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Review article

Molecular findings and approaches spotlighting *Mycobacterium bovis* persistence in cattle

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Abstract – *Mycobacterium tuberculosis* (*Mtb*) and *Mycobacterium bovis* (*M. bovis*) are the etiological agents of human and bovine tuberculosis (TB, bTB) respectively, and share genetic identity over 99% at the whole genome level. Progress has been made towards explaining how mycobacteria and their infected hosts remain in balance without producing clinical symptoms of disease, a phenomenon referred to as latency or persistence, which can be mimicked by certain in vitro conditions. Latency/persistence has mainly been studied using *Mtb*, where the two-component signalling system, *dosRS*, has been assigned an instrumental role, and even constitutes the current basis for development of new diagnostic methods and treatment addressing this particular stage of TB. *M. bovis* conserves homolog genes that in *Mtb* play a role in human latent TB infection and that, by analogy, would allow it to enter a persistent state in infected cattle; nevertheless, little attention has been paid to this stage in bovine hosts. We suggest that many of the advances acquired through the study of *Mtb* can and should be taken into consideration by research groups and veterinary professionals dealing with bTB. The study of the infection in bovines, paying particular attention to defining the molecular and cellular markers of a *M. bovis* persistent infection in cattle, presents great opportunities for the development and trial of new diagnostic tests and vaccines, tools that will surely help in promoting eradication of bTB in high-burden settings.

tuberculosis / *Mycobacterium* / persistence / latency / DosR

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1. INTRODUCTION

Tuberculosis is a mycobacterial infection that affects a wide range of mammals. Human and bovine tuberculosis (TB and bTB respectively)

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share key aspects such as developing similar lesions and immune responses, which often result in colonization and spread to the same organs, namely lungs and lymphatic tissues [12, 14, 73, 109]. Like in humans, aerosolized bacteria are the most common source of infectious organisms in cattle, and the primary site of natural infection is the respiratory tract [14, 21]. In bTB, airborne infection is considered the most common route of transmission, and more than 19% of cattle with bTB shed the mycobacteria [24, 111], mainly early during infection [68, 70]. The intermittent nature of bacilli shedding from infected animals, after a short initial post-infection period has been documented [67, 68]. Several studies indicate that between 8–26% of naturally infected animals release mycobacteria in clinical samples, but the precise duration of the secretion period is unknown [75, 95, 111].

Mycobacterium bovis (*M. bovis*) is a close relative of *Mycobacterium tuberculosis* (*Mtb*), and they share genetic identity over 99% at the whole genome level [34]. Different studies have supported the fact that although slight differences are found in the genome sequence of *Mtb* and *M. bovis*, the physiology and host range spectrum is different [36, 88]. In recent years, progress has been made regarding the molecular basis that might, at least partially, explain how mycobacteria are able to remain in a persistent or latent infection in its human host, and it has also been suggested that this particular stage of disease exists in cattle, by analogy with human TB [14, 42, 84, 109]; nevertheless, until now little attention has been paid to this form of infection in bovine hosts.

TB produced by *M. bovis* is an important human zoonosis associated with consumption of dairy products contaminated with the bacilli, and to labour risk (such as direct contact or droplet transmission) in farms or slaughterhouses [25, 39, 105, 108]. Close to 35% of the Latin American and Caribbean countries have reported the occurrence of TB due to *M. bovis*, and a conservative estimate would be that it causes 2% of the total pulmonary TB cases and 8% of extrapulmonary TB cases. Moreover, it is also estimated that 60% of the human population live in countries where cattle undergo no control or only limited control for bTB [20], constituting a potential infectious source. Ideally, all cattle that give positive readings to diagnostic tests should be killed, as they may well represent a source of ongoing infection. However, under financial constraints, for example in developing countries, programs to combat bTB require a more accurate targeting of cattle that represent the greatest threat of spreading infection [85]. We must consider the inability of current assays to discriminate between active and a likely latent infection in cattle, which could make the difference between sacrificing truly contagious animals or only suspected ones.

It was recently shown that *M. bovis* accounted for 45% of all culture-positive TB cases in children and 6% of adult cases in San Diego, California, United States, from 1994 to 2005 [93], whereas in the whole country, 1.4% of linked cases were identified as *M. bovis* [45]. On the other hand, surpassing a previously thought host preference [34], *Mtb* infected cattle has been reported in countries with high incidence of bTB [16, 105]. Defining the proportion as well as the clinical presentation of infected subjects (active or persistent) in affected countries is no doubt of enormous importance, as it is determining the same numbers for infected cattle [22].

It is worth noting that, contrary to TB, where active disease often occurs with clinical signs and symptoms (fever, weight loss, coughing, presence of acid-fast bacilli (AFB) in sputa or other clinical samples), during bTB, *M. bovis*-infected cattle do not present two well-defined clinical (active or latent) phases, nor does the immune reactivity test used nowadays (tuberculin skin test, TST) has the power to discern between these two likely states in infected bovines. Asymptomatic TST+ cattle have been found infected with either *M. bovis* or *Mtb* as well [105].

Clinically, latency is defined as the persistence of a tuberculous lesion with viable mycobacteria in a host without symptoms and has been demonstrated because of reactivation (defined as the onset of active disease upon, e.g., an immunosuppressive event) of apparently healthy people and/or reactivity towards

Mtb antigens despite lack of clinical manifestations and absence of AFB in clinical specimens [30, 35, 53, 54]. In contrast, in the context of bovines, persistence could perhaps be defined as the existence of a positive skin or IFN-γ test in cattle in which no lesions can be detected or when a culture is positive in the absence of lesions. However, the first approach presents the disadvantage of not directly defining whether reactivity is due to a present or past infection, or even because of contact with environmental mycobacteria. The latter approach is simply time consuming and again does not necessarily address which organisms are causing the infection unless more specific tests (PCR, hybridization, specific immune assays) are conducted.

Although individuals with latent infection theoretically do not transmit the disease, in countries where TB does not have a high incidence, it is estimated that there is a risk of 2–23% of reactivation of the disease during their lifetime, thus constituting a significant source of transmission upon reactivation [80]. Reactivation is thought to be derived from many events that lead to immunosuppression, from experimental evidence using *Mtb* infection in a mouse model [8]. Reactivation of latent tuberculosis produced by *M. bovis* infection has been documented in people with previous exposure to unpasteurised dairy products [54]. In addition, in TB cases associated with human *M. bovis* infection, the HIV/AIDS pandemic may lead to a greater susceptibility of people to overt TB [39], as has been shown with the use of anti-inflammatory treatment [54]. Envisioning a similar scenario, should persistent/latent bTB in cattle be present, reactivation could be associated with changes in cell-mediated immunity in tuberculous cattle as well as with viral immunosuppression, metabolic unbalance, corticosteroids and postpartum period [32, 82, 84, 102]. Nevertheless, the association of bTB with persistent and immunosuppressive viral diseases, such as bovine viral diarrhoea and bovine leucosis, has not been described in spite of its enzootic character in dairy cattle and deserves further attention.

Considering the scarce evidence demonstrating that *M. bovis* produces a latent/persistent infection in humans, and suggesting it can produce a similar state in cattle, as well as the few reports intended to decipher and precisely defining the existence of a chronic/persistent *M. bovis* infection state during disease, and its impact for uncontrolled bTB in several countries, we present this work, pursuing the following goals: (1) to present a brief summary of the current status and advances on tuberculosis persistence both in bovine and humans, and (2) to suggest paths to converge such knowledge for the benefit of the diagnosis and treatment efforts against bTB, for their application in the veterinary field.

2. IS *M. BOVIS* PERSISTENCE AFFECTED BY IMMUNE RESPONSES IN CATTLE?

In a histopathological setting, bTB spreads not only from animals with gross lung lesions, but also from those considered to represent a latent-like (persistent) infection without visible (gross) lesions [65, 69, 75, 84], although caution should be taken considering the arguments expressed above, for the need of clearly demonstrating (not suspecting) a latent bTB phase. Post mortem analysis of samples from cattle with a positive tuberculin skin test (TST+) and IFN-γ test reactors often correlates with gross lesions mainly in tracheobronchial, mediastinal, and retropharyngeal lymph nodes [76]. The majority of bTB cases occurring as natural infection are restricted to the lower respiratory tract, but no cavitation of lungs has been found, as opposed to some chronic human TB cases [19, 71, 75, 76, 118], raising exciting questions as to what is the nature of such differences. Exhaustive histopathological analyses of lungs have allowed the identification of lesions in 70% of bTB cases without visible lesions in this organ [65]. However, under low prevalence conditions, close to 60% of animals with TST+ have no visible lesions, and may thus constitute a persistently infected population, providing a good source of material for experimentally addressing the existence of such state in cattle.

In order to define the existence of a particular infection stage, a proper characterization and

classification of the lesions is necessary, as is being proposed for the different stages of granuloma development [76]. These are as follows: stage I/II early non-necrotic lesions; stage III infected core surrounded by multiple concentric layers of macrophages (including epithelioid and giant cells) and fibrous tissue; and stage IV central caseous necrosis. It is common to find type IV in combination with type I/II lesions [57]. Since caseous necrosis and calcification accompanied by a small number of positive AFB are common features of granulomas encountered in natural infections, this can be detected in animals with no visible or isolated histopathological lesions, independently of their TST status or IFN-γ responses [57, 69, 70]. Changes in the TST status, histopathological findings or cellular markers might prove to be helpful in estimating what type of infection a bovine has, either active or persistent, and are problems that surely deserve further exploration, as long as they can be improved to specifically determine *M. bovis* presence or any other mycobacteria as agent of the disease.

At a cellular level, once inside the host, *M. bovis* is ingested by macrophages and their activation aids limitation of or destruction of infecting mycobacteria [23, 86]. Moreover, differences in the antibacterial capacity of bovine macrophages, depending upon microenvironment and organ-specific factors or genetic background, may exist [87]. Experimental data suggests that an excessive immune response or a high virulence of the bacilli favour the pathogenesis of bTB [116, 117]. In susceptible hosts, the interaction of *M. bovis* with cells from the innate and adaptive immune response in the respiratory tissue and lymph nodes results in colonization and infection with development of several-stage lesions leading to the production of granulomas [76]. Granulomas represent the focal expression of the inflammatory response aimed at preventing mycobacterial growth, thus constituting a means whereby bacteria could potentially be directed or primed to enter a persistent state. During this interaction, T-cells are among the first cells involved in early granuloma formation [13]. All of the main T-cell subsets (γδ T-cells, CD4+ and CD8+ αβ T-cells) have been shown to be involved in the anti-mycobacterial immune response in cattle [85]. There, both CD4+ T-cells and CD8+ T-cells have an important involvement in controlling infected cells [85]. Interestingly, CD8+ cells may play a deleterious role by contributing to the immunopathology of bTB [110], while WC1+γδ T-cell may contribute toward the Th1 bias of the immune response in bTB, although they do not appear to be essential for the development of delayed-type hypersensitivity (DTH) [49, 50]. Within granulomas, there is a balance between mycobacterial killing and survival [99] that results in eradication, containment (persistence, latency) or progression of the infection, according to which side of the immune response is favoured [85, 86].

Within granulomas, the presence of AFB organisms might suggest these bacteria are in a replicative, rather than a dormant or persistent state, based on the lack of AFB staining in *Mtb* H37Rv under in vitro latent conditions, induced by nutrient deprivation [3]. However, we must point out that there is a lack of data about the acid-fast staining properties of bacilli assumed to be genetically impaired to enter a latent state (i.e. *dosRS* knock-out, a two component system involved in *Mtb* latency, described later), although it was recently demonstrated that the AFB phenotype is dependent on cell wall lipid modifications, controlled by *phoP* [115] and *kasB* [9]. PhoP was reported to affect expression of DosR-controlled genes [37], and for *kasB* either direct or indirect control by *dosRS* has not been demonstrated.

It was recently found that depending on the cytokine profile (either predominantly Th1- or Th2-type) cattle experimentally infected with *M. bovis* present different degrees of histopathological damage, thus strengthening the critical role of the immune response in the outcome of bTB [106]. As a consequence of hormonal changes, immunosuppression might occur during peripartum, diminishing cytokines and antibodies, along with Th2 polarization of immune response [63], which in persistently infected cows might promote reactivation. Further studies have indicated the role of additional factors in the onset and determining susceptibility to bTB, such as early production of IL-4 secreted by Th2 lymphocyte subsets [89, 90], and also

the early decrease of IL-10 secretion [106]. In mycobacterial infections, changes in the immune balance from a dominant Th1-type response towards a more prominent Th2 response are associated with disease progression, B-cell antibody production and anergy of cell-mediated immune responses [85]. Recent attempts to establish immune profiles associated with *M. bovis* infection status have been made in cattle suggesting innate immune gene repression in PBMC from bTB infected animals [66]. Despite this attempt, it is clear that immune phenomena in bTB are highly complex and hard to characterize in cattle. This outlines the need of modifying and improving the current strategies employed to determine bTB infection status, perhaps moving towards finding and validating specific molecular markers.

3. MYCOBACTERIAL FACTORS REQUIRED FOR LATENCY

The molecular mechanisms responsible for the onset of active or latent TB or reactivation from latency to active TB are not yet well understood. We must consider that mycobacteria have a complex life cycle and endure an ever-changing environment while trying to successfully establish and maintain an infection or even while surviving outside their host waiting for a new infective process to begin. Nevertheless, a factor that very likely contributes to the continued transmission of TB in humans and perhaps bovine hosts is the demonstrated capacity of mycobacteria to exist in a dormant, persistent or latent state for extended periods of time, particularly when under stress [80]. This often leads to dramatic changes in gene transcription, including those of particular interest in this review, namely genes related to latency, dormancy or persistence within a hostile environment. These include but are not necessarily limited to: (1) Hypoxia, (2) Reactive Nitrogen Species (RNI), (3) Reactive Oxygen Species (RON), (4) the host immune response (NK cells, activation of TLR and synthesis of host defence peptides among others). All these factors impose a combination of stresses that affect the pathogen [94, 101]. A function that would be expected to be up regulated during such attacks is the antioxidant response. In this regard, genes putatively involved in defence against oxidative stress can be found in mycobacterial genomes, such as *noxR1*, *noxR3,* and *ahpC*, which possess high identity with genes encoding for antioxidant enzymes widely distributed in prokaryotes. The *glbN* gene, which codes for a truncated haemoglobin (trHbN), is responsible for the nitric oxide (NO) metabolizing activity observed in mycobacterial stationary phase in vitro cultures [74]. Additionally, other components are required for adaptations occurring during persistent infection in animal or cellular models, where the protein α-crystallin (HspX, Rv2031c or Acr) from *Mtb* is required during the phase of steady growth within macrophages [104, 122].

Studies in both *Mtb* and *M. bovis* var. BCG grown under stress with low oxygen tension have shown that HspX is a protein expressed during these conditions, and is virtually the first protein required for a latency-like state functionally demonstrated to have an impact in both organisms under these particular conditions [33, 58, 103].

Different studies have approached the question of latency using alternative techniques. For example, Rustad et al. [97] suggest an extended hypoxic response regulon, and their study requires in vivo validation of the participation from genes found in their work. Saxena et al. [100] used a genetic screen for in vivo expressed technology; however, it is hard to directly extrapolate studies where not only transcription of hypoxia-responsive genes is assessed, but also translational effects (such as altered protein stability of GFP and/or kanamycin) likely occurring on the reporter genes should be considered.

Furthermore, the roles of MprAB [77], as well as PhoP [37] recently added another level of complexity solely to DosR regulation, and are not developed in this work in the idea of suggesting markers to define whether or not latency occurs in cattle, conversely to defining the mechanisms regulating an event that it is still unclear in bovines. Considering these arguments as well as the evidences linking in vitro to in vivo relevance for DosR and DosR-regulated genes, including the potential use of some

DosR-regulon proteins as new markers for latent/persistent infection, we discuss its participation in further detail below.

4. THE *dosRS* REGULON IS CONSERVED BETWEEN *M. BOVIS* AND *MTB*

The *dosRS* (also known as *devRS*) two-component system of *Mtb* regulates the expression of a set of 48 genes induced either by hypoxic shift-down or exposure to low doses of NO in vitro [114] and constitutes an operon with complex regulation that participates in bacterial response against a wide range of stresses [92]. DosR is the response regulator with DNA-binding activity, and DosS is one out of two Sensor Histidine Kinases (HK), along with DosT, which phosphorylates and thus regulates DosR DNA-binding activity. Its relevance in mycobacteria was independently confirmed by two groups, using *M. bovis* var. BCG (a *M. bovis* strain where a set of genomic deletions, insertions, and duplications have occurred as a consequence of in vitro passages) as a model organism [10], and then confirmed in *Mtb* [79], where mutants lacking either one or both components are affected in their capacity to survive a long-term culture under oxygen-limiting conditions. On the other hand, conflicting reports about its participation during infection in animal models have been published [62, 78], perhaps attributable to the different models used by each group. Indeed, Converse et al. [18] analyzed the participation of the *dosRS* system in four different models of *Mtb* infection (rabbits, guinea pigs, mouse hollow-fibre, and mouse), and found a growth defect only in mouse and guinea pigs. Kesavan et al. [51] analyzed expression of DosR-regulon and non-DosR controlled genes during *Mtb* persistent infection in rabbits. Another analysis of mycobacterial response to hypoxia and exposure to nitric oxide, including 14 different vaccine strains of *M. bovis* var. BCG, revealed the presence of a functional DosR-regulon similar to that found in *Mtb* [59]. Nevertheless, several BCG vaccine strains lacked *narK2* and *narX* induction and exhibit an altered phenotypes during dormancy [46]. We should notice the lack of immune responses to DosR regulon proteins following *M. bovis* var. BCG subcutaneous vaccination in a mouse model, and even in humans [35, 59], whereby such lack of induction might be the consequence, at least partially, of reduced bacterial survival with an inadequate space-temporal antigen production and presentation.

The genome of *M. bovis* strain AF2122/97 is virtually identical (99.95%) to that of *Mtb* strains H37Rv or CDC1551 [34]. By in silico analysis, comparing the predicted protein sequences derived from the AF2122/97 and H37Rv DosR antigens, we found an average 97% identity at the amino acid level in 41 out of 48 proteins, with only one amino acid difference in the reported sequences of DosS, within a predicted GAF domain. GAF presents haem-binding activity [98]. The other HK, DosT, already shown to phosphorylate DosR in vitro [91] and also relevant for survival under hypoxic conditions [52], also has one amino acid difference within a GAF domain, whereas DosR is 100% identical. This constitutes a strong indicator of the potential capacity of *M. bovis* to induce a bacterial survival mechanism that would enable it to remain present in cattle in a latent state. We can question whether or not differences in such GAF domains, or even in the rest of the proteins belonging to the DosR-regulon, have a biological role in mycobacterial adaptation to a different host.

5. CURRENT ASSAYS AND STRATEGIES FOR IMPROVING HUMAN LATENT TUBERCULOSIS DIAGNOSIS

For many decades, the TST assay, using a purified protein derivative (PPD), has been the most commonly used test to check for tuberculosis in cases of subclinical infection, both in humans and in cattle, with a little variation in the latter by comparison of reactivity toward PPD from *M. bovis* versus PPD from *Mycobacterium avium*. Mycobacterial PPD, as reported elsewhere, consists of a mixture of several hundred secreted proteins, obtained by trichloroacetic acid precipitation of cell-free culture filtrates derived from pathogenic mycobacteria. Due to

inherent genomic variations, it is tempting to speculate over differences in PPD constitution for *Mtb, M. bovis* and *M. avium*, for example, affecting specificity of detection, although a detailed analysis (in silico or proteomic) that ensures such a prediction should be conducted.

The use of PPD has a major drawback, as most antigens present in it are also present in *M. bovis* var. BCG, a bacterial strain used to vaccinate humans at an early age, which may affect the specificity of the test [6]. Despite this, TST is still considered the best tool for classifying individuals with no symptoms of clinical TB as latently infected [64]. Paradoxically, the proteins used as antigens in TST are produced by mycobacteria under conditions not experimentally defined as persistent. In spite of this fact, their use is considered in some developed countries as a valuable means of diagnosing both clinical (active) and subclinical (latent) TB [2, 6, 15]. It was not until very recently that new tests started to emerge for determining TB infection status [6, 17]. These are based on the detection of IFN-γ in blood samples after in vitro stimulation of blood cells with the so-called specific *Mtb* antigens ESAT-6 and CFP-10 [5], which are considered specific due to their absence in *M. bovis* var. BCG used in human vaccinations. This assay is the basis of a kit commercially available for determining human infection (QuantiFERON®-TB Gold; Cellestis Limited, Carnegie, Victoria, Australia). In humans, a study carried out in India based on measuring the cellular response (IFN-γ production) triggered by ESAT-6 and CFP-10 of *Mtb*, indicated a prevalence of 80% of latent TB infection in an apparently healthy population used in the study [53]. However, ESAT-6 and CFP-10 are conserved in wild-type *M. bovis*, and do not allow the determination of which pathogenic mycobacteria (*Mtb* or *M. bovis*) is infecting humans unless time-consuming procedures of microbiological culture, biochemical and antibiotic susceptibility tests or PCR approaches, not yet standard in some settings, have been conducted. In this regard, patients who had a negative microbiological cultures and normal lung histology were found to contain the insertion sequence (IS) 6110, a characteristic region of the tubercle bacilli, by in situ PCR [43].

On the other hand, proteins belonging to the DosR-regulon, which are expressed under experimental conditions considered to mimic aspects of latency, have also been evaluated as targets that differentiate TB infection status [30, 35]. HspX proved to be a good stimulator of the cellular immune response in patients with asymptomatic tuberculosis [30, 35] thus promising to be a new, good, and specific marker for latent TB infection that could replace the TST. However, this protein was immunogenic for B cells of patients with active pulmonary tuberculosis as well [119], and it is still unknown whether or not HspX could indeed be a good marker of latent TB infection at this point. Therefore, three proteins of the DosR-regulon, namely Rv1733c, Rv2031c (HspX), and Rv2626c, were evaluated in an independent study, and it was found that they induced a good cellular immune response in a mouse model of latency [96]. Furthermore, in asymptomatic, TST+ individuals from the Netherlands, a country with low incidence of TB (TST+, being a marker of latent infection), the immune response for four DosR-regulon proteins (Rv1733c, Rv2029c, Rv2627c and Rv2628c) was higher compared to that of cells from healthy people and patients with active disease [56].

In a different context, a role for cell types distinct to macrophages possibly linked to TB latency has started to emerge, based on in situ PCR evidence showing *Mtb* DNA in type II pneumocytes [43] or human adipose tissue [72]. Together, all this evidence expands alternatives for monitoring the infection status of subjects suspected to contain latent mycobacteria. Likewise, when bacilli are inside type II pneumocytes, the heparin binding haemagglutinin (HbhA, not part of the DosR-regulon), was found to be strongly expressed [28, 81]. A study measuring IFN-γ production in response to stimulation with native HbhA in 205 persons in Belgium (a country with a low incidence of TB where vaccination with BCG is not routinely used) gave a 92% sensitivity and a specificity of 94% using HbhA, compared to 90% and 70% obtained with PPD for detection of infected subjects, regardless of their BCG vaccination status as opposed to PPD reactivity. This indicates that in this setting, HbhA is a

good antigen for detecting individuals with asymptomatic infection [48]. As methylation of native HbhA is important for T-cell stimulation, a recombinant protein for wide use should possess this modification.

Thus, published evidences suggest that HbhA and DosR-controlled proteins might be good candidates for discriminating between active TB and latent infection. In fact, some DosR-regulated antigens have been either suggested or already used as new sub-unitary or DNA vaccines [7, 60, 96]. Perhaps it is about time to redefine the gold standard for TB infection status.

6. CHALLENGES IN DEFINING PERSISTENT BOVINE TUBERCULOSIS

In humans, active disease is clearly shown by clinical findings such as weight loss, night fevers, and coughing. Conversely, latent infection is demonstrated because of the reactivity to selected mycobacterial antigens with no signs of disease [30, 35, 53]. In cattle, there is no clear sign of a different status of active versus latent infection, and the concept of latent infection has remained more as a speculation than a proven fact [14, 42, 84, 109]. Perhaps this is influenced by the relatively short life span of cows compared to humans, or the lack of ethical concerns about sacrificing infected individuals, with greater concerns about economical losses and no attention to better understanding the infective process. However, this latter point does not consider continued transmission to animal and human hosts.

Latency has been suspected in animals that, upon exposure, turn positive to TST or an IFN-γ release assay using bovine PPD, purified ESAT-6/CFP-10 or the commercially available kit based on IFN-γ (BOVIGAM, Prionics, Zurich, Switzerland) [1, 120] that also uses PPD [14]. This complicates the interpretation and diagnosis of animals truly infected with human pathogens or environmental mycobacteria [47, 107]. In fact, PPD induced cross-reactions that varied according to the area, ranging from 2 to 20% [40, 41]. Evidence suggesting that diagnostic assays (TST and IFN-γ tests) can detect bTB in cattle soon after *M. bovis* infection regardless of the dose has been published [27, 70] thus, none of the tests currently available for the diagnosis of bTB allow a perfectly accurate determination of the *M. bovis* infection status of cattle [26]. Other authors indicate that during cow-to-cow *M. bovis* transmission, it takes about two months to present a TST+ reaction (ranging from 8 to 65 days) [38], potentially increasing the number of false negative results. Moreover, reversion from a TST+ result occurs at a 5% rate per year [38], possibly indicating eradication of infection or simply waning of immune memory.

As an alternative, an enhanced version of TST, which includes determination of MPB83-specific antibody responses, was developed and found to have a positive correlation with bacterial loads in infected cattle [61]. Denis et al. [31] used a different strategy by including immune modulators in an IFN-γ release assay, and found that anti-IL10 antibodies increased sensitivity of detection. However, these improvements are far from establishing whether or not latency occurs during bovine infection, thus further strengthening the need of either using antigens that allow this distinction for human TB and see how do they work in the veterinary setting, or finding new antigens that help to determine the status (active or persistent) of infection in cattle. We must also consider that animals naturally show variability in TST responses, and some show no clinical signs of disease or visible lesions when slaughtered, with microbiological cultures being negative or positive but from lymph nodes only. Some cows have the ability to resist overt infection despite exposure to *M. bovis*. The significant numbers of TST+ cattle that have no evidence of infection at abattoir meat inspections or on laboratory examinations would support, at least in part, the idea of a latent infection during bovine tuberculosis [84].

Although BCG has been shown to be an effective vaccine against bTB even using a low dose in cattle [11], it cannot be widely used unless a cost-effective laboratory test discriminates between infected and vaccinated cattle. IFN-γ responses to PPD are induced by BCG

vaccination of cattle, but their magnitude and duration are dose-dependent and short-lived [4]. Some antigens are either not present (i.e. ESAT-6 or CFP-10) or are only weakly expressed (i.e. MPB64 or MPB70) in BCG, and their use in the IFN-γ assay has been shown to offer an opportunity to discriminate between animals infected with *M. bovis* and those vaccinated with BCG [44, 83, 112]. Quite interestingly, T cell-responses against ESAT-6 correlated better with disease severity in cattle [113].

In addition to the lack of clinical symptoms, it is known that livestock is likely to present a TST+ reaction because of exposure to non-pathogenic, environmental mycobacteria [47, 107], thus increasing the rates of false positive detection. Recently, evidence of the effect of environmental strains of *M. avium* interfering with immune responses blocking BCG multiplication and induction of protective immunity to tuberculosis has been found [29, 121].

Further complications in defining which animals are indeed infected (actively or persistently) arise from the phenomenon of anergy. The origins of anergy in tuberculous individuals have not been completely defined but it is attributed to immunosuppression [55] and more recently to an undefined type of tolerance [47, 121]. In particular, in cattle under intensive production, the cases of anergy could be related to stressing factors such as transportation, handling, metabolic unbalance, and treatments with prostaglandins or corticosteroids, among others [32]. A post-mortem study with healthy, TST+ animals, with no visible injuries, indicated that the expression of interleukin IL-4 δ3 in these animals may be playing an important role in maintaining protection against infection after exposure to *M. bovis* [89, 90]. Therefore, this cellular response could be considered a particular hallmark of latent infection in cattle, constituting a plausible biomarker from the host's point of view.

Clearly the existence of animals with asymptomatic bTB imposes a great disadvantage to the veterinary field, further supporting the need for well established testing markers to distinguish active versus latent infection in cattle. These tests need to either directly measuring gene expression from *M. bovis* isolated from well-defined histopathological sections, or evaluating cellular markers from infected animals in low and high-incidence settings of bTB or experimentally infected in a time-course manner as already started by Thacker et al. [106]. Similar to latent tuberculosis in humans, in cattle, latency represents a significant challenge for the eradication of bTB, through the possible reactivation in an infected animal [14]. Despite the current progress achieved in the study of latent tuberculosis in humans, defining latency in bTB has practically and inexplicably been ignored, except for a recent call to define the participation of latency in the control of bTB [42]. It is tempting to envision that cattle may be the species in which persistent infection better resembles human latency. However, methods for experimental induction of persistent tuberculosis in cattle do not exist yet [109].

7. CONCLUDING REMARKS AND FUTURE DIRECTIONS

Latent tuberculosis infection remains a public health problem, because reactivation can happen many years after primary infection resulting in renovated transmission of strains previously thought to have been gotten rid of, or that existed thoroughly unsuspected. Despite latent TB being recognized in humans for a long time, persistent infection in cattle has largely been ignored. A comparative genomics point of view could be quintessential in providing better means to control, diminish or eradicate epidemiologically relevant events. It would be very interesting to investigate whether monitoring expression of genes belonging to the DosR-regulon in *M. bovis*-infected cattle would finally allow a description of a latent/persistent phase of bTB. Using this approach in combination with analysis of the expression and role of mycobacterial genes required for active growth would allow a more precise definition of temporal profiles during transition stages of the infection. One of the most active lines of basic research in the field of tuberculosis consists of deciphering what genes are turned on or off when mycobacteria move from

an active and replicative state to one of persistence.

Defining whether latency occurs in cattle and assessing the potential participation of different cell types as "incubators" of persistent bacilli can lay the groundwork for identifying genes and/or proteins that may be useful both for diagnosis and for the development of new vaccines against human and bovine tuberculosis. Alternatively, infected organs, from well characterized stages of infection in cattle are highly abundant in endemic areas and constitute an excellent source for which gene-specific or genome-wide expression profiles using ultrasensitive real time reverse transcription and quantitative PCR can be used to monitor the expression of genes required for active or latent infection based on *Mtb* studies, allowing dissection of their participation in a natural setting.

Due to their relevance for humans, studies of latency/persistence or isolated factors linked to it (i.e. hypoxia) have solely been studied in two slow-growing mycobacteria (*Mtb* and *M. bovis* var. BCG), but it might well be that other organisms within this genus are able to sustain such an infection, either in humans or their natural/preferred hosts. It would be interesting to look at different organs in chronically infected organisms, and perhaps conduct a species-wide analysis of the microbes found there, using markers such as ribosomal genes for example, or those that better and more accurately show the population represented in those environments.

On the other hand, addressing immune profiles or host susceptibility factors, either in experimentally- or naturally-infected cattle, would be a way to start unravelling the participation and balance between bacteria and bovine host immune system, whereby a persistent state and its contribution to continued transmission during long periods of time despite lack of evidence of ill animals could be ascertained. Thus, detailed histopathological or even molecular analyses are required to discern how many bTB lesions harbour mycobacteria in an active and/or persistent/latent state. Further experiments linking immunological profiles to the presence of mycobacteria in the different types of lesions, with demonstration of bacterial expression of latency markers are required. An immune response profile assessed in cows with diverse degrees of pathology, which correlates with contained infections of minimal tissue damage, might indicate which host factors are important for maintaining a persistent, non-progressive infection.

Considering the rapid accumulation and availability of genome sequences, we could use this information to answer whether there is any biological meaning to possible changes in DosRS sequences of vaccine and mycobacterial strains, and their likely impact on efficacy of protection and virulence, respectively. Given the variety of insertion, deletion and duplication events that occurred in BCG strains, the differences found in DosS and DosT, key components of a system developed to sense and respond to oxygen limitations or immune response-derived stressors, might have an impact in how mycobacteria adapts to its host. Mapping of *DosRS* and DosT sequences in different environmental pathogenic strains of *M. bovis*, for example, should be undertaken to assess genetic variability in these genes. Monitoring expression of these genes in the same isolates and/or infected tissues would reveal significant information about latency in cattle. We must consider that it is very likely that genes required for dormancy in *Mtb* which have a very close homolog in *M. bovis*, might be expressed in vivo during bTB.

The participation of genes other than the DosR-regulated ones cannot be ruled out, but their participation and relevance need to be further studied and validated in infected subjects. Studies showing the presence of humans latently infected with *M. bovis* indicate this bacterium may be able to persist in such a form in a non-preferred host, thus strengthening the notion of a possible persistent form in its natural host, the bovine, where it may also be latently harboured. Furthermore, *Mtb* has been isolated from asymptomatic cattle, suggesting the same capability of this bacterium to cause a silent infection in these animals. On the other hand, as no clinical symptoms are developed in infected bovines, this imposes a harder challenge for trying to establish evidence of latent/persistent infection in cows. The occasional

histological findings in slaughterhouses, joined to TST assays in cows, constitutes by no means a way to determine if *M. bovis* lies dormant in cattle and what the impact of such a state may have on the continued transmission of bTB. This clearly indicates that we need to focus our attention on its close relative and use the knowledge gathered there to speed up advances for the veterinary field dealing with tuberculosis every day.

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