Enhancing bTB Diagnosis: CXCL9 Detection via Quartz Crystal Microbalance

Marie Verdonck (r0808315)
KU Leuven - Cellular and Genetic Engineering

January 13, 2024

Abstract

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis* infection, poses a significant global economic and public health challenge. Conventional diagnostic approaches exhibit shortcomings in terms of specificity, sensitivity, and portability. This study proposes a novel approach using Quartz Crystal Microbalance (QCM) biosensor technology to detect the chemokine CXCL9 for enhanced diagnosis. The immunosensor utilizes *M. bovis*-specific antigens for blood sample stimulation (ESAT-6 and CFP10). It thereby aims to improve specificity and minimize cross-reactivity with environmental mycobacteria. By leveraging CXCL9 as a biomarker, the tool enhances sensitivity, particularly in early infection stages. The compact and user-friendly QCM device is designed to deliver rapid and reliable results, facilitating implementation with minimal training. Surface modification of the immunosensor involves the use of a biotinylated self-assembled monolayer to ensure reliability and consistency. The expected outcome is a sophisticated and cost-effective QCM immunosensor that overcomes the limitations of existing diagnostic methods. By improving early detection, the study anticipates a positive result on disease management, preventing the spread of bTB and minimizing economic losses in livestock farming. The envisioned impact is a transformative shift in bTB diagnostics, paving the way for more effective disease control globally.

1 Introduction

Bovine tuberculosis (bTB) is an infectious disease of cattle caused by intracellular pathogens of the *Mycobacterium tuberculosis* complex [1]. *M. bovis* has been recognized as the main source of infection for cattle. Lesion distribution and pathology in field cases indicate that the upper and lower respiratory tracts are primarily affected, suggesting inhalation is the most probable and important route of bovine infection [2]. Infection through the respiratory route via ingestion, either directly from infected animals or from contaminated pastures, water, or fomites, is considered the second major route. Other less common routes include congenital transmission via the umbilical cord, genital transmission during coitus if the sexual organs are infected, and udder infections leading to pseudo-vertical transmission of calves [3]. It is important to note that *M. bovis* is not the exclusive cause of bTB as other mycobacteria were also isolated from bTB cases. For instance, a study performed to isolate and identify the causative agents of bTB in Macedonia found that up to 25% of bTB-positive cattle were infected by *M. caprae* [4].

1.1 Pathogenesis and Clinical Manifestations

Pathogenicity in mycobacteria is a complex and multifactorial process intricately linked to several virulent factors within the cell wall and protein complexes. Their distinctive cell wall is characterized by a thick structure with a core composed of the mycolylarabinogalactan–peptidoglycan complex. This complex is further covered by a sheet-like surface layer of glycolipids such as mycosides, cord factors, and sulfolipids [5]. These components impart unique characteristics to the pathogen and influence its

pathogenicity in several ways. The hydrophobic nature of the lipid layer hinders nutrient penetration, resulting in slow growth and an extended incubation period for the disease. It also provides resistance against disinfectant agents, the host's immune system, and various anti-tuberculous drugs, introducing challenges in disease management [5]. Owing to the aggregation of complex lipids, mycobacteria exhibit cord formation which promotes the spread and virulence of the organism. Additionally, glycolipid complexes play a significant role in granuloma formation and contribute to the immune response during infection. Another concern is the environmental resistance of mycobacteria. For instance, *M. bovis* can live in slurry and soil for at least six months at normal temperatures and can persist for a longer time in damp, dark, and cold transport vehicles or buildings [6].

Upon entry into the host via ingestion or inhalation, bacteria reach the intestinal tract or alveolar spaces, respectively. Mycobacteria survive and reproduce within the macrophages, which serve as the primary site for intracellular replication [1]. They possess the ability to evade destruction by macrophages through the use of their unique mycobacterial lipids and proteins [5]. Thus, macrophages play a dual role in protecting the host against mycobacterial infections and promoting the establishment of early infection. This process of phagocytosis triggers a localized inflammatory response and the recruitment of mononuclear cells, resulting in the accumulation of a large number of phagocytes and the formation of granulomatous lesions, which characterize the disease [6]. Bacteria-containing phagocytes can also migrate to associated lymph nodes, leading to systemic spread of the infection. Other common symptoms of the disease include weakness, debility, and mild fluctuating fever. However, clinical signs are not always visible in the early stages of the disease, and many affected animals appear clinically normal. Another important feature of bTB is a phenomenon known as latency or persistence, which refers to the ability of *M. bovis* to persist in a host without causing apparent symptoms. This occurs when tuberculous mycobacteria are effectively contained within the human host and remain in a non-replicative state. Individuals with latent infection theoretically do not transmit the disease, but their capacity to perpetuate infection if they reactivate poses significant difficulties to bTB eradication [7].

1.2 Economic Impact

Besides being a concern for animal health, bTB has direct and indirect economic implications globally. The disease has a longstanding history of contributing to tuberculosis in humans, prompting governments to implement strict control such as test-and-slaughter and mandatory milk pasteurization. This significantly reduced disease prevalence but complete elimination has been difficult to achieve. The substantial cost of control efforts is evident in countries like the USA, Ireland, Spain, and Australia. In the USA, the total cost for TB surveillance from 1962 to 2017 amounted to 3 billion dollars, highlighting the financial commitment required for sustained control [8].

In Sub-Saharan Africa, insufficient resources for controlling bTB often lead to 90% of the population residing in countries with either no or only partial measures in place. With a cattle population of 60 million, Ethiopia boasts the largest herd in Africa, contributing significantly to the national GDP, agriculture GDP, and export earnings [9]. bTB causes increased mortality, reduced milk, lower fertility, and weight loss in cattle. Countries with high prevalence rates of bTB may also face trade restrictions due to concerns about disease transmission. This can have severe economic consequences, especially for countries, regions, or farmers heavily reliant on the export of livestock and animal products [10].

1.3 Public Health

M. bovis represents an important zoonotic risk to public health. Transmission primarily occurs through the consumption of contaminated unpasteurized dairy products or ingestion of undercooked meat. Additionally, airborne transmission can occur through direct contact with infected animals, resulting in an increased risk for farmers, veterinarians, slaughterhouse workers, and butchers [5]. Globally, there were an estimated 147.000 new cases of zoonotic TB in 2016. The disease claimed 12.500 lives, with

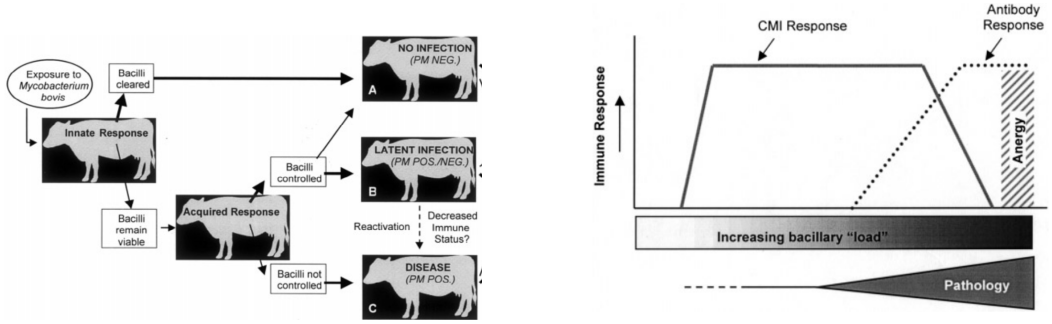
Africa being the most affected and South-East Asia following closely behind [11]. However, the shortage of routine monitoring data from most countries and the inability of the laboratory tests most frequently used to diagnose TB to distinguish between *M. tuberculosis* and *M. bovis* implies that the true burden of zoonotic TB is likely to be underestimated. Due to innate resistance to pyrazinamide, one of the main medications used in the treatment of TB, *M. bovis* poses difficulties for patient care and recovery [11].

In developed countries, the risk of transmission to humans has significantly decreased. However, *M. bovis* presents a serious risk to human health in underdeveloped nations, especially in the context of co-infection with HIV/AIDS [12]. Referred to as the 'Cursed Duet', the convergence of HIV infection and TB imposes an important burden on human health. HIV co-infection is the most important risk factor for the development of latent tuberculosis into an active disease. Additionally, *M. bovis* infection can accelerate the progression of HIV infection to AIDS [1]. The WHO identified TB as the global leading cause of death among people living with HIV worldwide with an estimated HIV-positive TB mortality of 167.000 people in 2022 [13]. This intersection between animal and human health underscores the importance of disease management.

2 State of the Art

2.1 Immune Response to *M. bovis*

The immune response in cattle to *M. bovis* infection involves an interplay of innate and adaptive immune mechanisms (Fig. 1a). Bacteria establish intracellular niches within macrophages, triggering a pro-inflammatory response [5]. Macrophages stimulate phagocytosis and activate microbicidal pathways to counteract the infection. Neutrophils are among the first responders, and contribute to the early immune response, while natural killer cells produce interferon-gamma (IFN-γ) and display cytolytic activity against infected cells [2]. In cases where innate mechanisms fail to contain the infection and mycobacteria successfully colonize macrophages, T-cells may be exposed to released antigens or breakdown products. The subsequent cell-mediated immune (CMI) response involves the sequential involvement of $\gamma\delta$, CD4, and CD8 T-cells [5]. The establishment of a Th1 immune response is characterized by the production of IFN-γ, primarily by CD4 T-cells [14]. This cytokine has several roles, including the activation of macrophages. Clonal expansion and differentiation of responding T-cells form the basis for immunological memory [15]. Immunohistological examination of early granulomatous lesions has confirmed T-cells to be among the first cells involved in the immune reaction [14]. While the CMI response dominates in less-advanced stages, the antibody response gains prominence in advanced infection (Fig. 1b).



(a) Most likely outcomes resulting from exposure of cattle to *M. bovis*. Adapted from [2]

(b) Immune responses in cattle following infection with *M. bovis* [2]

Figure 1: Immune Response to *M. bovis*

2.2 Ante-Mortem Diagnostics

Recognizing the predominant role of CMI during the early and intermediate stages of infection, the primary focus has been directed towards CMI-based diagnostics. Notably, there has been a recognition of the potential utility of antibody-based assays, especially in the identification of animals with chronic infections that might evade detection through CMI-based tests. The most commonly used ante-mortem diagnostic methods are the tuberculin skin test and the IFN-γ release assay, both assessing the CMI.

2.2.1 Tuberculin Skin Test (TST)

The tuberculin skin test has been the recommended standard procedure for ante-mortem diagnosis of bTB for more than 100 years, owing to its low cost and wide availability [16]. The test evaluates a delayed-type hypersensitivity reaction in response to intradermal injection of purified protein derivative (PPD) tuberculin from mycobacteria. After 72 hours, the skin fold thickness is assessed and compared to its pre-injection appearance. A swelling of more than 4 mm indicates a positive bovine response, whilst one of less than 3 mm is deemed negative [5]. The injection of PPD stimulates the CMI, causing T-cells sensitized by prior infection to accumulate at the site of injection. The release of cytokines induces local vasodilatation, edema, fibrin deposition, and recruitment of other inflammatory cells, leading to local swelling [17]. There are two common types of intradermal tuberculin tests: the Single Intradermal (SID) test, using PPD from *M. bovis*, and the Comparative Intradermal Tuberculin Test (CITT), which simultaneously injects bovine and avian PPDs (PPDb and PPDa, respectively) to enhance specificity. By injecting two types of tuberculin into the skin, the test can more accurately differentiate between animals infected with *M. bovis* and animals exposed to environmental mycobacteria that do not cause bTB [18]. Sensitivity and specificity ranges for TST are 55.1-96.8% and 75.5-100%, respectively [19].

Although it is widely used, TST presents notable drawbacks. The test requires a 72-hour incubation time and expertise is needed to administer the PPD correctly into the skin. Variability in test outcome is increased due to subjective interpretation by different operators [5]. Technical errors, including skin thickness measurement and PPD storage, can further compromise accuracy. False negatives may result from immunosuppression due to concurrent infection with viruses or drug treatments [16]. Cattle in advanced disease stages can experience anergy preventing their response to the TST. Even with widespread tuberculosis lesions throughout their internal organs, these anergic animals exhibit no external disease signs. Co-infection with or pre-exposure to environmental mycobacterium (e.g. *M. avium paratuberculosis*) or BCG-vaccination can lead to false positives due to cross-reactivity [18]. Moreover, most animals require 3-6 weeks post-infection to establish a complete immune response detectable through the TST. Testing during this early 'pre-allergic' phase leads to false negatives [5]. Another factor is desensitization, whereby skin reactivity to tuberculin is reduced for some time after a test. Administering another injection too soon without allowing adequate time for the immune system to recover may lead to a lack of reaction, hindering the identification of infected cattle. To prevent desensitization, a minimum 60-day interval between tests is maintained [20]. Lastly, TST cannot distinguish latent infection from disease [16].

2.2.2 IFN-γ Release Assay (IGRA)

The IFN-γ release assay is the most common laboratory-based test in bTB diagnosis. It is usually used in parallel with TST to maximize the detection of infected animals [5]. The *in vitro* blood test measures the release of IFN-γ in response to *M. bovis* antigens. First, blood is collected and transported to a laboratory where it is incubated at 37 °C for 24 hours and exposed to the test antigens. Typically, PPDb and PPDa are used. If the animal has encountered *M. bovis* or a similar mycobacterial infection, T-cells in the peripheral blood of cattle will release will produce IFN-γ upon antigen exposure. The amount of IFN-γ in the plasma is quantified by a sandwich enzyme-linked immunosorbent assay (ELISA). Infection with *M. bovis* is indicated by more IFN-γ production upon recognition of PPDb compared to PPDa or

the control. The results are interpreted using a threshold level of IFN-γ [5, 18]. IGRA sensitivity and specificity ranges are between 73.0–100% and 85.0–99.6%, respectively [19].

Advantages of the IGRA include the use of a more specific marker of *M. bovis* infection or previous exposure. It removes placement and reading errors compared to TST and enables a more objective, standardized interpretation of results [16]. The time to develop an IFN-γ response after infection takes between 1-5 weeks, which is shorter than for the TST. IGRA can thus detect earlier infections. It is more sensitive, faster, and requires only one farm visit [5]. Additionally, it allows for more rapid retesting since there is no interference with the host's immune system by injection of mycobacterial proteins [18].

The IGRA test also poses some challenges such as a higher cost and lower availability. There are some difficulties associated with collecting or transporting blood specimens in a stable environment. The samples need to be cultured and incubated with antigens within a few hours of collection as delays in the processing of the blood samples can significantly reduce the test accuracy [18]. Similar to TST, the use of PPD may lead to false positive results due to cross-reactivity with environmental mycobacteria. False negatives can occur at advanced stages where the humoral immune response increasingly takes over the CMI [5]. Lastly, IGRA cannot distinguish latent infection from disease [16].

2.2.3 Improvement Opportunities

At the core of effective disease management stands early and accurate diagnosis. The current diagnostic tools, TST and IGRA, are valuable for detecting *M. bovis* infections in cattle, but several challenges highlight the need for significant improvements in bTB diagnosis. Both methods suffer from reduced specificity due to cross-reactivity with environmental mycobacteria, leading to false positives. The main limitation is their low sensitivity, limiting the ability to accurately detect infected animals. Both tests suffer from the limitation of anergy, which can lead to false negatives. Furthermore, the inability of current diagnostic methods to differentiate between latent and active diseases presents a significant obstacle in effectively managing and controlling bTB.

The TST presents challenges in its administration and interpretation. Its 72-hour incubation time introduces logistical complexities because it necessitates multiple visits to the farm. The delayed immune response, taking 3-6 weeks post-infection for the TST, hinders the timely identification of infected animals. The subjective interpretation of TST results introduces variability dependent on the reader's expertise. Furthermore, result reliability of TST is impacted by technical errors and desensitization.

In contrast, IGRA addresses some logistical concerns by eliminating the need for a farm revisit. However, it presents its own set of challenges, such as the dependency on laboratory facilities for sample processing and the need for a rapid incubation process within 24 hours of blood collection, which poses practical constraints. Additionally, the higher cost and lower availability of IGRA further hinder its widespread adoption in bTB testing.

Given these challenges, there is an urgent need for the development of improved diagnostic tools that address the shortcomings of existing methods. Novel biomarkers are needed for better differentiation between healthy and infected animals, especially in cases where traditional methods fail to detect the infection. A more specific and sensitive diagnostic approach would greatly improve the efficiency and accuracy of bTB detection. One essential aspect is the integration of miniaturization, which allows for the creation of compact and efficient designs that are highly portable. This feature is especially important for disease surveillance in areas with limited infrastructure, as it enables rapid, on-site screening of numerous samples for targeted and timely interventions. The stability and standardization of diagnostic tools are crucial for ensuring reliable and consistent performance. Lastly, achieving economic feasibility and widespread availability is equally critical, especially in resource-constrained environments. By doing so, diagnostic tools can transcend geographical and economic boundaries and significantly contribute to global efforts in managing and eradicating bTB.

3 Objectives

This work outlines clear and targeted objectives aimed at advancing the field of bTB diagnostics by overcoming the current limitations in specificity, sensitivity, accessibility, and usability:

- **Use of novel antigens for enhanced specificity**: Current diagnostic tools lack specificity due to cross-reactivity with environmental mycobacteria. By using specific antigens, we aim to enhance the tool's ability to discriminate between *M. bovis* infections and non-specific reactions.
- **Discovery of early immune response biomarkers for improved sensitivity**: Current diagnostics have limited sensitivity, especially in detecting early-stage infections. Early detection is critical for timely interventions and preventing disease spread. Identifying early immune response biomarkers fills this gap in sensitivity.
- **Development of a compact and portable diagnostic tool for on-site screening**: Limited infrastructure in certain regions hampers the widespread use of current diagnostic tools. Portable tools facilitate ease of transportation and enable on-site screening, overcoming logistical challenges. This addresses the gap in accessibility and usability in diverse settings.
- **Enhancement of user-friendliness to facilitate wide adoption**: Current diagnostic tools require specialized expertise for accurate implementation and interpretation. The aim is to design the diagnostic tool to be user-friendly, requiring minimal training for accurate usage.
- **Achievement of economic feasibility for global accessibility**: Economic constraints limit the widespread adoption of the IGRA diagnostic tool. Affordable tools are more likely to foster global acceptance, particularly in environments constrained by limited resources.
- **Standardization and long-term stability**: Lack of standardization in current tools hinders consistent and reliable performance. Standardization improves result comparability across diverse settings, contributing to the overall reliability of the diagnostic tool. Long-term stability ensures sustained performance.

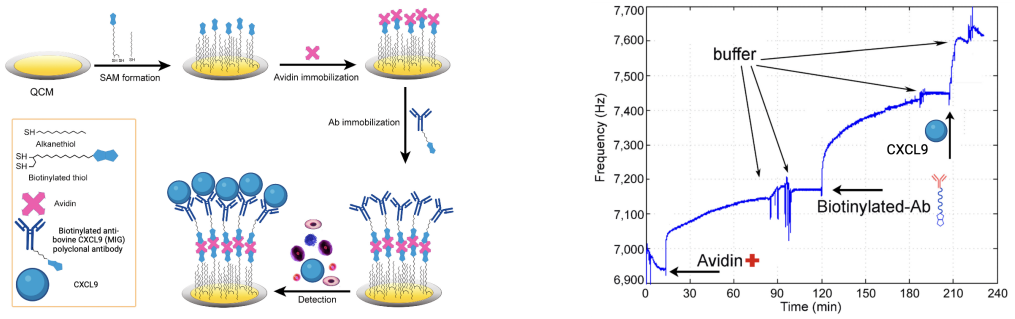
A novel diagnostic tool that incorporates specific antigens for enhanced specificity and early immune response biomarkers for improved sensitivity is expected to significantly outperform current diagnostic methods for bTB. A tool that is designed to be compact, user-friendly, economically feasible, standardized, and stable, ensures widespread accessibility and practical implementation in diverse settings.

4 Methodology

Here, I propose an innovative solution that addresses the challenges of diagnosis of *M. bovis* infection by detecting the chemokine CXCL9 using QCM, a highly sensitive and specific biosensor technology that measures changes in resonance frequency caused by mass changes on the quartz crystal surface (Fig. 2a). *M. bovis*-specific antigens ESAT-6 and CFP10 are used to stimulate blood samples. Subsequently, the samples are applied to the QCM. With this innovative approach, a quantitative measure of CXCL9 is provided which significantly improves the accuracy and efficacy of bTB diagnostics.

4.1 Quartz Crystal Microbalance (QCM)

QCM is an acoustic sensing technique that measures mass changes on its surface. The device consists of a thin, round quartz disc with electrodes that act as sensing surfaces at both sides of the crystal. Its operation is based on the piezoelectric effect whereby the crystal can be excited by applying an AC voltage across the electrodes [22]. The crystal exhibits a resonance frequency that varies upon surface



(a) Schematic illustration of the immunosensor. Adapted from [21]

(b) Frequency Shift Measurements. Adapted from [21]

Figure 2: QCM Immunosensor for the Detection of CXCL9

mass change. Leveraging the high quality factor of quartz crystals (up to 10^6), QCM enables precise sensing [22]. It offers direct label-free detection, using a real-time non-invasive approach at a low cost [23]. QCM is a versatile technique that can be applied to detect small molecules, viruses, bacteria, and eukaryotic cells [21]. Biosensing is possible by using surfaces functionalized with recognition elements. In the context of antibody–antigen interactions, the QCM transforms into an immunosensor.

The QCM technique ensures selectivity through precise surface modification. This modification involves the immobilization of active biomolecules designed to target a molecule of interest. Preserving the biological activity of capture biomolecules is essential for the efficacy of a biosensor. This is especially important for antibodies, as their specific binding competence could be compromised if immobilized on a solid support with a random orientation [21]. Establishing highly oriented antibody layers is thus needed to attain selectivity and sensitivity in antibody-based immunosensing. To enhance the sensitivity and reproducibility of the QCM immunosensor, CXCL9 antibodies are immobilized on the QCM surface using the self-assembled monolayer (SAM) method. SAM provides a simple yet effective approach to create a reproducible, ultra-thin, and ordered layer on the QCM surface. This enables the optimal immobilization of antibodies, thereby improving the sensitivity of the immunosensor [24]. The mixed biotinylated SAM provides a platform for the subsequent immobilization of avidin and biotinylated antibodies [21].

Once the surface is modified with CXCL9 antibodies, the QCM forms a sophisticated immunosensor. This configuration enables the selective capture of CXCL9, forming antibody-antigen complexes on the crystal surface. These binding events induce changes in mass and alter the resonance frequency (Fig. 2b). QCM immunosensors enable measurement of mass deposition down to 0.1 ng [21]. The variation in frequency is proportional to the change in surface mass, which can be calculated using Sauerbrey's equation [25]: $\Delta f = -\frac{2f_0^2 \Delta m}{\sqrt{\mu \rho_q} A}$. Here, Δf represents the frequency shift, f_0 denotes the fundamental resonance frequency of quartz, Δm is the mass change, μ corresponds to the shear stress of quartz, ρ_q to the quartz density and A to the surface area of the deposited film.

Other possible diagnostic techniques for the detection of *M. bovis* include PCR, ELISA, and lateral-flow assay (LFA). A comprehensive comparison of these methods is presented in Table 1 [5, 6, 19, 26, 27, 28].

4.2 C-X-C motif ligand 9 (CXCL9)

In addition to IFN-γ, several host biomarkers associated with CMI responses have emerged as potential candidates for blood-based TB tests for early diagnosis. One such candidate is CXCL9, also known as monokine induced by IFN-γ (MIG). Its ability to respond to specific *M. bovis* antigens suggests its potential as a reliable indicator of infection. Recent work has explored the CXCL9 responses to both specific (triple fusion protein consisting of ESAT-6, CFP10, and Rv3615c (E/C/Rv3615c)) and complex *M. bovis* antigens (PPDb and PPDa) for the development of whole-blood-based bTB diagnosis [29]. A ro-

Table 1: Comparison of Diagnostic Methods for *M. bovis* Detection

	ELISA	**PCR**	**LFA**	**QCM**
Procedure	Time-consuming	Time-consuming	Rapid	Rapid
Equipment	Laboratory	Laboratory	Portable	Portable
Requirement	Professionals	Professionals	Ease of use	Ease of use
Sample Volume	Large volume	Small volume	Small volume	Small volume
Cost	Costly	Costly	Low	Low
Storage Stability	Short	Short	Long	Long
Labeling	Antibodies	Label-free	Antibodies	Label-free
Multiplexing	Possible	Possible	Possible	Not Possible

bust CXCL9 mRNA response to E/C/Rv3615c was observed at two weeks post-infection (WPI) in *M. bovis* infected cattle. The response significantly exceeded the response by non-infected animals throughout the 16 weeks after infection. CXCL9 protein levels in *M. bovis*-infected cattle in response to E/C/Rv3615c and PPDb significantly exceeded the levels in non-infected cattle. The response to PPDb minus the response to PPDa was also significantly greater than for healthy cattle. Importantly, mean CXCL9 levels in non-stimulated plasma was not dependent on infection status and therefore did not interfere with the observed differential responses upon stimulation. Similarly, a study of CXCL9, CXCL10 and IFN-γ mRNA levels in African lions infected with *M. bovis* has shown that CXCL9 was the most strongly upregulated in ESAT-6/CFP-10 stimulated whole-blood samples compared to unstimulated samples [30]. Significantly high levels of CXCL9 mRNA were also found in early-stage TB granulomas in *M. bovis* infected cattle [31]. CXCL9 is suggested to play a role in attracting activated T cells and contributing to the development or maintenance of granulomas as part of the bovine anti-mycobacterial response [32]. This is particularly important during the early stages of infection when an increased number of cells is required to combat the pathogen. It was shown that the ratio of CXCL9 protein levels could be used for discriminating *M. bovis* naïve from *M. bovis* challenged animals. It could also significantly differentiate BCG-vaccinated cattle from *M. bovis* challenged ones. Cattle that were challenged with *M. bovis* after BCG vaccination were also differentiated from the naïve group [27].

Importantly, several studies have shown that stimulation with both ESAT-6 and CFP10 results in a higher test specificity compared to PPDb-stimulated samples. Especially in low or medium prevalence herds, the improved specificity of the ESAT-6 and CFP10 cocktail makes them valuable antigens for detecting *M. bovis* infections, addressing limitations associated with traditional antigens like PPDb [33, 34].

This evidence highlights ESAT-6/CFP-10-stimulated CXCL9 levels as a key biomarker for early detection of *M. bovis* infection, forming the basis for its incorporation into the QCM immunosensor for bTB diagnosis.

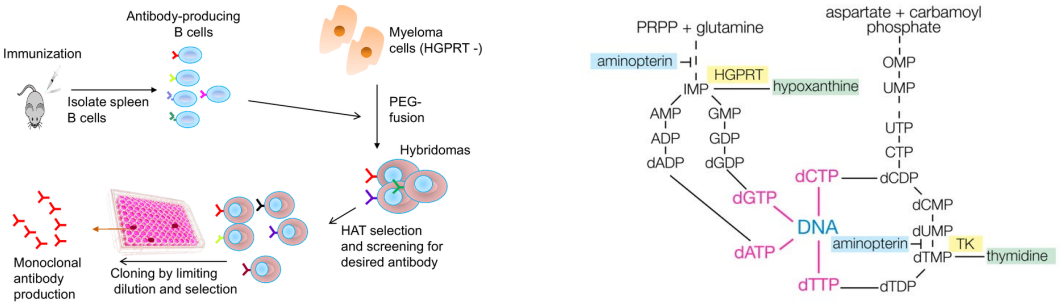
4.3 Antibody and Antigen Production

In developing an immunosensor, the choice between monoclonal and polyclonal antibodies (mABs and pABs) is pivotal. pABs, while advantageous for detecting a variety of epitopes and cost-effective short-term production, suffer from batch-to-batch variation and lack reproducibility. mABs, on the other hand, offer a homogeneous and predictable source with exquisite specificity. Their high specificity for a single epitope reduces the risk of cross-reactivity. They provide a reproducible and potentially inexhaustible supply, ensuring standardized systems for accurate *M. bovis* diagnostics. The decision to utilize mABs is thus grounded in their ability to meet the high standards of specificity and reproducibility required for the immunosensor, in combination with the quantification of CXCL9 proteins [35].

To generate mAbs, an approach involving immunization of mice and subsequent hybridoma formation is used (Fig. 3a). Before initiating the immunization process, serum is collected from mice to establish a baseline for ELISA screening. Then, 50 to 100 μg of bovine CXCL9 is administered to each mouse. The

immunogen is mixed with Alum adjuvant to enhance the immune response. After a two-week interval, a second injection of the adjuvant and immunogen is used to amplify the immune response. On day 21, tail bleeds are used to extract serum to ascertain the antibody titer for CXCL9 through ELISA. Administer regular boosting and perform test bleeds until the desired response is obtained. The final injection, 4 to 5 days before the fusion process, should only contain CXCL9 (no adjuvant) [36]. The best responder mouse is then selected and antibody-secreting spleen cells are extracted and fused to hypoxanthine guanine phosphoribosyltransferase (HGPRT)-deficient immortal myeloma cells using a fusing agent (polyethylene glycol) [37]. This results in hybridomas that combine the ability of B cells to produce specific antibodies with the continuous division and survival capabilities of myeloma cells. To eliminate unfused myeloma cells from the culture a selective medium containing hypoxanthine, aminopterin, and thymidine (HAT) is used (Fig. 3b). Aminopterin blocks the *de novo* pathway for nucleotide biosynthesis, and as a result, unfused HGPRT-negative myeloma cells die off. Hybridomas, however, survive this selection as the salvage pathway enzyme HGPRT is provided by their B cell counterpart [37].

Hybridomas undergo screening for selection based on antigen specificity through an ELISA-based assay. Bovine CXCL9 is coated on the plate to allow antibody binding. After incubating hybridoma culture supernatants, a secondary enzyme-labeled conjugate is introduced. The addition of a chromogenic substrate leads to a colored product for positive hybridomas [37].



(a) Hybridoma Technology [38]

(b) Principle of HAT selection [39]

Figure 3: Monoclonal Antibody Production

To produce bovine CXCL9 proteins for immunization, a yeast expression system will be used. The genetic manipulability of yeast allows for strain optimization and efficient protein expression. Unlike, bacterial expression systems, yeast can perform posttranslational modifications, ensuring proper folding and functionality of eukaryotic proteins. Moreover, yeast offers practical benefits, including faster, more cost-effective, and higher yield production when compared to mammalian cell culture expression systems [40]. Briefly, the bovine *CXCL9* gene is cloned into a vector containing yeast regulatory elements such as promoter and transcriptional termination sequences. For later purification steps, the protein will be expressed as a His6 fusion protein with an engineered Factor Xa site [41]. This vector is transformed into *Saccharomyces cerevisiae* using standard protocols (lithium acetate method or electroporation). Transformants can be selected using auxotrophic markers such as the *LEU2* gene. Successful transformation is confirmed through colony PCR and/or sequencing [42]. Once protein expression is confirmed through SDS-PAGE using anti-HIS antibodies, protein purification can be performed using affinity chromatography. The His6 fusion protein is retained on the column using nickel-nitrilotriacetic acid. CXCL9 can then be eluted and treated with Factor Xa for removal of the His tag [43]. The purified protein can then be used for mouse immunization.

4.4 Immunoassay Method Validation

Before implementing the proposed diagnostic test, its efficacy, reliability, and practicality will be rigorously assessed. The evaluation centers around the Receiver Operating Characteristic (ROC) curve (Fig. 4). The ROC curve shows the trade-off between sensitivity and specificity, allowing to evaluate

the discriminative power of the diagnostic method. Field trials involving diverse cattle populations will be conducted to evaluate test performance under real-world conditions. The assessment will consider various factors, including the stage of infection, co-infections, and potential environmental influences. Additionally, the diagnostic test's portability, ease of use, and cost-effectiveness will be assessed to ensure its feasibility for widespread adoption. Continuous monitoring and refinement will be crucial to obtain a tool that not only meets biotechnological standards but also aligns with practical needs for effective bTB management.

Diagnostic test result	Disease status	
	Present	Absent
Positive	a (TP)	b (FP)
Negative	c (FN)	d (TN)
Total	n_1=a+c	n_2=b+d

Summary Indices of Test Performance

TPF=True Positive Fraction (Sensitivity)= TP/ (TP+FN)= a/(a+c)

FNF=False Negative Fraction (1-Sensitivity)= FN/ (TP+FN)= c/(a+c)

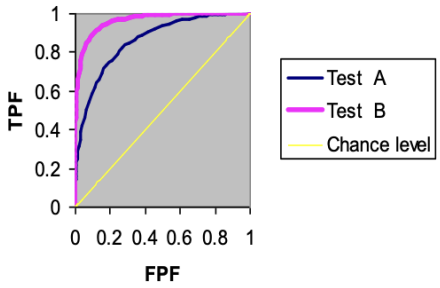
TNF=True Negative Fraction (Specificity)= TN/ (TN+FP)= d/(b+d)

FPF=False Positive Fraction (1-specificity)= FP/ (TN+FP)= b/(b+d)

PPV=Positive Predicted Value=TP/(TP+FP)=a/(a+b)

NPV=Negative Predicted Value=TN/(TN+FN)=d/(c+d)

(a) Summary of Test Performance [44]



(b) ROC curves of two diagnostic tests [44]

Figure 4: Diagnostic Test Evaluation

5 Significance and Innovation

The anticipated results of this research project include the development and validation of a highly specific, sensitive, and portable QCM immunosensor for the early diagnosis of *M. bovis* infection in cattle. The primary expected outcome is a robust diagnostic tool that surpasses the limitations of existing methods, providing rapid and reliable detection of bTB. Traditional diagnostic methods, such as TST and IGRA, exhibit limitations in terms of time consumption, cost, and variable performance. The proposed QCM immunosensor, with its rapid results, ease of use, and low cost, offers a transformative solution to these challenges. The high precision measurements, reproducibility, and potential reusability make this immunosensor a superior alternative to existing diagnostic techniques.

Firstly, the proposed diagnostic tool enhances specificity and sensitivity. By focusing on *M. bovis*-specific antigens, the risk of cross-reactivity with environmental mycobacteria is minimized. This approach is expected to provide a much-needed improvement over existing diagnostic methods, reducing false positives. Secondly, the compact and portable QCM immunosensor designed for on-site screening represents a breakthrough in terms of use. The user-friendly design and minimal training requirements aim to democratize bTB screening, making it accessible even in resource-constrained environments. The rapid, on-site screening capability of the proposed diagnostic tool makes it a practical and efficient solution. Furthermore, the economic feasibility and widespread availability of the tool contribute significantly to global efforts in managing and eradicating bTB.

In conclusion, early detection facilitated by the proposed diagnostic tool will have a positive effect on disease management. By providing a rapid, specific, and sensitive means of identifying *M. bovis* infections at early stages, the tool has the potential to prevent the spread of bTB within herds. This can minimize economic losses associated with reduced milk production, lower fertility, and weight loss in cattle. Leveraging QCM biosensor technology and specific *M. bovis* antigens, the study introduces a cutting-edge solution that combines precision, portability, and economic feasibility. The proposed diagnostic tool has the potential to set new standards for specificity, sensitivity, and ease of use in bTB diagnostics, contributing to the advancement of global livestock health and facilitating more effective disease control measures.

References

[1] Haobo Zhang, Mengda Liu, Weixing Fan, Shufang Sun, and Xiaoxu Fan. The impact of mycobacterium tuberculosis complex in the environment on one health approach. *Frontiers in Public Health*, 10:994745, 2022.

[2] JM Pollock and SD Neill. Mycobacterium bovis infection and tuberculosis in cattle. *The Veterinary Journal*, 163(2):115–127, 2002.

[3] FD Menzies and SD Neill. Cattle-to-cattle transmission of bovine tuberculosis. *The Veterinary Journal*, 160(2):92–106, 2000.

[4] Iskra Cvetkovikj, Slavcho Mrenoshki, Kiril Krstevski, Igor Djadjovski, Branko Angjelovski, Zagorka Popova, Aleksandar Janevski, Aleksandar Dodovski, and Aleksandar Cvetkovikj. Bovine tuberculosis in the republic of macedonia: postmortem, microbiological and molecular study in slaughtered reactor cattle. *Macedonian Veterinary Review*, 40(1):43–52, 2017.

[5] Mohamed Borham, Atef Oreiby, Attia El-Gedawy, Yamen Hegazy, Hazim O Khalifa, Magdy Al-Gaabary, and Tetsuya Matsumoto. Review on bovine tuberculosis: An emerging disease associated with multidrug-resistant mycobacterium species. *Pathogens*, 11(7):715, 2022.

[6] Joseph KN Kuria. Diseases caused by bacteria in cattle: tuberculosis. In *Bacterial cattle diseases*. IntechOpen, 2019.

[7] Angel H Alvarez, Ciro Estrada-Chávez, and Mario Alberto Flores-Valdez. Molecular findings and approaches spotlighting mycobacterium bovis persistence in cattle. *Veterinary research*, 40(3), 2009.

[8] Rea Tschopp, Jakob Zinsstag, Andrew Conlan, Gizachew Gemechu, James Wood, et al. Productivity loss and cost of bovine tuberculosis for the dairy livestock sector in ethiopia. *Preventive Veterinary Medicine*, 202:105616, 2022.

[9] Mebrate Getabalew, Tewodros Alemneh, and Derbie Zewdie. The milk processing: status, challenges and opportunities in ethiopia. *International Journal of Veterinary Science and Research*, 6(1):052–057, 2020.

[10] Rea Tschopp, Andrew JK Conlan, Gizachew Gemechu, Gizat Almaw, Jan Hattendorf, Jakob Zinsstag, ETHICOBOTS consortium, and James LN Wood. Effect of bovine tuberculosis on selected productivity parameters and trading in dairy cattle kept under intensive husbandry in central ethiopia. *Frontiers in Veterinary Science*, 8:698768, 2021.

[11] World Health Organization(WHO);Food, Agriculture Organization of the United Nations(FAO);World Organization for Animal Health (OIE); IUAT, International Union Against Tuberculosis, and Lung Disease (The Union). Road map for zoonotic tuberculosis, n.d. In Proceedings of the 48th Union World Conference on Lung Health, Guadalajara, Mexico, 11–14 October 2017.

[12] CJ Daborn and JM Grange. Hiv/aids and its implications for the control of animal tuberculosis. *British Veterinary Journal*, 149(5):405–417, 1993.

[13] World Health Organization. Tuberculosis profile: Global, 2023. `https://worldhealthorg.shinyapps.io/tb_profiles/?_inputs_&group_code=%22global%22&entity_type=%22group%22&lan=%22EN%22` [Accessed: 31 December 2023].

[14] JM Pollock, MD Welsh, and J McNair. Immune responses in bovine tuberculosis: towards new strategies for the diagnosis and control of disease. *Veterinary immunology and immunopathology*, 108(1-2):37–43, 2005.

[15] JM Pollock, J McNair, MD Welsh, RM Girvin, HE Kennedy, DP Mackie, and SD Neill. Immune responses in bovine tuberculosis. *Tuberculosis*, 81(1-2):103–107, 2001.

[16] Daniel V Vasconcelos-Santos, Manfred Zierhut, and Narsing A Rao. Strengths and weaknesses of diagnostic tools for tuberculous uveitis. *Ocular immunology and inflammation*, 17(5):351–355, 2009.

[17] Surajit Nayak and Basanti Acharjya. Mantoux test and its interpretation. *Indian dermatology online journal*, 3(1):2–6, 2012.

[18] R De la Rua-Domenech, AT Goodchild, HM Vordermeier, RG Hewinson, KH Christiansen, and RS Clifton-Hadley. Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculin tests, γ-interferon assay and other ancillary diagnostic techniques. *Research in veterinary science*, 81(2):190–210, 2006.

[19] I Schiller, B Oesch, HM Vordermeier, MV Palmer, BN Harris, KA Orloski, BM Buddle, TC Thacker, KP Lyashchenko, and WR Waters. Bovine tuberculosis: a review of current and emerging diagnostic techniques in view of their relevance for disease control and eradication. *Transboundary and emerging diseases*, 57(4):205–220, 2010.

[20] Michael Coad, Derek Clifford, Shelley G Rhodes, R Glyn Hewinson, H Martin Vordermeier, and Adam O Whelan. Repeat tuberculin skin testing leads to desensitisation in naturally infected tuberculous cattle which is associated with elevated interleukin-10 and decreased interleukin-1 beta responses. *Veterinary research*, 41(2), 2010.

[21] Ornella Maglio, Salvatore Costanzo, Rosaria Cercola, Gerardo Zambrano, Marco Mauro, Raffaele Battaglia, Gianluca Ferrini, Flavia Nastri, Vincenzo Pavone, and Angela Lombardi. A quartz crystal microbalance immunosensor for stem cell selection and extraction. *Sensors*, 17(12):2747, 2017.

[22] Ya Yang, Yifeng Tu, Xiaoshu Wang, Jinyin Pan, and Yun Ding. A label-free immunosensor for ultrasensitive detection of ketamine based on quartz crystal microbalance. *Sensors*, 15(4):8540–8549, 2015.

[23] Román Fernández, Pablo García, María García, José V García, Yolanda Jiménez, and Antonio Arnau. Design and validation of a 150 mhz hffqcm sensor for bio-sensing applications. *Sensors*, 17(9):2057, 2017.

[24] JE Ortega-Valencia, J Oliva-Ramírez, O García-Barradas, M Jiménez-Fernández, GH Rosas-Saito, JE Oseguera-Peña, and DV Melo-Máximo. Development of a qcm immunosensor with functionalized gold nanoparticles for the detection of salmonella typhimurium. *Materials Letters*, 314:131885, 2022.

[25] Günter Sauerbrey. Verwendung von schwingquarzen zur wägung dünner schichten und zur mikrowägung. *Zeitschrift für physik*, 155:206–222, 1959.

[26] Mayara Ingrid Sousa Lima, Emilly Caroline dos Santos Moraes, Jaqueline Diniz Pinho, Gustavo Henrique Corrêa Soares, and Ítalo Vinícius Cantanhêde Santos. Molecular and biotechnological approaches in the diagnosis of leprosy. In *Hansen's Disease-The Forgotten and Neglected Disease*. IntechOpen, 2019.

[27] Hamza Khalid, Louise Pierneef, Anouk Van Hooij, Zijie Zhou, Danielle De Jong, Elisa Tjon Kon Fat, Timothy K Connelley, Jayne C Hope, Paul LAM Corstjens, and Annemieke Geluk. Development of lateral flow assays to detect host proteins in cattle for improved diagnosis of bovine tuberculosis. *Frontiers in Veterinary Science*, 10, 2023.

[28] Shengmiao Liu, Xinyu Liu, Qianwen Pan, Zhihan Dai, Mingfei Pan, and Shuo Wang. A portable, label-free, reproducible quartz crystal microbalance immunochip for the detection of zearalenone in food samples. *Biosensors*, 11(2):53, 2021.

[29] Mitchell V Palmer, Tyler C Thacker, Meaghan M Rabideau, Gareth J Jones, Carly Kanipe, H Martin Vordermeier, and W Ray Waters. Biomarkers of cell-mediated immunity to bovine tuberculosis. *Veterinary immunology and immunopathology*, 220:109988, 2020.

[30] TT Olivier, IM Viljoen, J Hofmeyr, GA Hausler, WJ Goosen, ASW Tordiffe, P Buss, AG Loxton, RM Warren, MA Miller, et al. Development of a gene expression assay for the diagnosis of m ycobacterium bovis infection in african lions (p anthera leo). *Transboundary and emerging diseases*, 64(3):774–781, 2017.

[31] E Aranday-Cortes, NC Bull, B Villarreal-Ramos, J Gough, D Hicks, A Ortiz-Peláez, HM Vordermeier, and FJ Salguero. Upregulation of il-17 a, cxcl 9 and cxcl 10 in early-stage granulomas induced by m ycobacterium bovis in cattle. *Transboundary and emerging diseases*, 60(6):525–537, 2013.

[32] Katrin Smith, Léanie Kleynhans, Robin M Warren, Wynand J Goosen, and Michele A Miller. Cell-mediated immunological biomarkers and their diagnostic application in livestock and wildlife infected with mycobacterium bovis. *Frontiers in Immunology*, 12:483, 2021.

[33] S Flores-Villalva, F Suárez-Güemes, C Espitia, AO Whelan, M Vordermeier, and JA Gutiérrez-Pabello. Specificity of the tuberculin skin test is modified by use of a protein cocktail containing esat-6 and cfp-10 in cattle naturally infected with mycobacterium bovis. *Clinical and Vaccine Immunology*, 19(5):797–803, 2012.

[34] C Aagaard, M Govaerts, Virginia Meikle, JA Gutiérrez-Pabello, James McNair, Peter Andersen, Francisco Suárez-Güemes, J Pollock, C Espitia, and A Cataldi. Detection of bovine tuberculosis in herds with different disease prevalence and influence of paratuberculosis infection on ppdb and esat-6/cfp10 specificity. *Preventive veterinary medicine*, 96(3-4):161–169, 2010.

[35] Shikha Sharma, Hannah Byrne, and Richard J O'Kennedy. Antibodies and antibody-derived analytical biosensors. *Essays in biochemistry*, 60(1):9–18, 2016.

[36] Thermo Scientific Pierce. Antibody production and purification technical handbook. *Version*, 2:5, 2010.

[37] PN Nelson, GM Reynolds, EE Waldron, E Ward, K Giannopoulos, and PG Murray. Demystified…: monoclonal antibodies. *Molecular pathology*, 53(3):111, 2000.

[38] Mouldy Sioud. Phage display libraries: from binders to targeted drug delivery and human therapeutics. *Molecular biotechnology*, 61(4):286–303, 2019.

[39] Souphatta Sasorith Karima Cherouali, Mélissa Cambon and Marie-Paule Lefranc. Mammalian cell lines used in antibody engineering and expression system, 2015. `https://www.imgt.org/IMGTbiotechnology/Mammalian_cell_lines.html` [Accessed: 4 January 2024].

[40] Klaus H Nielsen. Protein expression-yeast. In *Methods in enzymology*, volume 536, pages 133–147. Elsevier, 2014.

[41] David S Waugh. An overview of enzymatic reagents for the removal of affinity tags. *Protein expression and purification*, 80(2):283–293, 2011.

[42] Diethard Mattanovich, Paola Branduardi, Laura Dato, Brigitte Gasser, Michael Sauer, and Danilo Porro. Recombinant protein production in yeasts. *Recombinant gene expression*, pages 329–358, 2012.

[43] Ying Xie, Xiao Han, and Yansong Miao. An effective recombinant protein expression and purification system in saccharomyces cerevisiae. *Current protocols in molecular biology*, 123(1):e62, 2018.

[44] Karimollah Hajian-Tilaki. Receiver operating characteristic (roc) curve analysis for medical diagnostic test evaluation. *Caspian journal of internal medicine*, 4(2):627, 2013.