Microbiological Research 252 (2021) 126853

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Revisiting tuberculosis screening: An insight to complementary diagnosis and prospective molecular approaches for the recognition of the dormant TB infection in human and cattle hosts

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ARTICLE INFO

Keywords:
Latent tuberculosis
Bovine tuberculosis
Host response
Latency antigens
Biomarkers
Ancillary tests
Molecular analysis

ABSTRACT

Tuberculosis (TB) is defined as a chronic infection in both human and cattle hosts and many subclinical cases remain undetected. After the pathogen is inhaled by a host, phagocyted bacilli can persist inside macrophages surviving intracellularly. Hosts develop granulomatous lesions in the lungs or lymph nodes, limiting infection. However, bacilli become persister cells. Immunological diagnosis of TB is performed basically by routine tuberculin skin test (TST), and in some cases, by ancillary interferon-gamma release assay (IGRA). The concept of human latent TB infection (LTBI) by *M. tuberculosis* is recognized in cohorts without symptoms by routine clinical diagnostic tests, and nowadays IGRA tests are used to confirm LTBI with either active or latent specific antigens of *M. tuberculosis*. On the other hand, dormant infection in cattle by *M. bovis* has not been described by TST or IGRA testing as complications occur by cross-reactive immune responses to homolog antigens of environmental mycobacteria or a false-negative test by anergic states of a wained bovine immunity, evidencing the need for deciphering more specific biomarkers by new-generation platforms of analysis for detection of *M. bovis* dormant infection. The study and description of bovine latent TB infection (boLTBI) would permit the recognition of hidden animal infection with an increase in the sensitivity of routine tests for an accurate estimation of infected dairy cattle. Evidence of immunological and experimental analysis of LTBI should be taken into account to improve the study and the description of the still neglected boLTBI.

1. Introduction

Tuberculosis (TB) is a chronic granulomatous inflammatory process that mainly affects the respiratory tract of an infected host. Etiologic agents *Mycobacterium tuberculosis* (*Mtb*) and *Mycobacterium bovis*, are members of the *Mycobacterium tuberculosis* complex (MTBC) that reside in the lungs and associated lymph nodes. Although the infection by *Mtb* is mainly a human infectious disease, humans are the suspected source of *Mtb* transmission to cattle, however, dissemination of *M. bovis* from cattle to man is very common (Wahdan et al., 2020). The pathogen has been isolated and identified in cattle attributed to close human-animal contact probably due to transmission of *Mtb* from pastoralists and dairy employees suffering from active TB process (Adesokan et al., 2019; Lombard et al., 2021). On the other hand, bovine infection by *M. bovis* cause a widespread mammalian infection that also occurs in other domestic and wildlife species, contributing to the disease persistence and spreading within populations by cross-infection between hosts as a zoonotic TB (zTB) particularly in low-income countries (Waters and Palmer, 2015; Kock et al., 2021). Transmission from cows to humans occurs *via* inhalation of aerosolized droplets disseminated by coughing, with a high prevalence of asymptomatic infection mainly among farmers, veterinarians, and abattoir workers exposed to infected cattle (Torres-Gonzalez et al., 2013; Rodriguez et al., 2020; Devi et al., 2021). Consumption of unpasteurized dairy products is the main transmission route to humans and has been associated with extrapulmonary TB cases (Dürr et al., 2013). Pulmonary zTB occurs very frequently among immunocompromised people.

Human TB cases due to *M. bovis* are equally prone to the same severity as cases due to *Mtb* and have been clinically indistinguishable unless molecular testing by genotyping and single-nucleotide polymorphisms (SNPs) analysis are used at species-level identification after culture and pathogen isolation (Bilal et al., 2010; Rodwell et al., 2010;

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https://doi.org/10.1016/j.micres.2021.126853
Received 22 June 2021; Received in revised form 21 August 2021; Accepted 22 August 2021
Available online 26 August 2021

Scott et al., 2016; Pérez de Val et al., 2021). DNA testing platforms, antigen-mining, and microarray technologies have potentiated biomarker discoveries that can be applied in searching the disease status by improving ancillary tests and favor parallel advances of gold-standard tests with new platforms for the diagnosis of human and cattle TB infections (Vordermeier et al., 2016; Wadhwa et al., 2012; García et al., 2019; Steinbach et al., 2019). The GeneXpert MTB/RIF assay is an automated real-time PCR system that simultaneously detects total DNA and resistance to rifampicin from live or dead bacilli in clinical samples. The test complements acute TB diagnosis and has been probed to be confirmatory for *M. bovis* recognition from bovine samples in natural settings (Hlokwe and Mogano, 2020).

The innate and adaptative immune responses against mycobacterial infection play a preponderant role in the location and development of associated lesions, contributing to the establishment and chronicity of bacterial infection. Human and cattle TB develop similar pathologies and immune responses, producing similar diseases (Russell, 2003; Waters et al., 2011; Blanco et al., 2021). This includes host granuloma formation, where an interplay of pathogen and immune factors of the host's innate and adaptative immunity exert pathognomonic signs of infection providing opportunities for extensive TB research (Pollock et al., 2005; Ramakrishnan, 2012; Waters et al., 2014; Kanipe and Palmer, 2020). Observations of human TB recognizes that most of the active TB cases may not be due to new infections, but rather to old chronic infections. It has been estimated that one-third of the world's population has latent tuberculosis infection (LTBI) and 10–20% of infected individuals will reactivate infection during their lifetime (Gupta et al., 2012; Cassidy and Martineau, 2014), a bacterial transition associated with a recrudescent disease characterized as "reactivation" which typically involve the weakening of the immune system (Ahmad, 2011). Latent infection is evidence of immunological control of TB where the pathogen becomes a slow-growing or growth-arrested persister cell changing its expression of metabolic pathway genes during early granuloma formation (Guirado et al., 2015; Fisher et al., 2017). This results in a Ghon complex, a tuberculous lung lesion accompanied by a granuloma in the thoracic lymph nodes and an exacerbated inflammatory hypoxic lesion after progression of infection, where mycobacteria persist residing inside phagocytic cells (Ganchua et al., 2020).

The most widely used test for the diagnosis of LTBI for contact tracing is the delayed hypersensitivity skin test (Mantoux) targeting the Purified Protein Derivative (PPD) in asymptomatic people (Fig. 1A). The test does not differentiate between infection, disease, and sensitization with non-tuberculous mycobacteria (NTM) or BCG vaccinated individuals because PPD is a crude mixture of antigens conserved in *Mtb*, *M. bovis*, BCG, and NTM. Using QuantiFERON-TB Gold and the T-SPOT. TB (Interferon-Gamma Release Assays — IGRA tests), it has been determined that IFN-γ produced by stimulated whole blood leukocytes with PPD, ESAT-6, CFP-10, and TB 7.7 antigens has higher specificity especially in populations vaccinated with BCG. Contact tracing investigation also involves clinical assessment, chest radiography, and microbiological evaluation of sputum. Pathogen and host biomarkers for the detection of active pulmonary and extrapulmonary TB have been

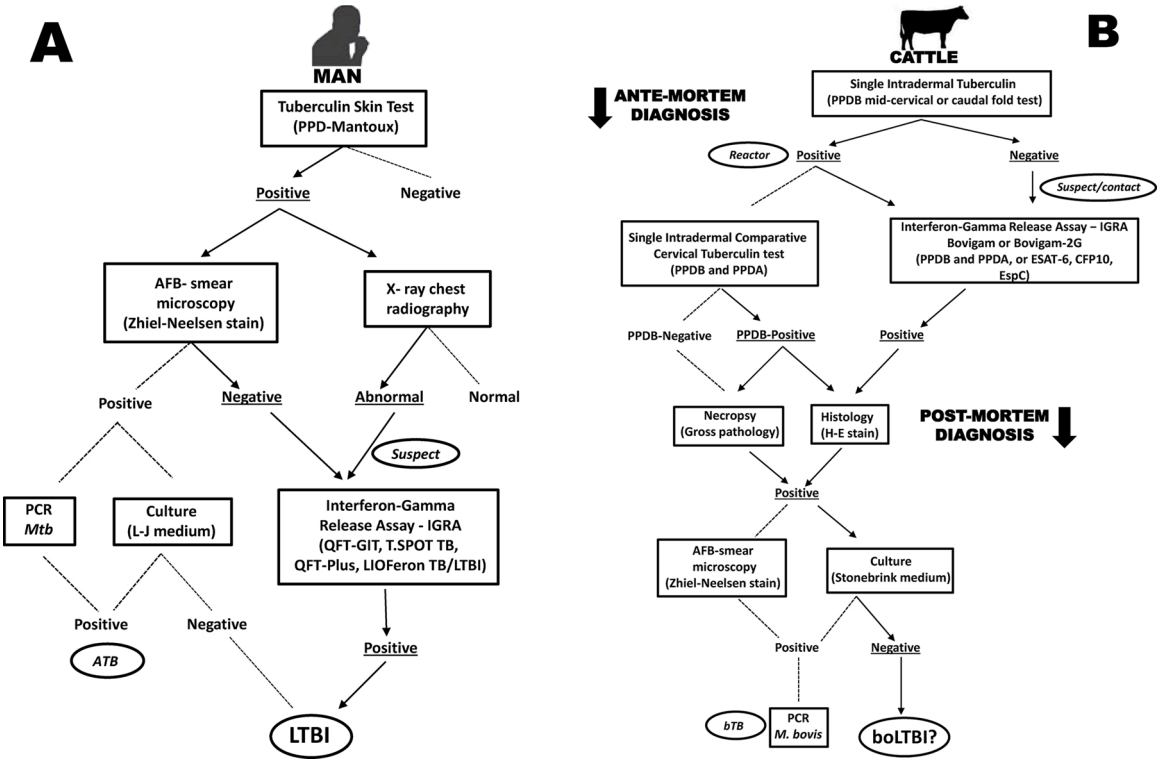

Fig. 1. Diagram of the routine workflow for the TB screening in human and cattle hosts. Scrutiny for the diagnosis of subclinical infection is highlighted. (A) Testing for suspected latent TB infection (LTBI) in asymptomatic people (suspects) is dependent on *in vivo* immune recognition of a complex mix of *Mtb* antigens (PPD) by the tuberculin skin test (TST) and with the *in vitro* ELISA-based blood test interferon-gamma release assay (IGRA). Besides, patients are subjected to clinical evaluation by sputum Acid-Fast Bacilli (AFB) smear and bacilloscopy, mycobacterial culture, PCR-based strain typing, and X-ray chest radiography for acute TB (ATB) assessment. (B) Similarly, bovine TB (bTB) diagnosis is performed with the ante-mortem tuberculin test (TST), making a reactor animal by positive standard interpretation. *In vivo* confirmation of probable *M. bovis* infection is performed by the single intradermal comparative cervical tuberculin test or with the ancillary IGRA (Bovigam^{TM}) with fresh blood sent to the laboratory, which also is applied to TST-negative animals (suspects/contacts), to confirm those TST-positive (or the misdiagnosed animals) to *M. bovis* PPD (PPDB) for next step of post-mortem examination for gross pathology at necropsy after slaughter. Lung and lymph node tissue samples are sent for bacteriological and histological analysis (Hematoxylin-Eosin staining), and bTB infection is confirmed by microscopy (AFB-smear), culture pathogen isolation, and PCR-species genotyping. Those clue points (underlined) with the most probable diagnostic data, which in sum may suggest a dormant stage of infection within the workflow are shown (arrows), and the hypothetical definition of bTB latent infection (boLTBI?) is proposed.

extensively reported in clinical specimens of affected people (Tucci et al., 2014; Yong et al., 2019). The diagnosis of LTBI has been supported with molecular data by the recognition of *Mtb*-specific gene sequences in tissue biopsies of deceased people with undefined causes of death (Hernández-Pando et al., 2000; Barrios-Payán et al., 2012; García-Basteiro et al., 2016). No significant immune responses to latency antigens have been observed in human–BCG vaccinated individuals by serology, not like that, by CMI-based tests (Lin et al., 2007). Also, the LTBI recognition is characterized by cell pro-inflammatory mediators in blood from subjects with no evident symptomatology, compared to healthy people, with an augmented Th1 type immune profile over a slight induced Th2 pathway to *Mtb* antigens (Banerjee et al., 2021; Mantri et al., 2021).

While early diagnosis of bTB with tuberculin skin test (TST) follows those principles of human TB assessment, animal disease is often misdiagnosed. This is due to animal conditions or false-negative interpretations in routine ante-mortem skin testing especially at low disease prevalence (Schiller et al., 2010). Similarly, animals regularly do not show signs until the infection has reached an advanced stage and active bTB is confirmed by gross pathology and pathogen isolation in culture after necropsy (Fig. 1B). Common observations in field examinations arise from asymptomatic animals with gross pathology at slaughter and importantly some with culture-negative to *M. bovis* (Menin et al., 2013; Tulu et al., 2021). As in humans, an ancillary IGRA test (Bovigam^{TM}) can be performed as a complementary test in cattle TB eradication schemes, and blood samples of aleatory suspicious animal contacts can be used to confirm infected (or misdiagnosed) animals within herds with variations in the sensitivity and specificity depending on cut-off point value adjustments of the test in regions with distinct bTB prevalences (Alvarez et al., 2012). Furthermore, the performance of the skin test is affected by host factors and the test itself, including the uniformity of PPDB from batch to batch in commercial preparations. In the generalization phase of infection, some animals may be “anergic” and show no reaction to the standard immunologic tests. Further complications with accurate bTB diagnosis arise with previous tuberculization or field coinfections with environmental mycobacteria that can lead to antigen cross-reactivity with *M. avium* subsp. *paratuberculosis* and other species of the MTBC that also have well-recognized subclinical states of infection, thus hindering immunological diagnosis (Lin et al., 2009; Barry et al., 2011; Roupie et al., 2018; Stabel and Bannantine, 2020; Wright et al., 2020). Besides, immunosuppressive coinfections with viral agents, as the bovine viral diarrhea virus (BVDV) with an impact on the herd’s immune status, may hinder a precise diagnosis of bTB (Charleston et al., 2001; Byrne et al., 2017).

Immunosuppressive events, as a wained immunity during old age, can render the reactivation of a potential subclinical bTB in cattle. By the diagnostic and pathological spectrum of animal infection during the bacterium-host interaction, the potential existence of a bovine latent TB infection (boLTBI) has been stated (Alvarez et al., 2009; Sabio y García et al., 2020). However, boLTBI has not yet been recognized due to the scarce experimental characterization of a subclinical animal infection that cannot be detected by standard ante-mortem diagnostic tests. Despite similarities between anti-TB immune responses, the response of bovines infected with *M. bovis* against individual LTBI-associated antigens is not as evident as the response of human hosts. A few studies were performed to diagnose natural bTB infection in cattle by antigenic stimuli of peripheral blood mononuclear cells (PBMCs) using the IGRA test adapted with selected recombinant latency-associated antigens from *Mtb*. However, classification of animals with a possible latent infection was difficult to assign because CMIs of blood that recognizes latency antigens do not precisely correlate with the expected phenotype of chronic infection gross pathology (Jones et al., 2011; Alvarez et al., 2017).

Regarding the relevance of opportune diagnosis of human LTBI for setting guidelines for precise chemotherapeutics oriented to the elimination of LTBI (Bastos et al., 2020; Godoy, 2021), bovines with *M. bovis* infection do not receive any treatment against bTB. Instead, a diagnosis of a persistent/dormant form of animal infection would represent an impact on adopting the best quarantine practices of infected cattle to diminish the spread of infection in herds and making decisions between isolating and vaccinating or sacrificing infected animals. Thus, a dormant phase of mycobacterial infection in bovines represents a serious complication for the eradication of the disease in herds. Recognition of the dormant stage of infection in cattle is difficult to assess because of the lack of specific phase biomarkers.

Genomics and next-generation high-throughput technologies have risen as promising tools in the infectious disease field as a whole and have created an opportunity for diagnosing bTB in cattle. These tools have shown the potential to describe new pathogen biomarkers, by genome-wide screening approaches, speeding the selection of the best mycobacterial antigenic mixtures with augmented potential for the characterization of infection-associated immune response of the host (MacHugh et al., 2009; Beltrán et al., 2011; McLoughlin et al., 2014; Coppola and Ottenhoff, 2018; Guerrero, 2019). Research in TB transcriptomics has been focusing attention on integrative genomics and molecular characterizations of host infection stages searching for those gene expression patterns perturbed upon *in vivo* infection or with *in vitro* studies of alveolar macrophage interactions (Blanco et al., 2012; Leisching et al., 2017; Shukla et al., 2017; Singhania et al., 2018a,b; Pisu et al., 2019; Hall et al., 2021). Moreover, analysis of cytokine and chemokine expression as mediators of the host immune response during natural TB infections is being considered for biomarker classification of infection with diagnostic purposes (Llibre and Duffy, 2018; Park et al., 2021; Smith et al., 2021). In addition, host specificities of *Mtb* and *M. bovis* are evidenced by the chemokine profiles of infected macrophages (Magee et al., 2014). Despite the physiological similarities between human and bovine TB, persistent mycobacterial infections are not yet fully characterized and equated. This review describes the diagnostic evidence of the current experimental strategies to improve molecular analysis of the subclinical form in both infectious diseases, with an emphasis on the study of the still undescribed bovine dormant infection.

2. Host immunity and cellular mediators contributing to latent infection

During persistent infection, live and virulent bacteria remain in host tissues in a state of equilibrium where bacteria do not trigger any mechanism to cause damage to the host cells that harbor them and the immune system does not eliminate them. Because of this, *Mtb* can survive long-term as a non-harmful cell to obtain environmental adaptation (Didelot et al., 2016). The infection can then reactivate at some point during the lifetime, resulting in active TB. An important factor of the development of LTBI is the formation of pulmonary granulomas, which are multicellular structures composed of fibroblasts, epithelioid cells, multinucleated giant cells, B-cells, neutrophils, macrophages, and effector T-cells that produce several chemokines, cytokines, and adhesion molecules that contribute to granuloma formation (Lin and Flynn, 2010; Cassidy and Martineau, 2014; Sia and Rengarajan, 2019).

The macrophages phagocyte and try to destroy *Mtb*, while the bacteria try to proliferate by manipulating macrophage functions to regulate host anti-*Mtb* responses inside these cells, circumventing killing and living inside these cells, to further spread and escape to infect new hosts (Awuh and Flo, 2017; Ehrt et al., 2018). *Mycobacterium tuberculosis* infects alveolar macrophages, and the formation of granuloma is a way to take control of bacterial growth, where is assumed that *Mtb* exists as an actively dividing cell or as a dormant bacillus depending on infection status inside its host (Pieters, 2008). Survival of *Mtb* within the macrophage activates only a certain set of genes related to cell wall modulation and lipid metabolization, such as mycolic acids, sulfolipids, lipomannan, lipoarabinomannan, and cholesterol contributing to the survival of the pathogen and to the suppression of immune responses of the host (Ghazaei, 2018; Sundararajan and Muniyan, 2021). This

facilitates the asymptomatic accumulation of mycobacterial antigens developing post-primary TB characterized by prolonged asymptomatic accumulation of host lipids and secreted mycobacterial antigens in alveolar cells, providing a niche for *Mtb* persistence with a long term carbon source of cholesterol from the host cell (Hunter, 2016; Martinot, 2018). Then, subsequent activation of a subset of effector cells is capable of producing IFN-γ, TNF-α, IL-1α, IL-1β, iNOS, and granzymes within the alveoli. Anti-*Mtb* functions including phagosomal maturation, inflammasome activation, autophagy, and apoptosis are cellular processes with extensive research focus for the elucidation of survival molecular strategies of *Mtb* inside the cytosolic environment persisting within "arrested" early endosomes (Bussi and Gutierrez, 2019; Chai et al., 2020a). Afterward, modulation of adaptative immunity occurs by manipulating host cytokine responses, downregulating antigen presentation with immune evasion inhibiting macrophage function, thus allowing persistence of mycobacteria within the macrophage (Young et al., 2008; Peddireddy et al., 2017; Ernst, 2018).

A complex interplay is evident between various immune-cell subsets of the adaptative immunity. These include CD8+, CD4+, γδT-cells, Th17, Tregs, and other immune cells like NKT, DCs, macrophages, B-cells, neutrophils, mucosal-associated invariant T (MAIT) cells, and myeloid-derived suppressor cells in TB onset (Urdahl et al., 2011; Prezzemolo et al., 2014; Lyadova and Panteleev, 2015; Loxton, 2019; Rao et al., 2019; Yamashita et al., 2019; Chai et al., 2020b; Luo et al., 2021). Immunological components of these are being studied to differentiate the acute from the latent stage of TB infection and discern their function to sustain infection (Zuñiga et al., 2012; Umemura et al., 2016; Coulter et al., 2017; Kumar et al., 2019; Medawar et al., 2019; Coppola et al., 2020; Estévez et al., 2020a,b). Thus, the host's cellular inflammatory response contributes to the maintenance of LTBI to the extent that expression of IL-17A has been associated with inhibited apoptosis of *Mtb* infected macrophages and increased neutrophil recruitment, promoting bacterial intracellular growth and showing potential for TB diagnosis (Cruz et al., 2015; Kamakia et al., 2017; Pollara et al., 2021).

Table 1
Studies published with the complementary and experimental diagnosis for TB/LTBI screening.

Authors	Nation	Sample size (cases/contacts)	Whole blood assay	*Mtb*-antigenic stimuli	Discriminatory biomarkers
Adankwah et al., 2021	Ghana	42	QuantiFERON-GIT, Multiplex cytokine	[a]ESAT-6, [a]CFP-10, [a]TB7.7, [b]Rv1733, [b]Rv2628	IFN-γ, IL-6, IL-22, IP-10
Akashi et al., 2021	Japan	233	QuantiFERON-GIT, Multiplex cytokine	ESAT-6, CFP-10, TB7.7	IL-1RA, IFN-γ, CXCL10/IP-10, CCL4/MIP-1β
Druszczynska et al., 2021	Poland	216	QuantiFERON-GIT, Multiplex cytokine	ESAT-6, CFP-10, TB7.7	IFN-α2, IL-12 (p40), IL-22, IL-28A, IFN-λ2, PTX-3, TSLP
Halliday et al., 2021	England	134	Flow citometry	Non-stimulated	HLA-DR^{+}IFNγ^{+} CD4 T-cells, CD45RA^{-}CCR7^{-}CD127^{-} IFNγ^{-}IL-2^{-}-TNFα^{+} CD4 T-cells
Estévez et al., 2020a	Spain	97	QuantiFERON-GIT, Multiplex cytokine	ESAT-6, CFP-10, TB7.7	IP-10, IL-7, BCA-1, TNF-α
He et al., 2020	China	90	Multiplex cytokine	ESAT-6	IL-23, IL-21, HGF, Bngf, IL-27, IL-31, IL-1β, IL-22, IL-18
Luo et al., 2019	China	149	T-SPOT.TB, Multiplex cytokine	ESAT-6, CFP-10	Eotaxin, MDC, MCP-1, IP-10, MIP-1α, IL-1α, IL-10, TNF-α
Manngo et al., 2019	South Africa	120	QuantiFERON-GIT, Multiplex cytokine	ESAT-6, CFP-10, TB7.7	IFN-γ, IL-2, IL-13, MCP-2, ITAC-1
Chowdhury et al., 2018	South Africa	28	QuantiFERON-GIT, Mass cytometry—CyTOF	ESAT-6, CFP-10, TB7.7	NADK, CXCL8, ADA, LRP11, DDC
La Manna et al., 2018	Italy	99	QuantiFERON-GIT, Multiplex cytokine	ESAT-6, CFP-10, TB7.7	IL-1β, IL-2, IL-8, IL-12p70, LIF, MCP-1, PDGF-BB, VEGF
Wang et al., 2018	China	304	QuantiFERON-GIT, Multiplex cytokine	ESAT-6, CFP-10	VEGF, IL-12p70, IP-10
Won et al., 2017	Korea	76	QuantiFERON-GIT, Multiplex cytokine	ESAT-6, CFP-10, TB7.7	EGF, GM-CSF, IL-2, IL-5, IL-10, IFN-γ, VEGF
Wu et al., 2017	China	92	Multiplex cytokine	[d]PPD	IL-2, IL-10, IFN-γ, IP-10, TNF-α
Yao et al., 2017	China	89	TB-IGRA, Multiplex cytokine	ESAT-6, CFP-10	IL-8, IP-10, MIP-1a, sIL-2Ra, MCP-3,VEGF
Bai et al., 2016	China	376	ELISPOT assay	[b]Rv2029c, Rv2628, [b]Rv1813c	IFN-γ
Chen et al., 2016	China	336	T-SPOT.TB, Multiplex cytokine	ESAT-6, CFP-10	Eotaxin-2, ICAM-1, MCSF, IFN-γ, IL-2, IL-4, IL-8, IL-11, IL-12p70, I-309, MIG
Suzukawa et al., 2016	Japan	70	QuantiFERON-GIT, Multiplex cytokine	ESAT-6, CFP-10, TB7.7	IFN-γ, IL-2, IL-5, IL-10, IL-1RA, MCP-1
Belay et al., 2015	Ethiopia	363	QuantiFERON-GIT, Cytokine-ELISA	ESAT-6, CFP-10, [b]Rv2031	IFN-γ, TNF-α, IL-10
Wergeland et al., 2015	Norway	164	QuantiFERON-GIT, Multiplex cytokine	ESAT-6, CFP-10, TB7.7	IP-10, sTNFr2
Essone et al., 2014	South Africa	30	QuantiFERON-GIT, Multiplex cytokine	ESAT-6, CFP-10, Rv2029c, [b]Rv2032, [c]Rv2389c	IL-1β, IFN-α2, IL-10, CRP, MIP-1β, MCP-1, MMP-2, MMP-9, SAP, VEGF
Hur et al., 2014	Malawi	39	ELISpot assay, QuantiFERON-GIT, Multiplex cytokine	ESAT-6, CFP-10, TB7.7, PPD	IL-5, IL-9, IL-13, IL-17
Mihret et al., 2013	Ethiopia	63	Multiplex cytokine	Non-stimulated	EGF, IFN-γ, IL-4, IP-10, MCP-3, Fractalkine
Chegou et al., 2012	South Africa	43	Multiplex cytokine	ESAT-6, CFP-10, Rv2389c, [b]Rv1737c, [b]Rv2032, [b]Rv0081, [c]Rv0867c	IFN-γ, IFN-α2, IL-12p40, IP-10, TNF-α, EGF, VEGF, IL-10, TNF-α, TGF-α, Fractalkine, RANTES
Frahm et al., 2011	USA	70	QuantiFERON-GIT, Multiplex cytokine	ESAT-6, CFP-10, TB7.7	IFN-α, IL-1Ra, IL-4, IL-15, MCP-1
Sutherland et al., 2010	Gambia	75	Multiplex cytokine	ESAT-6, CFP-10, PPD, [a]TB10.4	IFN-γ, TNF-α, IL-12p40, IL-13, IL-17
Chegou et al., 2009	South Africa	57	QuantiFERON-GIT, Multiplex cytokine	ESAT-6, CFP-10	IL-1α, EGF, sCD40 L, MIP-1β, TGF-α, VEGF

Sort order by decreasing publication year. aActive growth-phase antigens; blatency-associated antigens; creactivation/resuscitation antigens; dtuberculin-Purified Protein Derivative.

Importantly, treatment of immune-mediated inflammatory disorders with antagonists of TNF-α or CD4+ T-cell depletion by viral infections is associated with an increased risk of developing reactivation of disease (Larsen et al., 2008; Ahmed et al., 2016; Arbués et al., 2020; Khayat et al., 2021). A wealth of published data describes the measurement of a single or groups of chemokines and cytokines in a heterogeneous population as alternative biomarkers within IGRA tests (Mamishi et al., 2014; Petrone et al., 2018; Qiu et al., 2020; Wei et al., 2020). These include Apo-ACIII, CCL-1, CCL-8, CD56, CXCL1, CXCL8, CXCL9, EGF, NAD kinase—NADK, adenosine deaminase—ADA, fractalkine, IFN-γ, IL-1Ra, IL-2, IL-2Ra, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-13, IL-15, IL-17, IP-10, LIF, MCP-1, MCP-3, MIF, β-NGF, TNF-α, TNF-β, TRAIL, VEGF, GM-CSF, and SCF as biomarkers for improvement of diagnosis and discrimination between active TB patients, latent suspects, non-TB pulmonary infections, and healthy people by single and multiplex cytokine ELISA from sera samples after antigenic stimuli of whole blood (Table 1).

Alternatively, RT-PCR with qPCR and multiplex ligation-dependent probe amplification (dcRT-MLPA) techniques used with whole blood from subjects classified with TB or LTBI by the QuantiFERON-TB Gold test, associated several leukocyte mRNAs expression levels of effector molecules with a discriminative potential of infection stages. These effector molecules include IL-4, IL-10, IP-10, BPI, CCL19, EPHA4, FoxP3, FPR1, TGFB1, GBP1, IFITM3, GBP5, HLA-B, LOC400759, NPC2, DOCK9, S100A11, and STAT1 (Mihret et al., 2014; de Araujo et al., 2016; Korma et al., 2020; Perumal et al., 2021). Moreover, several species of RNAs (circRNA) and micro RNAs (miRNA) from PBMCs and plasma from TB suspects have also been associated with the activation of the host regulation of anti-TB immune responses with the capability to distinguish between those active and LTBI cases (Sabir et al., 2018; Liu et al., 2020; Lu et al., 2021).

2.1. Improved immunological approaches for diagnosis of LTBI

Advances in knowledge regarding LTBI have been made concerning the human TB infection model. Latent TB infection is defined by the detection of a specific immune response against antigens of MTBC by Mantoux (TST) or IGRA testing in a healthy subject with no symptoms or signs of active TB (Esmail et al., 2012). However, all patients with evidence of LTBI should undergo a clinical evaluation by sputum smear microscopy, culture, and X-ray chest radiography for active TB, plus recent reports of chest-computed tomography, should be evaluated to discern between active or latent TB process (Horsburg and Rubin, 2011; Muñoz et al., 2015; Uzorka et al., 2019; Bommart et al., 2021). On the other hand, distinct immunological tests for specific T-cell immunity targeting only detect exacerbated CMI responses by augmented IFN-γ production by memory CD4+ T –cells with *in vivo* tuberculin skin test (TST), or with *in vitro* QuantiFERON-TB Gold In-Tube—QFT-GIT, and T-SPOT.TB—IGRA tests (Zhou et al., 2020). These are not sufficiently accurate to distinguish between active TB and LTBI but may reflect the extent of pulmonary pathology (Wlodarczyk et al., 2014; Auguste et al., 2017). Moreover, these tests do not predict whether an individual with LTBI will develop active TB (McNerney et al., 2012; Salgame et al., 2015; Carranza et al., 2020; Mwaba et al., 2020). The immune response through IFN-γ from CD4+ T-cells against *Mtb*-specific antigens is not sufficient to control the severity of the disease or even to exactly categorize infection stages in TB suspects (Nunes-Alves et al., 2014). There is no gold-standard test that may help to assess the sensitivity of none available or potential test for LTBI diagnosis. However, LIOFeron TB/LTBI and QuantiFERON-TB Plus (QFT-Plus) are improved test formats with specific cocktails of the proteins ESAT-6, CFP-10, TB7.7 and AlaDH for a more precise distinction of suspects and tracing contacts (Sotgiu et al., 2019; Della Bella et al., 2020). Nonetheless, the QuantiFERON-TB Gold test has been used to support the diagnosis of subclinical TB infection. Some supporting studies used the IGRA test in combination with a similar TNF-α-release assay (TARA) (Kim et al., 2018), or even by the addition of LTBI-specific antigens of the dormancy survival regulator—DosR as blood antigenic reagents (Peña et al., 2015; Silva de Araujo et al., 2015; Meier et al., 2018).

The gene regulatory program that underlies the phenotype of latency of *Mtb* during disease is known as the Dormancy survival regulator (DosR) which consists of at least 48 *Mtb* genes allowing the mycobacteria to adapt for survival during extended periods of dormancy (Voskuil et al., 2003). This phenomenon occurs inside the hypoxic granuloma and is regulated by the Rv3133c/dosR transcription factor (Park et al., 2003; Hamidieh et al., 2021). It has been established that the bacterium itself plays an active role in the inflammatory process, where upregulation of DosR occurs in the immuno-competent host triggered by the advent of hypoxia resulting in long-term persistent infection (Mehra et al., 2015; Kundu and Basu, 2021). It is now assumed that this group of genes allows *Mtb* to survive intracellularly during LTBI, and their related proteins are accepted biomarkers for the diagnosis of LTBI with important immunogenic characteristics (Singh et al., 2014). Proteins Rv1733c, Rv1737c, Rv2031c, Rv2029c, Rv2627c, and Rv2628c are especially used as featured antigens for LTBI research (Demissie et al., 2006; Commandeur et al., 2011; Rakshit et al., 2017; Pandey et al., 2019; Adankwah et al., 2021). Moreover, multi-stage antigenic cocktails with ESAT-6/CFP-10 (acute phase), Rv2029c (dormancy phase), and Rv0867c/Rv2389c (resuscitation-promoting factor proteins—Rpf) have been used to potentiate LTBI diagnosis (Serra-Vidal et al., 2014; Rosser et al., 2017; Arroyo et al., 2018).

Multiplex microbead immune (MMI) assays with antigen-coated microbeads have been used to detect specific antibodies in the plasma of infected people (Khan et al., 2011). This has led to the recognition of antibodies to *Mtb* antigens Rv0934, Rv3881c, Rv1860 and Rv1827 in serum samples with discriminative potential between latent and active pulmonary TB (Li et al., 2021). With a group of 25 (out of 48) DosR antigens, detection of antibody titers to proteins Rv0079, Rv1738, Rv2029, Rv2030, Rv2624, Rv2627, Rv2628, Rv3127, and Rv3129 was achieved in an LTBI group (Shi et al., 2020), while in another study, 58/100 proteins from *Mtb* were detected more abundantly by IgG/IgM antibodies from acute TB (ATB) patients than from suspects with LTBI (Peng et al., 2020). Specific IgA memory B-cell subsets to latency antigen Rv2659c were also recognized by ELISpot with mAb anti-human IgA in fresh blood samples from an LTBI group but not from ATB patients (Soe et al., 2021). Augmented IgM levels of anti-HspX (Rv2031) response were detected between TB suspects with LTBI by Magpix bead-capture ELISA that enhance detection sensitivity compared to standard ELISA (Castro-Garza et al., 2017). Some differing observations regarding the serodiagnosis value of DosR proteins between healthy and TB diseased people are discouraging (Kimuda et al., 2017), and *Mtb* exposed subjects with an *Mtb*-specific IgG Fc profile to acute-phase antigens have been observed in certain non-IFN-γ responder cohorts (Lu et al., 2019). On the other hand, serum antibodies to proline-proline-glutamic acid PPE17 antigen (Rv1168c) or glycosylation patterns of antibodies may serve to distinguish LTBI suspects, showing signs of disease progression (Lu et al., 2016; Abraham et al., 2018). Thus, both Th1 and Th2-type anti-TB immunity have gained attention and are extensively revisited (Kozakiewicz et al., 2013; O'Shea et al., 2018; Simmons et al., 2018).

3. Integrative omics approaches in identifying biomarkers of TB infection stages

Data mining approaches in genomics and proteomics for deciphering the dormant-hypoxic pathway and the associated proteins of *Mtb* aimed at distinguishing pulmonary TB and LTBI have been extensively performed with *in vitro* and *in vivo* studies with pathogenic *Mtb* strains (Wang et al., 2011; Magombedze et al., 2013; Devasundaram et al., 2016; Sun et al., 2018; Kundu and Basu, 2021). Regulatory networks based on genome-wide transcription factor binding mapping and ChIP-Seq for searching of messenger RNAs, proteins, metabolites, and lipids profiles, proposed the DosR protein Rv0081 as a hub of the

dormant-associated hypoxic lipid metabolism (Galagan et al., 2013; Peterson et al., 2020). Besides Rv0081, proteins Rv1733c, Rv1737c, Rv2029c, Rv2031, and Rv2628 of DosR are among the most widely studied antigens with a discriminatory potential of TB infection stages (Meier et al., 2018). Other key proteins overexpressed during the hypoxic phase of intracellular survival are isocitrate lyase (Icl) and alanine dehydrogenase (AlaDH) which are now considered latency-specific targets (Campaniço et al., 2020).

On the other hand, attempts to dissect the genetic footprint of *M. bovis* infection have been performed by DNA-microarray platforms with transcripts of infected macrophages or peripheral blood mononuclear cells (Meade et al., 2007; Blanco et al., 2009a,b; Malone et al., 2018). Meta-analysis strategies include genome bioinformatic approaches using databases retrieved from clinical cases, and immunoproteome microarrays with the serum of TB suspects to assess whole antibody responses of infection stages against *Mtb* proteome in the search for new biomarkers (Kunnath-Velayudhan et al., 2010; Cao et al., 2018). An overall description of cytokine biomarkers during LTBI was reported including IL-2, IP-10, IL-5, IL-13, IFN-γ, IL-10, and TNF-α (Defelipe et al., 2016). The recent meta-genome analysis considered the whole MTBC lineage of mycobacteria to assess the influences of genetic variability on biomarker discovery of tuberculous infection, with particular significance in complex scenarios of natural settings (Kanabalan et al., 2021).

A deep validation study of blood transcriptomes with RNA-seq analysis of active and latently (with IGRA +ve and −ve criteria) infected suspects was performed using data from subjects with NTM infections, and a TB-like signature characterized by a group of IFN-inducible genes in those subclinical cases with increased risk of progression to active T was recognized (Singhania et al., 2018a,b). Similarly, in a controlled animal bTB infection setting, RNA-seq analysis of whole blood-cell transcriptomes identified two distinct gene expression clusters of 25 genes associated with the severity of infection (Wiarda et al., 2020). Similarly, a recognized transcription of a group of Th17-associated cytokines (IL-17A, IL-17 F, IL-22, IL-19, and IL-27) was detected in PPD-stimulated bovine PBMCs (Blanco et al., 2011; Waters et al., 2016). Controversy exists for consideration of detection of cytokines like IL-17A and IP-10 as potential biomarkers of bTB besides IFN-γ (Xin et al., 2018). Also, discordant observations by those T-cell subsets responsible for IL-17A production in infected cattle exist, where CD4+ T-cells look prone for gene expression of both IFN-γ and IL-17A (Elnaggar et al., 2018; Steinbach et al., 2018).

By immune-specific cDNA microarray, a differential cytokine gene expression pattern of PPDB-stimulated blood recognized groups of cattle with a false-positive ante-mortem test with no pathology at slaughter (Lim et al., 2012), suggesting that the immuno-transcriptomic approaches are a potential starting point of molecular characterization of animal infection stages. On the other hand, with microRNA-microarray analysis, overexpression of the miR-155 gene was found in PBMCs from *M. bovis* infected cattle with visible TB lesions and gross pathology, thus being described as a biomarker with prognostic value in animals with advanced pathology (Golby et al., 2014). This overall transcriptomic evidence highlights the great value of high-throughput meta-analysis technologies in starting to dissect the complex association of the host-pathogen interaction in resilient mycobacterial infections.

4. Immunological tests and biomarkers in the ante-mortem diagnosis of bTB

The single intradermal tuberculin (SIT) test, which includes caudal fold tuberculin (CFT), and derivated single intradermal comparative cervical tuberculin (SICCT) PPDB and PPDA-based tests are the main forms of bTB diagnosis worldwide. The Bovigam^{TM} assay (IGRA) is an ancillary blood-based test that can be used in parallel for the detection of CMI responses by stimulation with PPD-based antigens *in vitro* (Clegg et al., 2017). Simultaneous SICCT and IGRA tests can lead to incremental bTB detection in cattle, especially in herds with high proportions of false-negative skin tests (Clegg et al., 2019). These tests are considered for use in routine surveillance of bTB and have been the basis for continuous antigenic supplementation and field evaluations to differentiate tuberculous-infected animals from those of the NTM group. The antigens EspC, EsxG, ESAT-6, CFP-10, MPB70, and MPB83 are amongst those specific to MTBC that are selected for optimal recognition of the acute phase of bTB rather than a subclinical infection state (Encinas et al., 2018; Jenkins et al., 2018). Higher specificity of the IGRA test in herds having co-infection with NTM can be achieved with ESAT-6 and CFP-10 proteins instead of PPD antigens for differential diagnosis (DIVA diagnosis) of bTB (Waters et al., 2004; Vordermeier et al., 2011; Meng et al., 2015; Gutiérrez-Ortega et al., 2021).

In animals with advanced disease, the CMI responses tend to be diminished giving false-negative TST results (Lahuerta-Martin et al., 2015). IGRA testing of whole blood cultures with PPDB or ESAT-6/CFP-10 stimuli has been performed for simultaneous analysis of the cytokines IFN-γ, IL-1β, IL-2, IL-4, IL-10, IL-12, MIP-1, TNF-α, and IP-10 to achieve bTB diagnosis with superior sensitivity in cattle (Jones et al., 2010; Parsons et al., 2016). Additional detection of a set of host biomarkers such as TNF-α, CXCL10, and IL-22 was proposed to complement TST and IGRA tests in false-negative animals (Sánchez-Soto et al., 2017; Klepp et al., 2019). Replacement of PPD tuberculins with antigens of the acute phase of *M. bovis* or derivated peptides (peptide cocktail) has been described to give superior performance characteristics and improve immunological tests (Schiller et al., 2010; Srinivasan et al., 2019). An optimized Bovigam assay (Bovigam—2G^{TM}, B2G) with DIVA protein reagent-based stimulants has been determined to provide superior sensitivity in field applications (van der Heijden et al., 2016), but there have been conflicting results in studies using a more stringent cutoff value in the new B2G IFN-γ assay with an augmented diagnosis of false-positive cases (coinfected with NTM) between animals of herds considered to be free of bTB (Ghielmetti et al., 2021).

The chronic form of animal infection often behaves as an anergic state to CMI responses, thus identifying anergic animals by detection of specific anti-*M. bovis* antibodies has attracted new attention. Multi-antigen serological tests for detection of antibodies (IgG/IgM/IgA) include IDEXX *M. bovis* Ab-ELISA, TB-Luminex, Enferplex TB assay, MAPIA (Multi-antigen print immunoassay), and Lionex (POC—Point of Care test) using PPDB reagent or the individual antigens ESAT-6, CFP-10, LAM, MPB70, and MPB83 (Waters et al., 2017; Fontana et al., 2018; Kelley et al., 2020; Alonso et al., 2021). Even ELISA tests have shown promising results when used to detect CXCL9, CXCL10, IL-13, IL-21, and IL-22 in sera of infected animals in combination with the use of IGRA tests with DIVA or PPDB reagents for bTB assessment (Coad et al., 2019; Palmer et al., 2020). Performance of ante-mortem serological tests (IDEXX and Enferplex) in chronically infected herds complemented, but did not substitute, diagnosis with the SICCT test, allowing detection of additional *M. bovis* infected animals with increased specificity, probably in those anergic subpopulations (McCallan et al., 2021). Thus, a combination of standard and ancillary CMI tests with serological approaches may be appropriate for more accurate detection of bTB in the advanced stage of infection (Whelan et al., 2011; Lamont et al., 2014a,b; Garbaccio et al., 2019; Zewude et al., 2019; Carneiro et al., 2021).

5. Biomarkers at the post-mortem examination of infected cattle

Studies with laboratory animal models do not completely represent completely successful methods for analyzing the real phenomenon of LTBI because some of these models ultimately succumb to infection and others do not develop lesions like those found in natural infection. Thus cattle represent a long-lasting infection model for TB research where persistent bacillus grow slowly and cause similar granulomatous reactions as in humans (Singh and Gupta, 2018; Gong et al., 2020), which provides a reliable opportunity for a close understanding of LTBI (Van Rhijn et al., 2008; Alvarez-Herrera and Flores-Valdez, 2014). The

official diagnostic tests for surveillance of bTB are the tuberculin *in vivo* test, histopathology (with standard HE method), and the culture bacteriology post-mortem test (considered the gold-standard test). All of these prove to be insufficient in eradicating bTB. It is very common to find variations and different correlations of immunological and pathological parameters in the ante and post-mortem tests within animals under field conditions (Meikle et al., 2007; Okafor et al., 2014; Alvarez et al., 2017). This makes it difficult to associate a specific pathology to the stage of infection, as statistical latent class approaches for assessing effectiveness on diagnostics tests of hidden bTB infection have stated (Pucken et al., 2017).

It has been proposed that animals with IGRA +ve and TST −ve results that subsequently convert to TST +ve with scarce or NVL at slaughter support the concept of a boLTBI (Cassidy, 2006). When analyzing granulomatous lesions, molecular sieving by specific PCR is needed to precisely detect *M. bovis* from the MTBC or NTM-related infections within lymph nodes (Lázaro Sales et al., 2014; Michelet et al., 2020; Sánchez-Carvajal et al., 2021). Difficulties arise through histopathological observations of lesioned tissue samples where heterogeneous granuloma stages (with score pathologies from I to IV) are observed within the same tissue of naturally infected animals (Liebana et al., 2008; Domingo et al., 2014; Carrisoza-Urbina et al., 2019), suggesting different stages of infection within a single animal. In general, the infection stage from infected tissue is difficult to assign by the gross pathology of TB lesions. Bacterial burdens do not precisely correlate with the expression of Th1-type cytokines or lesion scores within bovine granulomas of experimentally infected cattle (Palmer et al., 2021). Stages III and IV of granulomatous lesions with transcripts of IFN-γ, TNF-α, and IL-10, but with low expression of IL-1β are associated with failure of immunologic control of infection and restoration of bacterial multiplication (Canal et al., 2017).

Augmented expression of mediators of both innate and adaptative immunity such as IFN-γ, IL-1α, IL-2, IL-10, IL-12, IL-17, TNF-α, IL-12p40, IL-22, CXCL8, CXCL9, CXCL10, and iNOS in PPDB stimulated PBMCs or pulmonary lymph nodes, has been described as a potential biomarker of disease progression in naturally or artificially infected animals (Meade et al., 2006; Thacker et al., 2007; Churbanov and Milligan, 2012; Aranday-Cortes et al., 2013; Blanco et al., 2014; Shu et al., 2014; Alfonseca-Silva et al., 2016; Fang et al., 2020; McLoughlin et al., 2021). Associated activation of CD4+, CD8+, and γδT-cell subsets suggest a cell transcriptional profile of the host response against infection (Blanco et al., 2009a,b; Nalpas et al., 2013). By real-time PCR, expression levels of chemokines CCL3, CCL4, CCL5, CXCL8, and CXCL10 were associated with pro-inflammatory cellular loads and lesion scores in bovine lymph nodes of experimentally infected cattle. This showed that early-stage lesions express fewer chemokines than late-stage granulomas, contributing to the maintenance of granuloma and control of infection (Widdison et al., 2009). Advanced stage granulomas also showed overexpression of TGF-β, type-I procollagen—PICP, IFN-γ, IL-10, and CCL2 by the activity of inflammatory cells, as γδT-cells, at the site of infection with gross pathology (Wangoo et al., 2005; Witchell et al., 2010; Rusk et al., 2017). However, locations of lymph nodes within the animal body are prone to express different patterns of IL-10, IL-17A, IL-22, IFN-γ, TGF-β, and TNF-α cytokines within infected cattle (Palmer et al., 2016), exposing complexities of histopathological analysis by tissue-specific differences in gene expression and protein profiles of individual tissue locations (Naranjo et al., 2007).

6. Molecular and experimental evidence suggesting the existence of the persistent dormant stage of *M. bovis*

The genome of *M. bovis* is 99.95% identical to that of the *Mtb* H37Rv strain, including all 48 dormancy-phase related DosR genes (Lin et al., 2009). Genomic evidence confirms that DosR genes are well conserved in MTBC, including BCG *M. bovis*, an attenuated strain that has demonstrated the ability to persist in the host for months or even years (Alvarez and Flores-Valdez, 2019; Mendum et al., 2019). These concepts represent important observations to support that *M. bovis* may have an active hypoxic metabolism. Observations with *M. bovis* BCG cultures described a dormant-like gene transcription of latency antigens Rv1733c, Rv2029, Rv2627, Rv2031, and Rv2628, a bacterial state of growth called the non-replicating persistence (NRP), performed by the inhibition of respiration with oxygen-limited Wayne culture system or with nitric oxide treatment in flasks mimicking the hypoxic environment (Lim et al., 1999; Boon and Dick, 2002; Flores-Valdez and Schoolnik, 2010; Alhusain, 2021). Besides, a differential expression of genes *narX* (Rv1736c) and *narK2* (Rv1737c) necessary for anaerobic nitrate respiration was observed during dormancy between different BCG strains, with variations in growth and survival (Honaker et al., 2008).

Diverse homologies to some DosR regulon members were also found in other bacterial genomes of the NTM group. However, neither of these studies described a transcriptional activity within stationary hypoxic states of growth (Selvaraj et al., 2012; Chen et al., 2013). Nonetheless, the opportunistic human pathogen *Mycobacterium marinum* (NTM) did exhibit transcriptional changes associated with dormant-resuscitating stages by RNA-seq analysis when using a different *in vitro* hypoxic dormancy mycobacterial model with oxygen-starving conditions (Jiang et al., 2020).

Encouraging results of a subclinical *M. bovis* cattle infection in natural settings came from the recognition of a T-cell memory response to the latency antigen Rv0188 in blood from a group of animals with limited or no pathology (Jones et al., 2011). Also encouraging, molecular studies by RT-PCR and qPCR for detection of expression of genes *hspX*, *pfkB,* and *mb2660c* (from DosR) in tissue samples suggested the activation of a probable dormant stage of *M. bovis* within bovine lymph nodes (Jiménez et al., 2013). Additionally, DosR recombinant HspX, PfkB, Mb1762c, and Mb2660c proteins, together with ESAT6 and CFP10, enhanced T-cell IFN-γ production in the blood of a group of animals with a culture-negative result (Alvarez et al., 2017), and in the same work, direct PCR analysis for nucleic acid amplification was performed in tissue samples of slaughtered cattle (with or without +ve IGRA test) to search for *M. bovis*-specific Rv2031c gene identifying cattle pathogen DNA sequence in lymph node samples with −ve bacteriological culture results in both IGRA groups (+ve and −ve) and with a non-visible lesion (NVL) at observation for granulomatous lesions. It has been largely speculated that animal samples with a chronic dormant stage of *M. bovis* may no have growth at culture, containing low bacterial loads.

In other studies of blood samples of infected animals, discriminatory meta-analyses by liquid chromatography and tandem mass spectrometry (LC–MS) with bovine serum collections was used to search for pathogen (MB1895c, MB2515c, and Pks5) and host (VDBP, A1AP, AGP, aGP1, RBP, IL-8, CRP, and fetuin-A) blood circulating proteins to propose potential biomarkers for subclinical animal infections (Seth et al., 2009; Lamont et al., 2014a,b; Gao et al., 2019).

7. Conclusions and outlook

Until now, the concept of LTBI has been considered an asymptomatic *Mtb* human infection based on associations of type IV hypersensitivity reactions to mycobacterial antigens with the Mantoux skin test or by *in vitro* QFT-GIT assay (IGRA) results of blood culture samples from people without clinical symptomatology. *In vivo* and *in vitro* infection models showed the participation of at least 48 *Mtb* DosR genes in the mycobacterial genetic activation of the latent TB phase, providing scientific evidence toward accepting mycobacterial DosR proteins as useful pathogen biomarkers. This complements the experimental diagnosis of latent stage TB infection employing IGRA tests. Discrepancies have recently been recognized between data from genome-wide transcriptome analysis and immunological tests that do not complement each other to precisely recognize those true LTBI cases in individuals.

On the other hand, in bTB diagnosis, the identification of new

antigenic compositions has increased the sensitivity of ante-mortem routine immunological tests. However, the post-mortem examination of tissue pathology and gold-standard testing with bacteriological pathogen isolation is a mandatory routine analysis for confirmation of animal infection, and the immunological testing is a suggestive diagnosis of an active infection. An extensive number of works in dairy cattle diagnosis have shown an increased detection of infected animals using the ancillary Bovigam-IGRA test after blood antigenic stimuli with different compositions of selected acute-phase antigens or derived peptides, and much fewer studies employing dormant phase proteins that are less antigenic in bovine blood. Thus, opportune identification of subclinical bTB infection nowadays represents a real challenge for epidemiological and control surveillance purposes. Proteomics studies of the host infectome by mycobacterial infections certainly will help to search for more antigenic and specific biomarkers for early diagnosis, laying the groundwork for a more comprehensive analysis of the immunopathology of bTB.

As supported by cumulative observations and difficulties in eradicating animal disease, boLTBI is likely to be a real but underestimated phenomenon due to a subclinical state of resilient infection in cattle. Animals with gross pathology lesion scores which may contain a dormant phase-related transcriptome of *M. bovis* and an active CMI response to DosR antigens in peripheral blood represent good candidates for further studies of boLTBI elucidation. Moreover, those tissue samples with positive *M. bovis* DNA but negative cultures of *M. bovis* represent a potential source material for genome-wide transcriptome studies of *in vivo* infection. Special considerations should be given to low culturable microbial loads in those granulomatous lesioned tissues, especially those in the center of the caseous lesions where more genetic material would be expected. The histopathological analysis could be improved with combined technologies such as laser-capture microdissection (LCM), *in situ* PCR, and semi-quantitative imaging scoring by computed tomography (MDCT).

On the other hand, a combination of circulatory and tissue host biomarkers in subclinical animal infection would consist of augmented TNF-α, IFN-γ, IL-1, IL-2, IL-10, IL-22, CXCL10, VDBP, and fetuin-A loads, among others. As human and cattle TB progress within the host, there is a gradual increase in both type1/type2 cytokine responses, *i.e.* IFN-γ and IL-2 (Th1), IL-4 and IL-5 (Th2), respectively. This clearly demonstrates a similar fate of infection pathogenesis and is very probably a reflection of progression through related dormant/active infection stages in natural hosts. It is assumed that control of TB infection is mainly due to efficient Th1 IFN-γ responses. With characterizations of γδT-cell subset and associated IL-17 production, its contribution to granuloma maturation through IFN-γ-independent mechanisms is evident, and at some point may be contributing to tissue damage in the advanced TB stage resulting in a wained Th1-immune response associated with observed immunopathology. While TNF is essential for granuloma formation, IL-10 production by B-cells in the proximity of granulomas may have the adverse effect of increasing disease severity by inhibiting the containment of *Mtb* and allowing the reactivation of infection, thereby increasing bacterial tissue burdens. Thus, there is a special interest in analyzing and comparing the cytokine profile during advanced chronic disease in both hosts to improve the diagnosis and recognition of signs of dormant/resuscitation stage of silent infection.

This revisited analysis of evidence related to the host immune responses and associated chemokines on human and cattle TB still shows the lack of specific biomarkers to associate an LTBI hallmark and more importantly in cattle diagnosis. There are found diverse immune mediators related to the latent stage of mycobacterial infection, however, they also fall into the group of cell mediators observed during the acute stage of infection within both hosts (Fig. 2), signaling the need for quantitative tools for a precise ascertainment. Multiplex bead arrays for multi-cytokine analysis of blood mediators in IGRA +ve and TST −ve

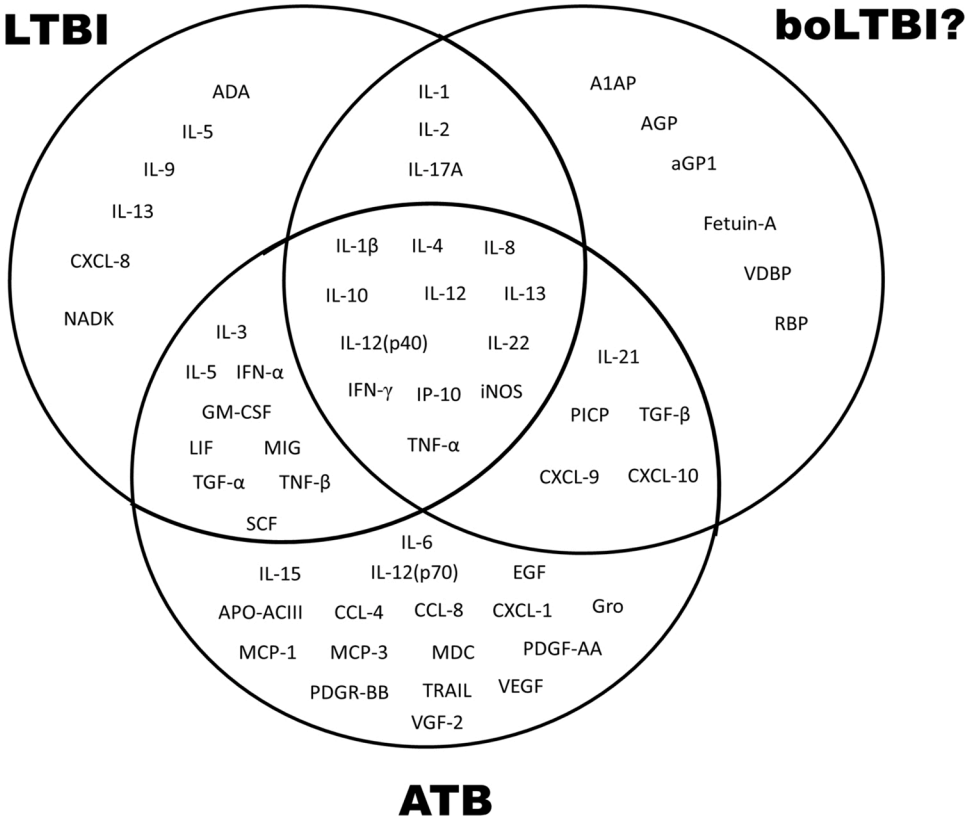

Fig. 2. Venn diagram showing most evident groups of host-cell mediators induced during TB infection stages. LTBI, human latent TB infection; ATB, acute TB infection; boLTBI?, hypothetical bovine LTBI.

animal cases undoubtedly would help to discern the best immunome match of boLTBI. Overall, the need is evident for multidisciplinary studies which undoubtedly must include associations of gold-standard tests with high-throughput molecular studies. Together, the application of next-generation transcriptomics, protein multi-array algorithms, LC/MS analysis, and high-throughput real-time qPCR for assessment of gene patterns of both acute and dormant states of *in vivo* infection would permit the identification of more biomarkers of bovine dormant infection.

In summary, what can be concluded from the experimental evidence of subclinical TB infection diagnosis in humans and bovines is:

- We know that human and bovine TB share key aspects such as developing similar chronic lesions and immune responses.
- Human and bovine hosts deal with infection with a similar Th1/Th2 phenotype, including related immune mediators.
- A genetic code related to the dormant phase of infection is well conserved in *M. tuberculosis* and *M. bovis* genomes.
- Gene transcription of *Mycobacterium* dormancy survival regulator (DosR) has been detected in infected tissues of both human and bovine hosts.
- Stimuli of CMI responses with stage-specific antigenic cocktails of *M. tuberculosis* permit the dissection of active and latent TB.
- Host cytokine signatures are descriptors of TB infection stages.
- Neglected boLTBI has not yet been described by standard testing.
- Omics platforms have started to reveal new suggestive stage-specific biomarkers of subclinical TB infections in human and animal hosts.

Theoretically, the boLTBI imposes a potential source for the dissemination of infection between herds from areas that were already considered free of bTB. Concerning the recognition of a dormant stage of *M. bovis* infection in cattle, some important questions need to be addressed to know its impact on the cattle industry. What are the consequences of misdiagnosis of latent TB at the animal or herd level? Do dormant infected animals transmit infection within a herd? Does hypothetical boLTBI persist in other natural reservoirs in wildlife? What other risk factors are involved in the establishment of latent infection in a herd? Persistent infection in cattle could be the main cause of recurrent infection and newly diagnosed animal cases. It is necessary to keep generating deeper knowledge on this resilient form of animal infection. Identifying new biomarkers will almost certainly reveal the hidden profile of boLTBI, opening a new perspective that may contribute to developing more accurate control strategies including diagnosis and vaccination strategies against this chronic animal disease.

Funding

This work has not received any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The author thanks the National Council of Science and Technology (CONACYT, México) for the early research support (Grants Nos. 81718, 83190, and 190426).

CRediT authorship contribution statement

Angel H. Alvarez: Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

The author especially thanks Mr. Brian Walsh (U.S. Peace Corps) for his help in improving the English language style in this manuscript and Mr. Javier Rodrígez Yáñez (CIATEJ) for his sustained support with the literature compilation. Also, the author expresses appreciation to anonymous reviewers for critically reading the manuscript and suggesting substantial improvements.

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