2015b, 2015a).

Cell mediated immunity (CMI)-based diagnostics used in eradication campaigns, namely, intradermal tests and interferon-gamma release assay (IGRA), have been deemed of poor specificity because the confirmatory tests (pathological examination and culture) fail to demonstrate the presence of lesions and the involvement of *M.*

bovis quite frequently (de la Rua-Domenech *et al.,* 2006). However, disagreements between confirmatory tests and official CMI-based methods are to be expected because their best sensitivity and specificity values are achieved at different immunopathological stages of the infection (de la Rua-Domenech *et al.,* 2006; O'Hagan *et al.,* 2015). Specificities above 99% have been estimated recently for the comparative intradermal skin test using surveillance tests results from officially TB free herds in Great Britain (Goodchild *et al.,* 2015). According to an observational case–control study on confirmed reactors from Northern Ireland, a substantial percentage of non-confirmed reactors could result from imperfect sensitivities of the confirmatory tests (O'Hagan *et al.,* 2015)]. The estimated specificity for the single intradermal test was also high in a study from Spain (Álvarez *et al.,* 2012) but infection cannot be confirmed in many slaughtered cattle. This situation raises a lack of confidence of farmers and field veterinarians in official *in vivo* tests (Ciaravino *et al.,* 2017). An epidemiological investigation in Spain pointed out that residual infection and interactions in the wildlife-domestic interface are probably the most relevant causes of bovine TB breakdowns (Guta *et al.,* 2014). In order to shed some light on this problem by giving additional reliable explanations for the occurrence of part of the non-confirmed reactor animals, we studied possible differences in the course of experimental TB infection in calves depending on the route of entry of *M. bovis* and its implications for diagnosis.

MATERIALS AND METHODS

Animal selection

This project was aimed at studying the interference of paratuberculosis vaccination on TB diagnosis (Serrano *et al.,* 2017a, 2017b), the effect of *M. bovis* inoculation route on the pathology and diagnostics of bovine TB (the present work) and the efficacy of an inactivated vaccine to protect them from oral and endotracheal challenge with *M. bovis* (unpublished work to be submitted). The data presented in this paper were obtained from two control groups belonging to two experimental infections

included in the same research project. For this project, thirty, two-month-old animals per experiment were preselected from the same feedlot that had originally purchased them from 13 different farms in northern Spain with no known history of TB. In order to confirm the absence of previous contact with *M. bovis* or other mycobacteria, an IGRA with avian and bovine purified protein derivative standard tuberculins (A-PPD, B-PPD) (CZ Veterinaria, Pontevedra, Spain) was performed in three different samplings at the feedlot. Finally, only 15 animals were recruited and transported to the biosafety level 3 (BSL-3) facilities in NEIKER for each experiment, each with three groups of five calves. In the present work, ten calves were used, five calves from the endotracheal challenge control group (experiment 1) and five calves from the oral challenge control group (experiment 2). Housing conditions, calf keepers and experimental procedures were exactly the same in both experiments.

All the experimental procedures involving animal housing and care were carried out in agreement with the European, National and Regional Law and Ethics Committee regulations. The experimental design underwent ethical review and was approved by NEIKER's Animal Care and Use Committee (OEBA-NEIKER-2015-010) and by the competent local authority, the Department of Agriculture of Diputación Foral de Bizkaia (PARAPATO-1264-BFA).

***M. bovis* challenge**

After their arrival to the BSL-3 facilities, calves went through a 2-week adaptation period. Afterwards, all calves were challenged with the same *M. bovis* field isolate suspended in 2 mL of phosphate-buffered saline (PBS) at the same dose of approximately 10^6 colony forming units (CFU). The isolate (SB0339 spoligotype profile) was originally obtained from a naturally infected wild boar. This strain has been used in previous experiments at NEIKER and its spoligotype is shared by domestic and wild *M. bovis* isolates. The chosen challenge route was different for each of the groups. For the orally challenged group (OC) the infective dose was administered with a syringe and the heads of the animals were maintained upwards until deglutition was observed and

no spilling was confirmed. In the group challenged by the endotracheal route (EC), firstly, the needle was introduced in the space between two consecutive tracheal rings located in the range of number 25 and 30 and air was aspirated to assure that the inoculum would be introduced inside the trachea and then the dose was injected. Only EC calves underwent previous intramuscular sedation with XILAGESIC® 2% (10 mg/50 kg) (Laboratorios Calier, S.A., Barcelona, Spain).

Interferon-gamma release assay (IGRA)

At the BSL-3 facilities, five samplings were performed at 0, 2, 4, 8 and 12 weeks after challenge and an additional one for three EC animals 20 weeks after challenge. Blood was collected from the jugular or caudal vein in tubes with lithium heparin. Stimulation of whole blood with A and B-PPD as well as with PBS (nil control) was carried out within 8 h of collection. The IDScreen® Ruminant interferon-gamma kit (IDvet, Grabels, France) licensed by the Spanish Government (Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente) was used for the detection of interferon-gamma in the stimulated blood supernatants according to the manufacturer's instructions. The standard cut-off of the kit was used to consider a sample positive (i.e., S/P% ≥ 35).

Skin test

The skin test was carried out in all animals 12 weeks after challenge and it was performed by inoculating 0.1 mL (2500 IU) of A- and B-PPD in the neck. The skin-fold thickness was measured before and 72 h after inoculation. The results of skin thickness increase were interpreted according to the standards of official criteria (EU Council Directive 64/432/CEE and Spanish RD 2611/1996) for both Single Intradermal Test (SIT) and Comparative Intradermal Test (CIT). A calf was considered SIT positive, inconclusive or negative when the increase was 4 mm or greater, between 2 and 4 mm or less than 2 mm, respectively. For CIT, animals were deemed positive, inconclusive or negative when the bovine injection site exceeded the avian site by greater than 4, 1-4 and 1 mm or less, respectively.

Necropsies

All animals were systematically and thoroughly necropsied. Necropsies from the entire EC group were carried out 12 weeks after challenge. However, animals from the OC group were slaughtered at two different time points. Initially, necropsies began 12 weeks after challenge and gross lesions were absent in the first two animals. Considering that lesions could be at the initial stages of disease and not visible at this time point, the duration of the experiment with the three remaining calves was extended for 8 additional weeks. At the end of the study, animals were sedated by an intramuscular injection of XILAGESIC® 2% (2.5 mg/50 kg) and intravenously injected with T61 (4–6 mL/50 kg) (Intervet International GMBH, Unterschleissheim, Germany). Organs were thoroughly inspected for TB-compatible lesions and collected samples were distributed within five body areas as follows: head (nasal turbinate, palatine tonsils and mandibular, parotid and retropharyngeal lymph nodes (LN), thorax (tracheal, prescapular, tracheobronchial and mediastinal LN), lung (right and left cranial and caudal lobes and medium and accessory lobes), abdomen (hepatic, jejunal and ileocecal LN as well as liver and spleen) and others (prefemoral and popliteal LN).

Gross pathology

Organs and tissues were visually inspected for the presence of lesions and all of them were thoroughly palpated and sliced in search of deeper lesions. The TB compatible lesions found were classified according to Palmer et al. (Palmer *et al.,* 2007). Briefly, two scoring systems were established to measure the severity of the lesions in lung and in LN. The scoring scale for lungs was as follows: 0, no visible lesions; 1, no external gross lesions, but lesions seen upon slicing; 2, less than 5 lesions of < 10 mm in diameter; 3, more than 5 lesions of < 10 mm in diameter; 4, more than 1 distinct gross lesion of > 10 mm in diameter; 5, coalescing gross lesions. Lesion classification of the LN was ranked as follows: 0, no visible lesions; 1, small focal lesion (1–2 mm in diameter); 2, several small foci; 3, extensive lesions.

Culture

Samples were processed to confirm the presence of mycobacteria in solid (Coletsos-Difco, Francisco Soria Melguizo SA, Madrid, Spain) and liquid culture (BBL Mycobacteria growth indicator tubes (MGIT), Becton–Dickinson, Franklin Lakes, NJ, USA) according to the protocol described previously by Garrido et al. (Garrido *et al.,* 2011). Briefly, 2 g of tissue was homogenized in 10 mL of sterile distilled water. Five milliliter were decontaminated in hexadecyl-pyridinium chloride 0.75% (w/v) for 12–18 h for solid culture. Samples were centrifuged at 2500 × g for 5 min; pellets were cultured in Coletsos tubes at 37 °C for 4 months. The remaining 5 mL were decontaminated and processed for liquid culture in BBL MGIT tubes supplemented with BBL MGIT PANTA and BACTEC MGIT growth supplement according to the manufacturer's instructions. BBL MGIT tubes were incubated for 42 days in a BACTEC MGIT 960 System.

Colonies were visualized under a stereomicroscope. According to the number of colonies in each tube, a culture score was defined in order to categorize the infection level of each tissue. Score categories were as follows: 0, no growth; 1, less than 10 colonies; 2, between 10 and 50 colonies; 3, over 50 colonies (Garrido *et al.,* 2011).
DNA was extracted from all positive cultures and a *M. tuberculosis* complex-specific PCR (Sevilla *et al.,* 2015) was performed to confirm that *M. bovis* was responsible for the growth. All isolates were confirmed as *M. bovis* SB0339 by spoligotyping (Kamerbeek et al., 1997).

Statistics

The number of affected tissues and culture positive samples, as well as scores for gross lesions and cultures were calculated per area and per animal. Differences in the distribution of lesions and positive culture results between groups were assessed using Chi square test. Mann-Whitney U-test was used to study the differences between lesion and culture scores of the different areas of both groups. Correlation between skin test results and lesion scores as well as between skin test results and number of

tissues with lesions was performed by Spearman. Statistical significance was considered at p values < 0.05. Statistical analysis was completed using R Commander.

RESULTS

No mortality was recorded during the experiments. All animals completed the trials without showing TB-compatible clinical signs.

Ante mortem diagnosis: SIT, CIT and IGRA tests

Reactivity to both ante mortem diagnostic techniques was confirmed in both groups (see Table 1). Two weeks after challenge, all EC animals were categorized as reactors to the IGRA test. This positive reactor state remained throughout the experiment for all animals except for one calf that tested negative 12 weeks after challenge. In the case of the OC group, reactivity was first detected 4 weeks after challenge in three of these animals and all five became positive 8 weeks after challenge. However, in the following sampling (12 weeks after challenge) two of them were IGRA negative again.

Table 1. Results for IGRA and skin tuberculin tests performed

Group	Calf ID	IGRA test						Skin test				
		Weeks after challenge						Δ(mm) skin thickness			Interpretation	
		0	2	4	8	12	20	PPD-A	PPD-B	PPD-B – PPD-A	SIT	CIT
EC	1	N	P	P	P	P	-	1	5	4	P	I
	2	N	P	P	P	P	-	1	12	11	P	P
	3	N	P	P	P	N[a]	-	1	8	7	P	P
	4	N	P	P	P	P	-	0	9	9	P	P
	5	N	P	P	P	P	-	2	9	7	P	P
OC	6	N	N	N	P	P	-	3	16	13	P	P
	7	N	N	N	P	N[b]	-	9	22	13	P	P
	8	N	N	P	P	N[C]	P	3	10	7	P	P
	9	N	N	P	P	P	P	2	8	6	P	P
	10	N	N	P	P	P	P	5	17	12	P	P

SIT: Single Intradermal Test, CIT: Comparative Intradermal Test, EC: endotracheally challenged, OC: orally challenged, Δ: increase, A-PPD: avian PPD, B-PPD: bovine PPD, P: positive, I: inconclusive, N: negative.

[a] IDscreen S/P% = 28.06.

[b] IDscreen S/P% = 20.17.

[c] IDscreen S/P% = 22.26.

—: these animals were necropsied 12 weeks after challenge. The remaining three were tested for IGRA once more before being necropsied 20 weeks after challenge.

On the contrary, all calves from both groups came out as clearly reactors to the SIT and CIT except for one EC calf with an inconclusive CIT result. This animal was exactly at the uppermost skin thickness increase limit (4 mm) to be considered inconclusive but was clearly positive to all IGRA tests from week 2 after challenge on.

Skin test (PPD-B–PPD-A) was negatively although not significantly correlated with lesion scores (rho = −0.446, $p = 0.197$) and the number of tissues with lesions (rho = −0.588, $p = 0.074$).

Post mortem analysis

All animals from the EC group presented gross lesions compatible with TB (Table 2). Almost all of the affected tissues found in the EC group (22/28, 79%) were located in the thoracic LN and in the lungs: all five EC animals (5/5, 100%) presented lesions in the thoracic area and three of them (3/5, 60%) also in the lung lobes. Macroscopic lesions in head and abdominal tissues were only found in one of the EC calves (1/5, 20%). Only one affected prefemoral LN was observed and it belonged to an EC animal.

In the OC group no gross lesions were observed in the two calves slaughtered 12 weeks after challenge. The three remaining OC animals (3/5, 60%) (Table 2) necropsied 20 weeks after challenge presented macroscopic lesions that appeared in two of the five defined areas. In the thoracic region only one affected tissue was detected in one animal. The abdomen was the most affected area (Table 2). More precisely, one of these OC calves showed macroscopic lesions in the proximal, medium or distal jejunal LN while another animal had visible lesions only at the proximal and medium jejunal LN.

Table 2. Distribution and score of confirmed tuberculous lesions in the tissues of the studied groups.

			Head				Thorax				Lung					Abdomen			Others	
Group	Calves ID	Necropsy: weeks A.C	Palatine tonsil	Parotid LN	Retropharingeal LN	Mandibular LN	Tracheobronchial LN	Prescapular LN	Tracheal LN	Mediastinal LN	Left craneal lobe	Left caudal lobe	Right craneal lobe	Right caudal lobe	Accesory lobe	Hepatic LN	Jejunal LN	Spleen	Prefemoral LN	Total Lesion Score
EC	1	XII					2		7	4	2	2	2		2					21
	2	XII						3	8	2				1					1	15
	3	XII							2	2				1						5
	4	XII					6	3	10	9										28
	5	XII	1	1	1		4	3	9	6						2		1		28
OC	6	XII																		0
	7	XII																		0
	8	XX								3										3
	9	XX															6			6
	10	XX															10			10

One LN affected (right or left): dotted; two LN affected (both right and left or two of three, cranial, caudal, or medial): light gray; three LN affected (cranial, caudal, and medial): dark gray. Total Lesion score is the sum of the scores of all tissues per animal.

R: right, L: left, EC: endotracheally challenged, OC: oral challenged, AC: after challenge, LN: lymph node.

Group comparison shows that EC calves presented more macroscopic lesions than OC animals ($p < 0.001$). These differences were also significant for the thoracic and lung areas ($p < 0.001$ and $p < 0.05$, respectively). However, OC animals presented more tissues with macroscopic lesions at the abdomen although differences were not significant and only a tendency was observed ($p = 0.23$).

Lesion scores (shown in Table 2) were always lower for the OC group compared to the EC group in all areas, except for the abdomen (Table 2 and Figure 1), being five times higher in this area for the OC group (16 vs. 3 respectively) ($p = 0.35$). EC animals had significantly higher pathology scores in the thoracic and lung areas ($p < 0.001$ and $p < 0.05$, respectively).

Figure 1. Tuberculous lesion scores in *Mycobacterium bovis* challenged calves. Dot plot representing lesion score distribution in head, thorax, lung, abdomen and total (sum of all areas) for each animal. Horizontal lines show the median values. Significant differences were found in the thorax ($p < 0.001$) and lung ($p < 0.05$). EC: endotracheally challenged, OC: orally challenged.

Tissue culture results are shown in Table 3. Culture and/or histopathology confirmed the involvement of *M. bovis* in all tissues presenting gross lesions and additional positive tissues were detected in those showing no macroscopic lesion. In the EC group, more animals (3/5, 60%) yielded positive cultures in the head area than those displaying macroscopic lesions (1/5, 20%). Similarly, in the OC group not only the three animals (3/5, 60%) presenting gross lesions at different sites were confirmed by culture but also the two calves with no macroscopic lesion (5/5; 100%) yielded *M. bovis* isolates from abdominal tissues. EC animals had significantly more positive cultures than OC calves in the thoracic and pulmonary regions ($p < 0.001$ and $p < 0.05$, respectively). Significant differences were also found in relation to the abdominal area where OC animals showed more tissues with positive culture ($p < 0.05$).

Table 3. Distribution of *M. bovis* positive cultures and culture scores in the tissues of each group.

Group	Calves ID	Necropsy: weeks AC	Palatine tonsil	Parotid LN	Retropharingeal LN	Mandibular LN	Tracheobronchial LN	Prescapular LN	Tracheal LN	Mediastinal LN	Left cranial lobe	Left caudal lobe	Right craneal lobe	Right caudal lobe	Hepatic LN	Jejunal LN	Ileocecal LN	Spleen	Prefemoral LN	Total Culture Score
			Head				Thorax				Lung				Abdomen				Others	
EC	1	XII			(dotted)		(dotted)	3 (dark gray)	3 (dark gray)	1 (dark gray)	1 (dark gray)	2 (light gray)	1 (dark gray)							11
	2	XII			(dotted)		(dotted)	1 (dark gray)	3 (dark gray)	(dotted)			(dotted)						(dotted)	4
	3	XII							1 (light gray)					1 (light gray)						2
	4	XII					2 (dark gray)	3 (dark gray)	3 (dark gray)	2 (dark gray)										10
	5	XII	(dotted)	(dotted)	1 (dark gray)		2 (dark gray)	3 (dark gray)	2 (dark gray)	1 (dark gray)					1 (dark gray)			(dotted)		10
OC	6	XII													1 (light gray)	1 (light gray)	1 (light gray)			3
	7	XII														1 (light gray)				1
	8	XX				(dotted)				3 (dark gray)										3
	9	XX														3 (dark gray)				3
	10	XX														3 (dark gray)				3

MGIT culture positive: dotted; solid culture positive: light gray; both MGIT and solid culture positive: dark gray. Culture score is the sum of the scores of all tissues per animal.
R: right, L: left, EC: endotracheally challenged, OC: oral challenged, AC: after challenge, LN: lymph node.

Detailed culture scores are shown in Table 3 and Figure 2. Culture scores were always lower in the OC group (thorax $p < 0.05$), except for the abdominal area (Figure 2), where the OC group presented total culture scores ten times higher (10 vs. 1) although significant differences were not observed ($p = 0.68$).

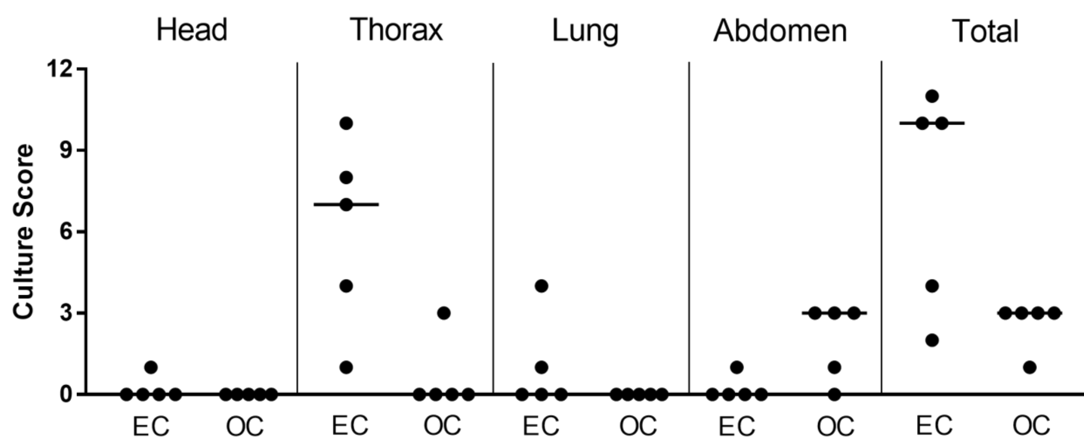

Figure 2. Culture scores in *Mycobacterium bovis* challenged calves. Dot plot representing culture score distribution in head, thorax, lung, abdomen and total (sum of all areas) for each animal. Horizontal lines show the median values. Significant differences were found in the thorax ($p < 0.05$).
EC: endotracheally challenged, OC: orally challenged.

DISCUSSION

In this experiment, infection was confirmed in all ten animals by culture and histopathology regardless of the challenge route. Despite the small size of study groups, anatomic distribution of gross lesions, culture results as well as pathology and culture scores showed significant differences between both groups. In the EC group, macroscopic lesions and isolation of the etiological agent were observed in all defined areas. However, most of the tissues presenting gross lesions and positive culture, as well as the highest lesion and culture scores, appeared in the thorax and lungs. In contrast, the OC group presented a different picture showing the abdomen as the most affected area, with 4 positive animals out of 5. Furthermore, infection was confirmed by culture in four abdominal LN belonging to the two calves with no visible TB-compatible lesions that were slaughtered at the same time point as the EC group (12 weeks after challenge).

As could be expected (Gormley and Corner, 2018; Pollock *et al.,* 2006), these experimental results suggest that depending on the route of infection the distribution of lesions can vary; that is, after an endotracheal challenge, lesions are more likely to be found in the respiratory tract and associated tissues whereas the digestive tract is the most affected area after an oral challenge. Although the number of animals used was small, these findings are consistent with the results obtained in previous studies where it was stated that after inhalation of *M. bovis* most lesions appear in the nasopharynx and lower respiratory tract, including the lungs and associated LN and that *M. bovis* ingestion usually causes lesions in the mesenteric lymph nodes (Collins and Grange, 1983; Domingo *et al.,* 2014; Pollock *et al.,* 2006). Moreover, the results obtained by Fitzgerald et al. (Fitzgerald *et al.,* 2016) in tuberculous cows housed together and in calves and cats fed with waste-TB-milk showed the same tendency in the number and distribution of lesions as well as in the areas where positive cultures were detected. Lesions were detected in the thorax in cows infected by airborne transmission and abdominal area for the calves and cats fed with waste milk.

Although the thoracic and lung areas were the primary site of confirmed infection for the EC group, isolation of bacteria from the head, abdominal area or prefemoral LN occurred in three out of five of the animals. This extrapulmonary and extrathoracic dissemination of the etiological agent could be due to oropharyngeal exposure and/or swallowing of tracheobronchial secretions carrying bacteria as mentioned in previous studies (Pérez De Val *et al.,* 2011). By contrast, the primary affected site in the OC group was the abdomen instead of the thoracic or lung areas. One animal showed no positive culture for the jejunal LN. The same animal was the only one with a positive culture in the thoracic and head areas. The mandibular and mediastinal LN of this animal could have become infected through oropharyngeal exposure and/or inhalation during challenge or with bacteria shed by the other infected OC calves.

According to the outcomes of this experimental study, the time needed for the development of macroscopic lesions seems to differ depending on the infection route. All animals from both groups were clearly reactors to the skin test 12 weeks after challenge, and all five EC calves showed gross lesions during the necropsies. However, lesions were not visible in all OC animals despite positive skin tests and cultures. No macroscopic lesions were found in the two OC calves slaughtered at the same time point as the five EC animals. The three OC calves slaughtered 20 weeks after challenge showed visible lesions. A recent cross-sectional study suggested that tuberculin reaction size (PPD-B–PPD-A size) was significantly positively associated with maximum TB lesion number and size (Byrne *et al.,* 2018). In contrast to this report studying natural TB cases, we could not see such an association in our experimental setting. In fact, we have seen a negative, although not significant correlation and the greatest tuberculin reaction sizes recorded belonged to the two OC calves only confirmed by *M. bovis* isolation from abdominal lymph nodes (Tables 1, 2 and 3). As stated in other studies, the cellular immune response gets activated during the very early stages of the infection (Ritacco *et al.,* 1991; Welsh *et al.,* 2005) and our data suggest that 12 weeks after an oral challenge, the etiological agent may have been

able to spread into different abdominal LN as seen by culture results but the time required for visible lesion development seems to take longer.

In line with this, IGRA was able to identify all infected animals in the EC group as early as 2 weeks after challenge. In contrast, the OC group displayed positive animals (3/5) for the first time, 4 weeks after challenge, suggesting that mounting a CMI response capable of producing detectable interferon-gamma levels could need more time in animals infected through the oral route. One EC and two OC animals turned to a negative IGRA status after being positive, but they were clear reactors to the skin test. This phenomenon may simply be related to the performance of the diagnostic kit used or to the dynamics of the immune response to infection of these calves. Despite this, a recent study reported significantly lower sensitivities for IDScreen® compared to the Bovigam® Kit (Casal *et al.,* 2017). These authors introduced an additional cut-off point (S/P% ≥ 16) following information provided by the manufacturer (Casal *et al.,* 2017). The three samples that turned negative would be deemed positive if this alternative cut-off point was used in our study. Further studies are needed to assess the performance of the different IGRA tests.

We believe that there is not a fully established habit of inspecting tissues from the digestive system as thoroughly as other anatomic sites in abattoirs. Submitting samples from these tissues to the laboratory has not been made common practice unless macroscopic lesions were observed. Taking this into account, in this study we aimed to explore the possibility of attributing a more relevant role to oral transmission in the epidemiology and diagnosis of bovine TB in light of recent research dealing with this widespread wildlife-livestock-multi-host infectious disease (Barasona *et al.,* 2017; Cowie *et al.,* 2016; Fitzgerald *et al.,* 2016; Good *et al.,* 2018; Gortázar *et al.,* 2015; Guta *et al.,* 2014; Santos *et al.,* 2015b, 2015a) and the relatively low confirmation rate of skin test reactor cattle (Byrne *et al.,* 2018; Ciaravino *et al.,* 2017; de la Rua-Domenech *et al.,* 2006; Goodchild *et al.,* 2015; Gormley and Corner, 2018; O'Hagan *et al.,* 2015). Our results demonstrate that the so-called nonconfirmed reactors can be

composed of animals in early stages of the infection, animals able to contain the spread of bacteria or animals with difficult to detect lesions or lesions in tissues seldom affected and inspected, among others. Reasons for unspecific CMI-based test results have been carefully described in previous reports (de la Rua-Domenech *et al.,* 2006).

These results may contribute to explain the scenario often found in slaughterhouses where animals that are reactors to the ante mortem techniques show no TB-compatible gross lesions during inspection. The sensitivity of visual inspection for lesion detection can be severely compromised because of the difficulty of distinguishing small lesions or infection foci; this issue increases as the dimension of the animal and samples to be checked increases (Gormley and Corner, 2018). At population level scales, Byrne et al. (Byrne *et al.,* 2018) showed that a significant proportion of reactor cattle do not present visible lesions at abattoir inspection. In Northern Ireland not all reactor animals are subjected to laboratory testing depending on some epidemiological and pathological parameters (O'Hagan *et al.,* 2015). Usually, bulked retropharyngeal, bronchial and mediastinal LN from up to five reactors without macroscopic lesions and at least one lesion from three reactors with visible lesions are submitted to histological examination and/or culture. Under this TB breakdown management strategy, visible lesions were found in 43% of the reactors, although TB could not be confirmed in 0.2% of these cases (O'Hagan *et al.,* 2015). On the contrary, the 95.7% of reactors with no visible lesions (the remaining 57% of reactors) could not be confirmed as infected by histology and/or culture of the LN mentioned above. This insensitivity can be accentuated when only the digestive tract is involved in infection. Lesions in this location can readily go unnoticed because these tissues are normally removed at the beginning of slaughtering without being as carefully examined as the thoracic and head tissues. This is of particular relevance in a context of multi-host infection and likely oral inter-species transmission (Barasona *et al.,* 2017; Cowie *et al.,* 2016).

Although additional research on challenge dose and time required for visible lesion development is necessary, our results indicate that depending on the route of infection, the distribution and development of lesions may vary, and this can have implications for TB diagnostics in terms of confirmation of skin test reactor calves. Therefore, since *M. bovis* persists in cattle population and non-confirmed reactors arise, there is an urgent need to improve current control and inspection protocols. Further studies with more animals per group are needed to assess the impact of the route of infection on TB transmission, pathology and diagnosis also under natural conditions.

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6. GENERAL DISCUSISON

Nowadays tuberculosis continues to be a worldwide distributed disease affecting humans and animals. It is considered as one of the top 10 causes of death among humans and also it is the infectious disease with the greatest mortality rate (World Health Organization, 2018). In addition, tuberculosis together with paratuberculosis are two of the diseases that cause the greatest economic losses to the livestock sector. Although the tuberculosis bacillus was one of the first infectious agents discovered and the implementation of eradication programs throughout the world further underlined its importance, there are still aspects of the pathogenesis that remain unclear, difficulting the diagnosis of subclinical cases. Also infections with *Map* and other mycobacteria may interfere with the diagnostic tests against bovine tuberculosis being the reason for paratuberculosis vaccination not to be allowed in countries undergoing bovine tuberculosis eradication programs in cattle.

The main objective of this dissertation was to contribute to improve knowledge in two fundamental aspects of bovine tuberculosis: diagnosis and pathogenesis. This main objective was split into three specific questions: 1) Is it possible to avoid paratuberculosis vaccination interference in the detection of *M. bovis* carrriers? 2) Does paratuberculosis vaccination provide some degree of heterologous protection against tuberculosis? 3) Does tuberculosis infection route affect the diagnostic test results? As a consequence, the first study aims to find a way to avoid the interference paratuberculosis vaccination may cause with the official diagnostic techniques for bovine tuberculosis. The two remaining articles address the evolution of the course of the disease under different situations. While the effect of *Map* vaccination on *M. bovis* infected animals was assessed in the second study, possible differences in the pathogenesis of bovine tuberculosis depending on the *M. bovis* infection route were studied in the third assay as well as its implications for the diagnosis.

Each study has been reported independently and, therefore, its results have been discussed separately. However a joint discussion of the results obtained in each

study can also contribute to integrate related knowledge and add new perspectives to the management of both tuberculosis and paratuberculosis control.

Accurate diagnosis of bovine tuberculosis may be influenced by the antigenic similarity of the pathogen to other non-tuberculosis mycobacteria such as *Map* or environmental mycobacteria as well as by the use of paratuberculosis vaccines. During the first experiment an infection with *M. bovis* was carried out under experimental conditions on cattle previously vaccinated against *Map*. Because vaccination against *Map* in cattle is not allowed in most countries due to interference with the current diagnostic techniques for bovine tuberculosis, both: alternative interpretation diagnostic criteria to those officially established and more specific *M. bovis* antigens were tested.

Over the last decades significant progress has been made determining more specific or DIVA diagnostic *M. bovis* antigens. Two of the major antigenic targets identified in cattle and humans are ESAT-6 and CFP-10 (Pollock and Andersen, 1997; Vordermeier *et al.*, 2011; Meng *et al.*, 2015). In addition Rv3615c usefulness as a DIVA diagnostic reagent has also been confirmed after identifying *M. bovis* naturally infected bovines categorized as negative for ESAT-6/CFP-10 (Sidders *et al.*, 2008). Furthermore the effect of paratuberculosis infection or vaccination in the detection of bovine tuberculosis using the specific antigens has been assessed in previous studies. When used in the IFN-γ release test, ESAT-6, CFP-10, and Rv3615c showed high specificity values in cattle and goats infected with paratuberculosis or vaccinated with different heat-killed *Map* vaccines (Stabel *et al.*, 2011; Flores-Villalva *et al.*, 2012; Jones *et al.*, 2012; Pérez de Val *et al.*, 2012; Coad *et al.*, 2013).

However results obtained in our first study did not show high specificity values. Reactive calves to both tuberculins, as well as to the specific antigens (ESAT-6/CFP10 and Rv3615c) were found in the *Map* vaccinated *M. bovis* non-infected group from the second sampling upon arrival to BSL-3 facilities. This could have been caused by a change in their microbial environment that would have induced a temporarily rising of the unspecific cellular immune response over the cut-off. However all non-infected

calves became non-reactors for both specific antigens before the end of the experiment, while a fraction remained reactive for the official antigens. The ESAT-6/CFP10 combination showed the highest specificity since it only missclassified three animals as false positive results during the whole experiment.

As for the sensitivity of the IFN-γ test our data were in line with previous experiments (Aagaard *et al.*, 2006; Flores-Villalva *et al.*, 2012). Results showed that the ESAT-6/CFP10 antigen combination was more sensitive than the official tuberculins. All calves from the most problematic group (*Map* vaccinated, *M. bovis* infected) were correctly identified by the ESAT-6/CFP10 antigen combination from the second sampling post-infection until the end of the experiment.

Results obtained from the first study in relation to the tuberculin skin test show that the CIT is a more specific approach (Sp=100%) of the technique, rightly classifying as negative *Map* vaccinated *M. bovis* non-infected calves, than the SIT (Sp=40%). These outcomes match with previous studies carried out under field conditions in cattle (Garrido *et al.*, 2013) and goats (Chartier *et al.*, 2012). However the SIT showed a higher sensitivity (Se=100%) than the CIT (Se=60%) after classifying all calves correctly from both infected groups. Three out of the four misclassified animals by the CIT as non-reactors belong to the most problematic group (*Map* vaccinated *M. bovis* infected). Similar outcomes were obtained previously in goats (Pérez de Val *et al.*, 2012). This situation implies that our approach measuring the relative increase of the skin thickness produced by the avian and bovine antigens was more reliable and could provide a substantial improvement in the interpretation of the CIT in cases where an heterologous sensitization has occurred. Indeed, all animals from both vaccinated groups were correctly classified after applying this alternative criterion to the SIT and CIT boosting their specificity and sensitivity from 40% and 60% up to 100% respectively.

In addition, a protein and a peptide cocktail formed by the above mentioned three specific antigens were also tested. To the best of our knowledge, this is the first

time that the skin test has been performed with these three specific antigens combined in both cocktails in paratuberculosis vaccinated calves subsequently infected with *M. bovis*. Nevertheless in previous studies minimal false-positive responses to the Rv3615c antigen alone and to the combination of ESAT-6 and CFP-10 in goats and calves were observed (Flores-Villalva *et al.*, 2012; Jones *et al.*, 2012). As for the avian and bovine antigens different cut-offs were tested and, once again, the relative increase of the skin thickness criteria displayed the most encouraging results. Furthermore after applying this criterion with the protein cocktail, all animals from the three groups were correctly classified achieving a specificity and sensitivity of 100% respectively.

In this first paper the existence of the interference produced by the vaccine against paratuberculosis with the diagnostic techniques of tuberculosis is once again confirmed. However, our results indicate that the use of the vaccine against *Map* does not have to be at the expense of an accurate diagnosis of tuberculosis, since no *Map*-vaccinated and *M. bovis* infected animals were overlooked. Moreover the further development and progressive incorporation of these specific antigens into the routine diagnosis of tuberculosis could represent an impulse for the control strategies of both tuberculosis and paratuberculosis through vaccination, without harming the detection of infected animals.

The restriction of *Map* vaccination in cattle leads to a lack of knowledge about the effect that this effective vaccine against *Map* (Juste *et al.*, 2009; Juste and Perez, 2011; Alonso-Hearn *et al.*, 2012), pathogen which shares a very similar antigenic composition with *M. bovis*, could have on the establishment and evolution of bovine tuberculosis.

To the best of our knowledge the effect of a *Map* heat-inactivated vaccine on the pathogenesis of the bovine tuberculosis has only been studied in one previous experiment in goats (Pérez de Val *et al.*, 2012). The second study making up this dissertation would be the first assay examining this topic in bovines under experimental

conditions. In this case the perspective changed from diagnostic interference to protection against bovine tuberculosis which has already been described at a certain degree in goats (Pérez de Val *et al.*, 2012). This hypothesis could be confirmed since vaccination led to a reduction of the pathological and bacteriological results in the lung and the thorax area. Although no statistical differences were found between groups, the degree of protection observed can be considered as epidemiologically relevant. Lung benefits clearly from this containment. Compared to the unvaccinated animals the vaccinated group presented a reduction of 89% and 71% in the number of visible lesions and their severity respectively at the lung, as well as 80% less positive culture results and a 60% reduction of the bacterial burden.

Although the degree of protection conferred is probably not enough to impede the establishment of the disease it may contain it to some extent. In addition being the respiratory system the main excretion route of *M. bovis*, the reduction of the bacterial load in the lung may lead to a reduction of the presence of the pathogen in the environment and consequently diminish the transmission rates of the disease. It is well known that vaccination coverage or efficiency does not need to be 100% in order to keep diseases under control in a population (Johni and Samuell, 2000).

Unlike what was observed by Pérez de Val and co-workers (Pérez de Val *et al.*, 2012) lesions in the vaccinated group were also found in areas other than the lung and corresponding lymph nodes such as the head and abdomen. This could be due to pulmonary dissemination to the oral cavity of mycobacterial shedding by tracheobronchial secretions and subsequent ingestion after the endotracheal challenge, as previously hypothesized in another study (Pérez De Val *et al.*, 2011). However the wider spreading of the lesions can be explained by other reasons too: the selected dose and the infection route. In our study the selected dose was minimum 3 logs higher than in previous studies and the inoculum was introduced in the host through the trachea instead of being directly deposited in the lung (Bezos *et al.*, 2010; Pérez de Val *et al.*, 2012).

The protective effect of the *Map* vaccine described in our second study, despite having used such a high infective dose, is very promising: higher protection levels could be expected under field conditions knowing that the amount of *M. bovis* that free animals are exposed to is most probably many logs lower.

The reported spread of lesions by the second study hints at the infection route as a possible reason for the lack of visible lesions in many skin test positive animals. The complexity of the bovine tuberculosis epidemiology combined with gaps in knowledge and poor specificity deemed diagnostic techniques have raised a lack of confidence of the farmers and field veterinarians in the official *in vivo* tests (Ciaravino *et al.*, 2017). Therefore, the third study included in this dissertation is a comparison of the spread of lesions and infection according to two experimental routes: oral and intratracheal.

However significant different results obtained in the third study indicate that the lack of specificity associated with the techniques may not be responsible for the non-confirmed reactors. This study sustained the hypothesis that the oral route could play a more relevant role in transmission, diagnosis and disease persistence than previously thought. Wildlife species contribute to the maintenance and spread of the disease and the indirect transmission by ingestion of contaminated food and water by cattle has been accepted as the most important transmission route between feral animals and cattle. We aimed to explore pathological differences between calves infected through the oral and the respiratory route. Previous assays have stated that lesion distribution seems to be determined by the route of entry of the pathogen. After the ingestion of bacilli, lesions usually appear in lymph nodes and tissues of the abdominal system and in the respiratory system and associated lymph nodes after a respiratory infection (Collins and Grange, 1983; Pollock *et al.*, 2006; Liebana *et al.*, 2008; Domingo *et al.*, 2014). The fact that the aerogenous transmission is considered to be the most frequent in cattle has biased the carcasses visual inspection at the slaughterhouses focusing mostly on the target organs after a respiratory infection.

Results confirmed that the CMI response got activated during the first stages of infection as stated in previous studies (Ritacco *et al.*, 1991; Welsh *et al.*, 2005). However despite the positive IFN-γ status being reached by all animals during the experiment, our results showed that displaying a full active CMI response producing detectable amounts of IFN-γ may take longer after an oral than a respiratory infection.

Outcomes of this study suggest that time needed for the development of macroscopic lesions seems to depend on the infection route. All animals appeared as clearly skin tuberculin reactors 12 weeks after challenge. The endotracheally challenged calves were sacrificed at the skin test lecture day and all of them presented gross lesions. On the other hand two orally challenged calves were categorized as non-confirmed reactors since no macroscopic lesions were observed during the necropsies at the same day. Necropsies of the three remaining calves challenged through the oral route were not carried out until week 20 post challenge and compatible lesions with bovine tuberculosis were found in all of them. Our data confirmed that 12 weeks after an oral challenge the pathogen has spread into different abdominal lymph nodes as seen by positive culture results. Nevertheless lesion development has been proven to take longer than after a respiratory challenge since isolation from the two non-confirmed reactors were obtained from macroscopic lesion free tissues.

These results may contribute to explain the scenario often found in slaughterhouses where no bovine tuberculosis compatible lesions are found in animals clearly reactive to the CMI response techniques and, on the other hand, to regain the farmers' confidence on the *in vivo* tests.

In the present doctoral dissertation applied and fundamental knowledge advances have been made regarding bovine tuberculosis diagnosis and pathogenesis, respectively. The improvements of knowledge regarding the development of the disease as well as the ability to reach an accurate diagnosis regardless of the different immunological status are crucial elements in preventing the transmission of the disease. Even more specifically, it must be underlined that the conclusions of these

studies may have a direct application in tuberculosis control programs as well as in the postmortem inspection in the slaughterhouses. In addition control of paratuberculosis could also benefit from the outcomes obtained since the use of the *Map* vaccines could be facilitated by them.

7. CONCLUSIONS

1. Paratuberculosis vaccination interference on the bovine tuberculosis diagnostic tests can be overcome if alternative diagnostic criteria or new more specific antigens are taken into account. The use of the defined ESAT-6/CFP10 antigen in the INF-γ test and the inoculation of three specific antigens (ESAT-6/CFP10 and Rv3615c) as skin test reagents in form of a proteinic cocktail avoid the interference linked to *Map* vaccination.

2. Vaccination against paratuberculosis confers some degree of heterologous protection against experimental *M. bovis* infections by reducing the severity of the lesions and the bacterial burden. The containment of the infection benefits the lung above all. This could be of great importance since the respiratory pathway is the main excretion route of *M. bovis* and a reduction of the bacterial load in the lung may lead to a reduction of the pathogen presence in the environment.

3. The development of a fully active cellular immune response capable of producing detectable levels of IFN-γ needs a longer period of time after an oral infection than after a respiratory infection.

4. The route of infection plays a critical previously unrecognized role in the location and bacterial burden of tuberculous lesions. Both parameters remain restricted to the abdominal area after an oral infection whereas the respiratory system is the main target after an aerogenous infection. In addition time required for gross lesions development seems to be longer after an oral infection.

8. SUMMARY

8.1. SUMMARY

Animal tuberculosis caused by infection with *M. bovis* is a global concern involving Animal and Public Health with derivations on conservation and economy. Cattle are the main domestic host both because of the disease epidemiology and the importance of this species in the livestock industry. Therefore bovine tuberculosis is the main objective of disease control programs throughout the world. However other livestock species including goats, sheep and pigs as well as several wild hosts contribute to maintain the disease. Official bovine tuberculosis eradication programs in cattle are based on intensive test and cull strategies. Nevertheless the limitation of the *in vivo* diagnostic methods, partly due to the complex development of the disease and to cross-reactions with other mycobacteria, together with the existence of wildlife reservoirs severely hampers success of the current control efforts.

The main objective of this Doctoral Thesis was to contribute to improve the knowledge in two fundamental research areas of the bovine tuberculosis: diagnosis and pathogenesis.

Due to the possible interference with the official bovine tuberculosis diagnostic methods, vaccination against *Map* in cattle is banned although its effectiveness has been proven in different species. Therefore the first study focused on searching alternative interpretation criteria for the standard intradermal tuberculin test and on assessing new more specific antigens in order to overcome the vaccination interference. Study I outcomes highlight that this interference can be completely avoided if new testing strategies such as specific antigens or new diagnostic criteria for the traditional comparative avian and bovine tuberculin test are established.

The banning of paratuberculosis vaccination in cattle means that little is known about the effect that this effective vaccine against *Map* could have in the development of a bovine tuberculosis infection. The second study was carried out to evaluate the

effect of paratuberculosis vaccination on preventing experimental *M. bovis* infection in calves. For that purpose pathological and bacteriological changes associated with the use of the vaccine were assessed. Results obtained show that the development of an experimental *M. bovis* infection can be modified by the use of a paratuberculosis vaccine. A decrease in the severity of the lesions and the bacterial burden was observed suggesting that a certain degree of heterologous protection against *M. bovis* may be conferred by the *Map* vaccine. The level of protection may not be enough to impede the establishment of the disease or transmission within a herd, but it may be helpful to contain the infection to some degree.

Although the presence of bovine tuberculosis in cattle has been drastically reduced since the implementation of eradication programs, many countries still have a residual infection rate. The aerogenous entry is accepted as the most frequent transmission route in cattle, affecting mainly the respiratory system. On the other hand different wildlife species contribute to the maintenance and spread of the disease. In addition indirect transmission by ingestion of contaminated food and water by cattle has been described as the most important transmission pathway between wildlife and livestock. However, only few experiments have been carried out in order to assess the oral route as a transmission pathway. Therefore the third study aimed to explain the appearance of reactive animals to the official CMI-based diagnostic tests but not presenting any macroscopic lesions. The hypothesis of the paper was that the oral route could be playing a more relevant role in transmission, detection and disease persistence. Differences in the development of the disease after an oral and a respiratory infection under experimental conditions were assessed. All animals were reactors to the tuberculin skin test. However results suggest that after a respiratory infection lesions are more likely to be found in the respiratory system and associated lymph nodes whereas after the oral infection lesions tend to appear in the abdominal area. In addition outcomes of the last study suggest that after an oral infection time

required for the development of gross lesions seems to take longer than after a respiratory infection.

Results obtained in this Thesis may have a direct application in the tuberculosis erradication programs and could facilitate the use of the paratuberculosis vaccine.

8.2. RESUMEN

La tuberculosis es una enfermedad infecciosa causada por bacterias pertenecientes al complejo *Mycobacterium tuberculosis* (MTC). De acuerdo con datos recientemente aportados por la Organización Mundial de la Salud, la tuberculosis continúa siendo a día de hoy una de las diez principales causas de mortalidad a nivel mundial, además de estar considerada como la principal causa de muerte por un agente infeccioso. Actualmente se estima que 1,7 billones de personas están infectadas de manera latente por el patógeno, de las cuales el 5-10% acabarán desarrollando la enfermedad. Además de los humanos, diferentes especies animales se pueden ver afectadas también por esta enfermedad.

Las micobacterias más estudiadas en animales han sido *Mycobacterium bovis (M. bovis)* y *Mycobacterium*. Caprae. La tuberculosis animal ha sido descrita mayoritariamente en ganado bovino, considerado como el hospedador mayoritario debido a la situación epidemiológica que presenta esta especie, así como por su importancia en la industria ganadera. Sin embargo se ha demostrado que otras especies domésticas rumiantes (cabras y ovejas) y no rumiantes (cerdos, caballos, gatos…) así como animales silvestres (ciervos, gamos, jabalíes, tejones…) pueden verse afectados también por la enfermedad. Por otra parte, *M. bovis* está clasificado como microorganismo zoonótico, por lo que la tuberculosis bovina está considerada como un objetivo primordial de control no sólo por la Sanidad Animal sino también por la Salud Pública.

Los programas de erradicación se centran en el ganado bovino y en el caprino bajo determinadas circunstancias. Sin embargo existen otros animales domésticos como las ovejas o cerdos que, junto con la fauna silvestre, principalmente ciervo y jabalí, pueden contribuir al mantenimiento de la infección ya que se encuentran exentos de seguimientos rutinarios. Los datos observados en España desde el inicio de las campañas de saneamiento bovino en la década de los 80 han sido satisfactorios. Sin embargo hay comunidades autónomas, principalmente aquellas con

mayor número de animales en extensivo y con mayor densidad de fauna silvestre, cuyas prevalencias a nivel de explotación siguen siendo altas.

El diagnóstico *in vivo* de la enfermedad se fundamenta en la prueba de la intradermotuberculinización. Esta técnica diagnóstica se basa en la inyección intradérmica de derivados proteicos purificados de tuberculina y en su capacidad para detectar una hipersensibilidad retardada en animales infectados. Los programas oficiales de erradicación de la tuberculosis en el ganado vacuno se basan en estrategias de detección y eliminación. Sin embargo las limitaciones de los métodos de diagnóstico *in vivo*, en parte debido al complejo desarrollo de la enfermedad y a las reacciones cruzadas con otras micobacterias, junto con la existencia de reservorios de fauna silvestre, comprometen el éxito de las campañas de saneamiento bovino frente a la tuberculosis.

Una de la micobacterias próximas a *M. bovis* y que puede interferir con las técnicas de rutina en el diagnóstico de la tuberculosis es *Mycobacterium avium* subsp. *paratuberculosis* (*Map*), agente etiológico de la paratuberculosis. La paratuberculosis es una enteritis granulomatosa crónica que afecta sobre todo a explotaciones de rumiantes y cuya patogenia no ha sido claramente definida. Aunque históricamente la enfermedad estaba ligada a los rumiantes domésticos, se ha demostrado que *Map* también está presente en rumiantes silvestres (ciervos, gamos…) y no rumiantes (conejos, liebres, jabalíes. tejones, algunas aves…). La vacunación se muestra como el método más efectivo para controlar la enfermedad. Varios estudios han confirmado la reducción de la carga de *Map* excretada en heces. Esto se traduce en una menor exposición de los animales al agente pudiendo disminuir de esta manera el número de casos clínicos. Además el uso de la vacuna también reduce la extensión de las lesiones propias de la enfermedad así como la colonización de tejido intestinal por el patógeno. Teniendo en cuenta que la principal ruta de infección es la fecal-oral, debido a la contaminación de los alimentos con las heces, la reducción de la excreción es fundamental para minimizar el riesgo de infección de nuevos animales. Sin embargo,

la vacunación frente a paratuberculosis está restringida por posibles interferencias con el diagnóstico de la tuberculosis.

El objetivo principal de esta tesis ha sido contribuir a mejorar el área de conocimiento de dos aspectos fundamentales de la tuberculosis bovina: el diagnóstico y la patogénesis. Para encontrar respuesta a las cuestiones que nos planteábamos antes de comenzar la Tesis, el objetivo principal se dividió en tres preguntas específicas:

1) ¿Sería posible evitar la interferencia de la vacunación contra la paratuberculosis en la detección de animales infectados con *M. bovis*?

2) ¿Podría proporcionar la vacunación contra la paratuberculosis algún grado de protección heteróloga contra la tuberculosis?

3) ¿Cómo afecta la vía de infección a la distribución de las lesiones y al tiempo de generación de respuesta de los animales?

En consecuencia, el primer estudio se centró en encontrar una forma de evitar la interferencia que la vacunación contra la paratuberculosis puede causar en las técnicas oficiales de diagnóstico de la tuberculosis bovina. Los dos artículos restantes abordaron la evolución del curso de la enfermedad en diferentes situaciones. Mientras que en el segundo estudio se evaluó el efecto de la vacunación frente a paratuberculosis en animales posteriormente infectados de manera experimental con *M. bovis*, en el tercer ensayo se estudiaron las posibles diferencias en la patogenia de la tuberculosis bovina en función de dos vías de infección diferentes con *M. bovis*, así como las implicaciones que esto podría acarrear para un diagnóstico certero.

Aunque la eficacia de la vacuna frente a paratuberculosis ha sido probada en repetidas ocasiones en diferentes especies, su uso en ganado bovino no está permitido debido a la posible interferencia con los métodos oficiales de diagnóstico de la tuberculosis. El primer estudio se centró en la búsqueda de criterios alternativos de

interpretación para la prueba de la intradermotuberculinización oficial y en la evaluación de nuevos antígenos más específicos para evitar la interferencia de la vacunación. Los resultados de este primer estudio destacan que esta interferencia puede evitarse por completo si se establecen nuevas estrategias como el uso de antígenos específicos o nuevos criterios de interpretación en la prueba de la intradermorreacción.

La prohibición de la vacunación frente a la paratuberculosis en el ganado bovino tiene como resultado un bajo conocimiento sobre el efecto que esta vacuna podría tener en el control de la tuberculosis bovina, siendo ambas enfermedades ocasionadas por patógenos con una composición antigénica similar. Por esta razón el segundo estudio se llevó a cabo para evaluar el efecto de la vacunación contra la paratuberculosis en la prevención de la infección experimental por *M. bovis* en terneros. Para ello se evaluaron los cambios patológicos y bacteriológicos asociados al uso de la vacuna. Los resultados obtenidos tras una infección experimental con *M. bovis* permitieron observar una disminución del nivel de gravedad de las lesiones así como de la carga bacteriana, lo que sugiere que la vacunación frente a *Map* puede conferir cierto grado de protección heteróloga frente a *M. bovis*. Es cierto que el nivel de protección puede que no sea suficiente para impedir el establecimiento de la enfermedad o la transmisión dentro de un rebaño, pero puede ser útil para contener el desarrollo lesional y el riesgo de transmisión.

Aunque la presencia de tuberculosis bovina se ha reducido notablemente desde la introducción de los programas de erradicación, la mayoría de los países todavía cuentan con una tasa de infección residual. La entrada aerógena del patógeno es aceptada como la vía de transmisión más frecuente en el ganado vacuno. Sin embargo, diferentes especies de fauna silvestre contribuyen al mantenimiento y la propagación del agente infeccioso, siendo la transmisión indirecta por ingestión de alimentos y agua contaminados por parte del ganado bovino la vía de transmisión más

importante entre las especies silvestres y el ganado vacuno. Sin embargo la importancia de la ruta de transmisión oral en la tuberculosis bovina sólo ha sido abordada en un número reducido de experimentos. Por lo tanto el objetivo del tercer estudio fue encontrar una explicación a la presencia de animales reactivos a las pruebas diagnósticas oficiales basadas en la respuesta inmune celular, pero carentes de lesiones macroscópicas. La hipótesis presentada en este tercer trabajo fue que la vía oral podría estar jugando un papel relevante en la transmisión, detección y persistencia de la enfermedad. Para ello se evaluaron las diferencias en el desarrollo de la enfermedad en función de si la ruta de entrada había sido la oral o la respiratoria bajo condiciones experimentales. Todos los animales mostraron reacciones positivas a la prueba de la intradermotuberculinización. Sin embargo los resultados obtenidos sugieren que la localización de las lesiones varía en base a la ruta de entrada del patógeno. Aquellos animales infectados por vía respiratoria presentaron la mayoría de las lesiones en el sistema respiratorio y ganglios linfáticos asociados, mientras que las lesiones se concentraron en el área abdominal en aquellos animales que fueron infectados por la vía oral. Además, los resultados de este último estudio sugieren que no sólo la localización de las lesiones difiere en relación a la vía de infección sino también el tiempo requerido para el desarrollo de las mismas, ya que la aparición de lesiones macroscópicas tras la infección oral requirió de un mayor espacio de tiempo que tras la infección por vía respiratoria.

Como conclusión final cabría señalar que los resultados obtenidos en esta Tesis podrían tener una aplicación directa en los programas de erradicación de la tuberculosis además de facilitar el uso de la vacuna contra la paratuberculosis en ganado bovino.

8.3. LABURPENA

Mycobacterium bovis (*M.bovis*) animalien tuberkulosia sortzen duen bakterioa da eta ekonomian eta kontserbazioan duen eraginagatik Osasun Publikoko kezka orokortzat jotzen da. Abeltzaintzan duen garrantziagatik eta bakterioaren epidemiologiagatik, tuberkulosiaren ostalari nagusia abelgorria da. Izan ere, behi-tuberkulosia munduan zehar ezartzen diren kontrol programetan garrantzi gehien duena da, nahiz eta beste ostalari batzuek gaixotasuna mantentzeko ahalmena izan; adibidez, ardiek, ahuntzek, txerriek eta zenbait animali basatiek. Abereen behi-tuberkulosia errotik erauzteko programa eraginkorrak animalien diagnosian eta hiltzeko estrategia zorrotzetan oinarritzen dira. Hala ere, gaur egungo metodo diagnostikoak mugak ditu. Lehenengoa, gaixotasunaren eboluzioak duen konplexutasuna; bigarrena, beste mikobakteria batzuekin erakusten duen erreakzio gurutzatua eta hirugarrena, animali basatiek gaixotasunaren ostalari izateko duten gaitasuna.

Doktorego tesi honen helburu nagusia behi-tuberkulosiaren bi funtsezko ikerketa arloetan ezagutza hobetzea izan da: diagnostikoa eta patogenia.

Paratuberkulosiaren aurkako txertoak zenbait espezietan eraginkortasun ona erakutsi duen arren, bere erabilera debekatuta dago behi-tuberkulosiaren diagnostikoan erabiltzen den metodoarekin interferentzia egin dezakeelako. Hori dela eta, lehenengo azterlanak dermis barneko tuberkulina probaren estandarra eta antigeno espezifiko berrien ebaluazioa aztertzen du, txertoak ematen duen erreakzio gurutzatua saihesteko. Lehenengo azterlanaren (Study I) emaitzen arabera, interferentzia hori guztiz saihestu daiteke, espezifikoagoak diren antigeno berriak sortuz edota hegazti eta behi-tuberkulina konparatze proba interpretatzeko irizpide berriak sortuz.

Abereen paratuberkulosiaren txertoa behietan erabiltzearen debekuak, tuberkulosiaren infekzioaren garapenean duen efektua ezagutzen ez dela aditzera

ematen du. Bigarren azterlanean (Study II) txahalak esperimentalki txertatu eta M. bovis-ekin infektatu ziren tuberkulosiaren garapenean paratuberculosiaren aurkako txertoaren ondorioak ikertzeko. Helburua betetzeko txertoari lotutako aldaketa patologikoak zein bakteriologikoak aztertu ziren. Lortutako emaitzek, paratuberkulosiaren aurkako txertoa erabiliz M. *bovis*-ek sortzen duen infekzioa moldatu daitekeela erakutsi zuten. Lesioen larritasuna eta bakterio-karga murriztu zirenez, paratuberkulosiaren aurkako txertoak *M. bovis*-en infekzioaren aurkako zenbait babes heterologo eman dezakeela adierazten du. Babes maila ez da nahikoa gaixotasuna edo transmisioa guztiz eragozteko, baina lagungarria izan daiteke infekzioa neurri batean menderatzeko.

Nahiz eta erauzte programa ofizialei esker behi-tuberkulosia asko gutxitu den, oraindik gaixotasunaren hondarrak herrialde askotan aurki daitezke. Bakterioaren transmisio ohikoa aire-bidez ematen da, arnas aparatuan kalte gehien egiten. Bestalde, zenbait espezie basatiek gaitz hau mantentzen eta barreiatzen laguntzen dute. Gainera, elikagai eta ur kutsatuen bidez gertatzen den zeharkako transmisioa, aho-bidez gertatzen dena, abeltzaintza eta espezie basatien arteko transmisio modu nagusitzat hartu da. Hala ere, esperimentu gutxi burutu dira aho-bidezko transmisioa balioesteko. Hori dela eta, hirugarren azterlanean lesio makroskopikorik gabeko baina dermis barneko tuberkulina proban erreakzioa izan zuten animaliak aztertu ziren. Adierazi zen hipotesia honako hau da: aho-bidezko transmisioak tuberkulosiaren iraupenean, detekzioan eta transmisioan orain arte pentsatu dena baino eragin handiagoa du. Horretarako, aho eta aire-bidezko infekzioak konparatzeko esperimentua burutu zen eta gaixotasunaren garapenean agertu ziren desberdintasunak aztertu ziren. Animalia guztiek dermis barneko tuberkulina proban erreakzioa izan zuten. Bestalde, aire-bidezko infekzioaren ondorioz arnas-aparatuko gongoil linfatikoetan eta birikietan lesio gehiago agertu ziren; aho bidezkoan; berriz, digestio aparatuko organoetan. Gainera, aho-bidez infektatu ziren animaliek denbora

gehiago behar izan zuten lesio makroskopiko larriak garatzeko aire-bidez infektatu zirenek baino.

Doktorengo tesi honen aurkikuntzak tuberkulosiaren erauzketa programan eta paratuberkulosiaren txertoaren erabileran aplikazio zuzena izan dezake.

8.4. ZUSSAMENFASSUNG

Tiertuberkulose, die durch eine Infektion durch *Mycobacterium bovis* (*M. bovis)* verursacht wird, ist ein weltweites Problem, das Tiergesundheit, *Artenschutz,* die Volkswirtschaft und sogar die öffentliche Gesundheit betrifft, da sie als *Zoonose* klassifiziert ist. Rinder stellen den größten Anteil an *Schlachtvieh* dar und sind deshalb das Hauptziel der Seuchenbekämpfung. Jedoch tragen andere Nutztiere wie Ziegen, Schafe und Schweine genau sowie verschiedene Wildtiere zum Fortbestand der Krankheit bei. Effektive Programme zur Auslöschung der bovinen Tuberkulose bei Rindern basieren auf intensiven Kontroll- und Keulungsstrategien. Jedoch könnten die aktuellen Anstrengungen zur Kontrolle durch die beschränkten Möglichkeiten der *in vivo* Diagnosemethoden, die teilweise aufgrund des komplexen Krankheitsverlaufs und Kreuzreaktionen mit anderen Mikobakterien beruhen, negativ beeinträchtigt werden. Ein zusätzlicher negativer Einfluss resultiert aus dem Fortbestand der Krankheit bei Wildtieren.

Das Hauptziel dieser Doktorarbeit war es, das Wissen in zwei Gebieten der Grundlagenforschung zur Rindertuberkulose zu erweitern: Diagnose und Pathogenese.

Wegen der möglichen Beeinträchtigung der amtlichen Methoden zur Diagnose der bovinen Tuberkulose ist die Impfung gegen *Mycobacterium avium* subsp. *paratuberculosis* (*Map)* bei Rindern untersagt, obwohl ihre Effektivität in anderen Tierarten bewiesen ist. Daher setzte die erste Studie den Akzent auf die Suche nach alternativen Interpretationskriterien des intradermalen Tuberkulin- Standardtestes und auf die Bewertung neuer, noch spezifischerer Antigene, um die Störung der Diagnose von Rindertuberkulose durch die *Map* Impfungen zu vermeiden.

Die Ergebnisse der ersten Studie heben hervor, dass die Beeinflussung komplett vermieden werden kann, wenn neue Test-Strategien wie spezifische Antigene oder

neue Diagnose-Kriterien für die herkömmlichen Vogel- und Rindertuberkulin eingeführt werden.

Das Verbot der Paratuberkulose-Impfung bei Rindern hat zur Folge, dass nur wenig über die möglichen Auswirkungen dieses wirksamen Impfstoffs gegen *Map* bei der Entwicklung einer Rindertuberkulose-Infektion bekannt ist. Die zweite Studie wurde durchgeführt, um den Effekt einer Paratuberkulose-Impfung bei experimentell mit *M. bovis* infizierten Rindern evaluieren zu können. Zu diesem Zweck wurden pathologische und bakteriologische Veränderungen in Zusammenhang mit dem Einsatz des Impfstoffes untersucht. Die hieraus gewonnenen Ergebnisse zeigen, dass die Entwicklung einer experimentell herbeigeführten *M. bovis* Infektion durch den Einsatz des Paratuberkulose-Impfstoffes modifiziert werden kann. Ein Rückgang in der Schwere der Läsionen und bei der Bakterienbelastung war zu beobachten, was andeutet, dass ein bestimmter Grad an heterologem Schutz gegen *M. bovis* durch die *Map*-Impfung erreicht werden kann. Das Schutzniveau ist wohl nicht ausreichend, um den Ausbruch der Krankheit oder die Übertragung innerhalb einer Herde zu verhindern, könnte aber nützlich sein, die Krankheit bis zu einem gewissen Grad einzudämmen.

Obwohl das Vorkommen der bovinen Tuberkulose bei Rindern seit der Einführung von Auslöschungsprogrammen drastisch reduziert wurde, weisen viele Länder immer noch eine hohe Infektionsrate auf. Die Atemwegsübertragung wird allgemein als die häufigste Übertragungsart angesehen.

Bei dieser Übertragungsart ist vor allem das System der Atemwege betroffen. Darüber hinaus tragen verschiedene Wildtiere zu Erhalt und Verbreitung der Krankheit bei. Dazu kommt die indirekte Übertragung durch die Inkorporation verseuchter Nahrung und Wasser durch Rinder, was als der häufigste Übertragungsweg zwischen Vieh und Wild beschrieben wird.

Jedoch wurden nur wenige Experimente durchgeführt, die die Atemwege als Übertragungsweg zum Untersuchungsgegenstand hatten.

Aus diesem Grund zielte die dritte Studie darauf ab, die Symptome von bei CMI-basierten Diagnosetests positiv getesteten, aber keine makroskopischen Läsionen aufweisenden, Tiere zu erklären. Die Hypothese dieser Arbeit war es nun, dass der orale Weg eine größere Rolle bei Übertragung, Detektion und Persistenz der Krankheit spielen könnte als bisher angenommen. Unterschiede im Krankheitsverlauf bei oraler Infektion bzw. Infektion über die Atemwege wurden untersucht (unter Experimentalbedingungen). Alle Tiere reagierten positiv auf den Tuberkulin-Hauttest. Die Ergebnisse zeigten jedoch Unterschiede beim Ort des Auftretens infektiöser Läsionen: Nach einer Atemwegsinfektion traten diese eher im Atemwegssystem und den verbundenen Lymphknoten auf, wohingegen sie nach einer oralen Infektion eher im abdominalen Bereich auftraten. Zusätzlich zeigten die Ergebnisse der letzten Studie, dass nach einer oralen Infektion die Ausbildung sichtbarer Läsionen längere Zeit in Anspruch nahm als nach einer Atemwegsinfektion.

Die Ergebnisse dieser Arbeit könnten direkte Anwendung in Tuberkuloseausrottungsprogrammen finden und könnten den Einsatz der Paratuberkulose-Impfung erleichtern.

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