EXPERIMENTAL AND *IN SILICO* STUDIES ON BIOENGINEERED POLYESTER NANOBEADS FOR LATENT TUBERCULOSIS DIAGNOSIS

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2025

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by

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Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

August 2025

ACKNOWLEDGEMENTS

First and foremost, I extend my deepest gratitude to Allah SWT for granting me the strength and perseverance to complete this thesis. I am immensely thankful to my family for their unwavering support and understanding throughout my lengthy PhD journey.

I am profoundly grateful to my supervisory and advisory team at USM: Dr. Ezzeddin Kamil Mohamed Hashim, Prof. Dr. Norazmi Mohd Nor, Prof. Armando Acosta Dominguez, and Prof. Dr. Maria Elena Sarmiento. Their exceptional guidance, insightful consultations, and consistent encouragement have been invaluable.

I would also like to express my heartfelt thanks to my friends, Nadiah, Fatihah Ahmad, Bibi Nur Bazlini, Fatin Marzuki, and many others, for their moral support, advice, and companionship during this journey. Additionally, I am thankful to the staff at USM, both in the laboratory and administration, for their assistance in easing many tasks during my time at the university.

Finally, I extend my gratitude to my sponsor and the project grants that made my PhD study possible.

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LIST OF SYMBOLS

Symbols	Meaning
OD	optical density
mM	milli molar
mg	milligram
mm	millimeter
ng	nanogram
pH	quantitative measure of the acidity or basicity of aqueous or other liquid solutions
pg/mL	picogram per milliliter
rpm	revolutions per minute
v/v	volume to volume
w/v	weight by volume
wt/vol	weight/volume concentration
°C	degree celcius
×g	the g force or relative centrifugal force (RCF)
µg/mL	microgram per milliliter
pg/mL	picograms per milliliter

LIST OF ABBREVIATIONS

Abbreviations	Meaning
aa	amino acids
APCs	antigen presenting cells
BCA-1	human B cell-attracting chemokine 1
BCEs	B cell epitope(s)
BCG	Bacille Calmette-Guérin Vaccine
BMI	body mass index
BMTB	MTB-antigen-coated biopolyester nanobeads
BSA	Bradford assay
BamHI	a type II restriction endonuclease recognising G/GATCC
CCL-11	C-C motif chemokine 11
CFP-10	10-kDa culture filtrate protein
COPD	chronic obstructive pulmonary disease
CXCL-3 (and -11)	C-X-C motif chemokine -3 (and -11)
DCs	dendritic cells
DR-TB	drug resistant tuberculosis
DosR	dormancy survival regulator
E. coli	*Escherichia coli*
ESAT-6	early secreted antigenic target of 6 kDa protein
Fe^{2+}	ferrous ion
Fe^{3+}	ferric
GC/MS	gas chromatography–mass spectroscopy
GM-CSF	granulocyte-macrophage colony-stimulating factor
GRAS	generally regard as safe
GrB	granzyme B
GD	green diluent
H_2S	hydrogen sulphide
HEG	highly expressed genes
HLA	human leukocyte antigen
HPUSM	Hospital Pakar Universiti Sains Malaysia

HSP	high score pairing
IEDB	Immune Epitope DDatabase and Analysis Resource
IFN-γ	Interferon gamma
IL-12, -21	interleukin e.g.,IL-12, -21
IL-1ra	iinterleukin-1 receptor antagonist
IL-2R□	interleukin-2 receptor alpha
IP-10	Interferon-inducible protein-10
IPTG	isopropyl β- d-1-thiogalactopyranoside
IR	immunogenic region
IgG1	immunoglobulin e.g., IgG1, IgG2b, IgG2c
KIRs	killer-cell immunoglobulin-like receptors
LAMP	loop-mediated isothermal amplification
LILRB1	leukocyte immunoglobulin like receptor B1
LMIC	low and middle income countries
LPS	lipopolyssacride
LTBI	latent TB infection
MAIT	mucosal-associated invariant T cells
MALDI-TOF-MS	matrix assisted laser desorption ionization-time of flight mass spectrometry
MAMPs	microbe-associated molecular patterns
MDR	multidrug resistant strains
MHC	major histocompatibility complex (Class -I or -II)
MTB	*Mycobacterium tuberculosis*
NCBI	US National Center for Biotechnology Information
NK cells	natural killer T cells
Na_2HPO_4	sodium phosphate dibasic
NaCl	sodium chloride
OD	optical density
PB	plain biopolyester nanobeads
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PC	population coverage
PCC	the Pearson correlation coefficient

PCR	polymerase chain reaction
pET14b	a vector carries an N-terminal sequence
PHA	polyhydroxyalkalanoate
PHB	poly(3-hydroxybutyrate)
PLWHIV	people living with HIV
pMCS69	plasmid multiple cloning sites number 69
PPD	purified protein derivative standard
PRR	pathogen recognition receptors
PTB	pulmonary TB
PhaA	β-ketothiolase
PhaB	acetoacetyl coenzyme A (CoA) reductase
PhaC	polyester synthase
QFT-GIT	QuantiFeron Gold-In-Tube
QFT-Plus	QuantiFERON-TB Gold Plus
RD	region of difference
RD-1	regions of difference 1
RR	rifampicin resistant
RT	room temperature
Rv ID	common MTB identification
sCD40L	soluble CD40L
	standard deviation
SDS-PAGE	sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SEA	Southeast Asia
sigB (and -D,-F,-I,-Jz)	sigma factors B (and -D,-F,-I,-Jz)
SpeI	a type of restriction enzyme recognising A/CTAGT
TB	tuberculosis
TCEs	T cell epitope(s)
T_{CM}	central memory T cells
TCRs	T cell receptors
T_{EFF}	recruited T effector cells
T_{EM}	T effector memory cells
TEM	transmission electron microscopy

TNF□	tumor necrosis factor alpha
T_{RM}	tissue-resident memory cells
Th1	type 1 T helper
Th2	type 2 T helper
T_{inn}	innate-like T cells
Tris-HCl	tris hydrochloride
USFDA	US Food and Drug Administration
VEGF	vascular endothelial growth factor
XDR	extremly drug resistant
XbaI	a type of restriction enzyme recognising T/CTAGA
γδ	gamma delta T cells

LIST OF APPENDICES

Appendix A: Research Ethical Clearance

Appendix B : Supplementary Materials

KAJIAN EKSPERIMEN DAN IN SILICO TERHADAP NANOBID POLIESTER YANG DIBIOJURUTERAKAN UNTUK DIAGNOSIS TUBERKULOSIS LATEN

ABSTRAK

Mycobacterium tuberculosis (MTB) ialah agen penyebab bagi penyakit tuberkulosis (TB) dan kekal sebagai salah satu daripada sepuluh penyebab utama kematian, dengan 1.4 juta kematian pada tahun 2021 sahaja. Dengan kadar kematian sebanyak 50% jika tidak dirawat, jangkitan TB aktif perlu didiagnos secepat mungkin. Setakat ini, dianggarkan bahawa satu perempat daripada populasi dunia dijangkiti dengan jangkitan TB laten (LTBI), yang menjadi reservoir MTB. Kaedah diagnostik semasa bagi LTBI, seperti Ujian Kulit Tuberkulin Mantoux (TST) dan Ujian Pelepasan Interferon Gamma (IGRA), mempunyai kelemahan: TST menunjukkan spesifisiti yang rendah, manakala IGRA adalah mahal untuk digunakan secara meluas terutamanya di kawasan jangkitan TB yang tinggi. Bermatlamat untuk mengurangkan kos kaedah diagnostik bagi jangkitan TB, kajian kebolehlaksanaan pada platform baru (iaitu, biokejuruteraan pembuatan nanobid biopoliester) menggunakan teknik IGRA telah dijalankan. Platform ini telah berjaya diuji untuk mendiagnosis jangkitan *M. bovis* pada lembu. Tiga antigen MTB yang sama seperti dalam IGRA (iaitu, ESAT-6, CFP-10, dan TB7.7) telah digunakan dalam platform nanobid biopoliester-tersalut-antigen MTB (BMTB). Kadar kepekatan optimum BMTB diuji menggunakan sampel manusia. Kepekatan optimum BMTB ditentukan pada 10 ng. Analisis perbandingan kepekatan IFN-γ antara BMTB dengan kit QuantiFERON (QFT), seperti yang disyorkan oleh pengilang, telah dijalankan. QFT menunjukkan prestasi lebih baik daripada BMTB dalam perbandingan kepekatan IFN-γ. Untuk meningkatkan lagi keupayaan pembezaan BMTB dalam tiga kumpulan sampel: TB pulmonari (PTB), TST positif (TSTp), dan TST negatif (TSTn), kepekatan dalam enam sitokin terpilih (iaitu, IFN-γ, IP-10, IL-2, TNF-α, CCL-3, CCL-11) dianalisis. IL-2 dan CCL-11 adalah sitokin individu yang menunjukkan prestasi terbaik dalam perbezaan antara PTB terhadap kumpulan TSTn, TSTp, dan individu sihat (TSTp dan TSTn), dengan mencapai sensitiviti dan spesifisiti melebihi 80%. Kombinasi sitokin terbaik ialah IFN-γ+IL-2 apabila PTB dibandingkan dengan kumpulan TSTn, TSTp, dan individu sihat (TSTn+TSTp). Kemudian, untuk meningkatkan lagi sensitiviti platform BMTB, pengenalpastian antigen tambahan yang mempunyai sifat imunogenik tinggi menggunakan pendekatan *in siliko* (imunoinformatik) dilakukan. Sifat ini dinilai berdasarkan enam kriteria epitop sel T (TCE): padanan dengan antigen MTB H37Rv, liputan merentasi 273 strain MTB, padanan dengan gen MTB yang terekspresi tinggi, epitop promiskus, liputan populasi, dan pertindihan dengan epitop sel B (BCE). Setiap TCE mempunyai skor kumulatif berdasarkan kesemua enam kriteria tersebut. Antigen-antigen MTB kemudiannya disenaraikan mengikut jumlah skor-skor TCE di dalamnya yang telah diperbetulkan. Antigen yang disenaraikan teratas kedudukannya dibandingkan dengan literatur untuk potensi mengagak diagnostiknya. Antara antigen yang disenarai pendek ialah antigen IGRA yang sedia ada (ESAT-6 dan CFP-10), antigen berpotensi yang baru dikenalpasti seperti protein-seakan-ESAT-6 (iaitu., EsxJ, EsxK, EsxM, EsxP, EsxW), keluarga Ag85 (iaitu Ag85A, Ag85B, dan Ag85C), dan lain-lain (contohnya: HspX, PfkB, dan Rv1733). Kesimpulannya, BMTB telah menunjukkan potensi sebagai platform diagnostik berkos rendah, walaupun sensitivitinya masih boleh ditingkatkan dengan menambah antigen MTB berimunogenik tinggi.

EXPERIMENTAL AND *IN SILICO* STUDIES ON BIOENGINEERED POLYESTER NANOBEADS FOR LATENT TUBERCULOSIS DIAGNOSIS

ABSTRACT

Mycobacterium tuberculosis (MTB) is the causative agent for tuberculosis (TB) and remains one of the top ten causes of mortality, with 1.4 million deaths in 2021 alone. With a 50% mortality rate if left untreated, active TB infection needs to be diagnosed as soon as possible. Currently, it is estimated that a quarter of the world's population is infected with latent TB infection (LTBI), that acts as an MTB reservoir. Current diagnostic methods for LTBI, such as the Mantoux Tuberculin Skin Test (TST) and the Interferon Gamma Release Assay (IGRA), have limitations: the former exhibits low specificity, while the latter is expensive to be widely use especially in high TB burden areas. Aiming to decrease the cost of TB infection diagnostic methods, a feasibility study on a new platform (i.e., bioengineered biopolyester nanobeads), using the IGRA technique was conducted. This platform was already successfully tested to diagnose *M. bovis*-infected cows. The same three MTB antigens (i.e., ESAT-6, CFP-10, and TB7.7) used in IGRA, was utilised to bioengineer the MTB-antigen-coated biopolyester nanobeads platform (BMTB). Optimisation of the BMTB concentration was performed using human samples. The optimised concentration of BMTB was determined to be 10 ng. A comparative analysis of IFN-γ levels between BMTB and QuantiFERON (QFT) kit, as recommended by the manufacturer, was performed. QFT demonstrated superior performance compared to BMTB in the IFN-γ release assays. In order to evaluate the discriminatory power of BMTB in three sample groups: pulmonary TB (PTB), TST positive (TSTp), and TST negative (TSTn), the concentrations of six selected cytokines (i.e., IFN-γ, IP-10, IL-2, TNF-α, CCL-3, CCL-11) in them were analysed. IL-2 and CCL-11 were the best-performing individual cytokines for distinguishing PTB against TSTp, TSTn, and healthy individual (TSTp and TSTn) group, achieving greater than 80% sensitivity and specificity. The best cytokine combination is IFN-γ+IL-2 when PTB is compared to TSTn, TSTp, and to healthy individual (i.e., TSTn + TSTp) groups. Then, to further improve the sensitivity of the BMTB platform, additional antigens that have high immunogenic properties were identified using *in silico* (immunoinformatics) approaches. These properties are based on six T cell epitope (TCE) criteria: matching with MTB H37Rv antigens, coverage across 273 MTB strains, association with highly expressed MTB genes, promiscuous epitopes, population coverage, and overlap with B cell epitopes (BCEs). Each TCE has a cumulative score of all six criteria. MTB antigens were then ranked based on their corrected total TCE scores within them. Top -ranked antigens were compared to literature for their inferred diagnostic potential. Among the shorlisted antigens are the existing IGRA antigens (ESAT-6 and CFP-10), newly identified potential antigens like ESAT-6-like proteins (i.e., EsxJ, EsxK, EsxM, EsxP, EsxW), the Ag85 family (i.e., Ag85A, Ag85B, and Ag85C), and several others (e.g., HspX, PfkB, and Rv1733). In conclusion, the BMTB showed potential as a low-cost LTBI diagnostic platform, although its sensitivity can be further enhanced by incorporating additional highly immunogenic MTB antigens.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Prior to the COVID-19 outbreak, *Mycobacterium tuberculosis* (MTB) was the leading causative agent for infectious disease. The World Health Organisation (WHO) estimated that a quarter of the human population was already infected with MTB (Houben and Dodd, 2016; WHO, 2023). The tuberculosis (TB) epidemic has been found to be larger than previous estimates suggested. In 2022, there were an estimated 7.5 million new TB cases worldwide, with men, women, and children accounting for 55%, 33%, and 12% of the cases, respectively (WHO, 2023). To date, TB is a major threat to global public health, second only to COVID-19, despite significant efforts to control it (Zellweger *et al.*, 2020).

Patients with HIV, those with silicosis, young children under the age of 5 years old who are in close contact with TB patients, prisoners, drug users, and people living in high-incidence endemic areas, including Africa and Southeast Asia, are more susceptible to getting TB (Matteelli *et al.*, 2018; Paton *et al.*, 2019; WHO, 2023). In 2022 alone, an estimated 1.13 million TB deaths occurred among HIV-negative people and 167,000 HIV-positive people (WHO, 2023). These figures are nearly equivalent to pre-pandemic statistics from 2019.

Latent TB infection (LTBI) represents an enormous reservoir of the disease with significant present and future implications as a source of new cases of TB. The current landscape of the disease is characterised by a lack of highly effective but affordable diagnostic methods, the increasing prevalence of multi-drug-resistant strains (MDR), inadequate therapeutic coverage, co-infection with HIV, and the absence of an effective vaccine (Fatima *et al.*, 2020; Letang *et al.*, 2020; Sotgiu *et al.*, 2019; WHO, 2023).

Rapid and accurate diagnosis has been the core of the international strategy to control TB, but existing diagnostic tests result in millions of undiagnosed and untreated cases. Despite significant advances in technology, the current diagnostic methods (e.g., sputum smear and culture) still in use were introduced over 100 years ago. The sensitivity of these methods is approximately 46% (Nasiri *et al.*, 2019), with lower sensitivity in patients co-infected with HIV. To control the TB epidemic, it is essential to ensure the availability of suitable diagnostic methods. Following the introduction of the international strategy for TB diagnosis and treatment, only a small proportion of the 7.5 million new TB cases each year receive a laboratory-confirmed diagnosis. Many are detected only after they have developed advanced stages and transmitted the infection to their close contacts (WHO, 2023).

Diagnostic test manufacturers have not developed a new diagnostic test targeting the needs of developing countries, where approximately 90% of all TB patients live (WHO, 2023). High-technology methods (e.g., GeneXpertMTB/RIF), that can detect TB with a lower bacterial load in clinical specimens, have been introduced in developed countries. However, these methods are challenging to implement in high-burden developing countries mainly because of their cost, that make their routine application infeasible (Matteelli *et al.*, 2018). Post-primary or reactivation of TB in latently infected individuals has been an important source of new TB cases. This is particularly relevant for individuals infected with HIV, as well as those with associated conditions and non-communicable diseases (e.g., diabetes mellitus), that are on the rise globally (Matteelli *et al.*, 2018; WHO, 2023). In developing countries, where these associations with TB pose a significant challenge to infection control, addressing them could be key to preventing the reversal of progress made in the fight against TB (Matteelli *et al.*, 2018; WHO, 2023).

As previously discussed, the diagnosis of LTBI is paramount for the control of TB. Following the diagnosis of LTBI, a preventive treatment course lasting up to 6 months with isoniazid or rifapentine can effectively reduce the risk of reactivation (Foreman *et al.*, 2020). Currently, there is no gold standard for the diagnosis of LTBI. Existing methods, i.e., the Tuberculin Skin Test (TST) and the Interferon Gamma Release Assay (IGRA), by indirectly detect immune responses against MTB antigens (Matteelli *et al.*, 2018). Due to the limitations of the TST, the use of IGRA, e.g., QuantiFERON (QFT), is widely accepted and has been recommended by the WHO, (2023) over the TST. The main drawback of this method is the high cost of the kit. Therefore, ways to reduce the cost were explored by applying this concept to a more affordable platform, i.e., biopolyester nano beads.

Alternatively, encouraging results have been observed with *M. bovis*-antigens-coated biopolyester nanobeads for diagnosing *M. bovis* infection in cows and in experimental TB vaccines. These findings suggest the potential for evaluating these nanobeads for the stimulation of whole blood for human TB diagnostics. MTB-antigen-coated biopolyester nanobeads (BMTB) represent a novel platform with potential as an alternative for TB infection diagnosis. This approach offers the potential for a feasible, cost-effective, and custom-made diagnostic method. It can be modified to adapt to different geographic regions within a short development period, depending on the needs. Other advantages include the ability to accommodate multiple antigens in the design, low production costs, the ability to be developed in a short time, and a long shelf life. These advantages make it an attractive technology for development as an alternative TB diagnostic method.

It has been demonstrated that antigens expressed in the biopolyester nanobeads elicited Type 1 (Th1) and 2 (Th2) T helper cell immune responses against different antigens and induced protection against experimental TB antigens (Gonzalez-Miro *et al.*, 2019; Parlane *et al.*, 2012, 2009). There are reports that MTB 85A and ESAT-6 antigens expressed on biopolyester nanobeads induce higher cellular responses than soluble antigens. Several antigens from the MTB, e.g., ESAT-6 (Rv3875), CFP-10 (Rv3874), and Rv3615c, have been expressed in biopolyester nanobeads produced in *Escherichia coli (E. coli)* (Gonzalez-Miro *et al.*, 2019; Parlane *et al.*, 2012, 2009). These biopolyester nanobeads were evaluated in comparison to the TST method in cattle, that yielded an excellent result (Chen *et al.*, 2014). Biopolyester nanobeads also have significant potential for improvement through the addition of new antigens that have not yet been utilised. This can be achieved by selecting or shortlisting potential antigens using multiple methods, e.g., literature, database-searching, and immunoinformatics.

Overall, biopolyester nanobead technology offers the opportunity to quickly develop a custom-made diagnostic reagent using bioengineered *E. coli* to coat selected antigens on the surface of biopolyester nanobeads (Rehm, 2010). Besides the short product development timeline and the ability to accommodate multiple antigen in the design, the biopolyester nanobeads could be cost-effectively produced. The product has been shown to be effective in TB vaccination experimental models and in field evaluations for TB infection detection in cattle (Arif *et al.*, 2021; Karimah and Pambudi, 2020; Rehm, 2010). Evaluating biopolyester nanobeads coated with MTB antigens for MTB infection in humans is a promising strategy for developing cheaper and more effective IGRA methods for the detection and diagnosis of MTB.

1.2 Problem Statement

The current diagnostic methods for LTBI cannot effectively discriminate between active TB patients, LTBI, and non-MTB infections at a lower cost. The need for improved diagnostic methods to accurately discriminate between TB infection stages is undeniable. Developing cheaper and better diagnostic methods is one of the crucial strategies to fight the infection. However, current IGRA diagnostic techniques, such as QFT, are quite expensive, particularly for high TB burden areas, which are predominantly in developing countries. This limits their usage, which may contribute to TB's further spread, and remains a major global health concern. This could result in inappropriate disease management and treatment. By investigating the potential use of MTB-antigen-coated biopolyester nanobeads (BMTB) as a novel TB diagnostic approach, this study aims to address the high cost of current IGRA techniques. The study aim to develop a lower-cost and more effective platform for differentiating TB infection among PTB, TSTp, and TSTn groups by optimising the loading capacity of these nanobeads and examining both individual and combined cytokine responses.

Due to the feasibility of the BMTB platform, which allows the incorporation of multiple antigens at a consistent cost, it presents a valuable opportunity to add or modify antigens to improve performance. Thus, in hope to improve the sensitivity of the BMTB methods by assessing additional antigens based on their T cell epitopes (TCEs) and their specific immunogenic criteria. Through immunoinformatics, it hope to the shortlist candidate antigens or epitopes, as an effort to find synergistic complementary antigens to be incorporate into BMTB in the future. By enhancing TB diagnostics, this research can hopefully contribute to the improvement of the TB diagnostic methods.

1.3 Research Questions

a) How effective is the performance of biopolyester nanobeads coated with MTB-specific antigens to diagnose TB infection?

b) Can the BMTB be used to effectively discriminate between PTB, TSTp, and TSTn groups as an alternative of QFT?

c) Which potential cytokines can be utilised to effectively discriminate between different stages of TB infection?

d) Can a combination of cytokines improve the ability to discriminate between different stages of TB infection?

e) Using immunoinformatics techniques, are there additional suitable epitopes or antigens that can be used to improve the sensitivity of BMTB diagnostic platform?

1.4 Theoretical framework

This study adapted the versatility of biopolyester nanobeads coated with *M. bovis* antigens, a technology that is already established (patent no: WO 2011/013097 A2) and owned by PolyBatics Ltd, where Prof. Bernd Rehm (the collaborator in this project) serves as both a shareholder and the Chief Scientific Officer (Parlane *et al.*, 2014). In the referenced study, the biopolyester nanobeads were used as a delivery platform for *M. bovis*-specific antigens in a bovine revaccination strategy against bovine TB (Parlane *et al.,* 2014). In parallel, the group also conducted research on utilising the biopolyester platform for bovine TB diagnostics. This work, reported by Chen *et al.*, (2014) and Parlane *et al.*, (2016), demonstrated that a combination of ESAT-6, CFP-10, Rv3615c, and Rv3020c antigens could be used to develop a new test for diagnosing bovine TB.

Given the successful application of biopolyester nanobeads in bovine TB vaccination and diagnostics, it is theoretically feasible to adapt this approach for diagnosing human TB. This represents a novel application in humans, and the use of different antigens to detect different TB strains in different subjects poses challenges, particularly in detecting LTBI. In this study, the platform is benchmarked against a commercial LTBI diagnostic kit, the QuantiFERON® test.

In addition, several studies have suggested the use of other individual cytokines (e.g., IL-2, TNF-α, IP-10, CCL-3, and CCL-1) and their combinations as biomarkers for discriminating human TB infections (Alvarez, 2021; Chendi *et al.*, 2021; Kumar *et al.*, 2021a; Sudbury *et al.*, 2020; Zhuang *et al.*, 2024). The screening of these biomarkers is conducted using the Luminex Multiplex platform. To improve the sensitivity and specificity of the new diagnostic platform, additional antigens were shortlisted using bioinformatics approaches, which facilitated the identification of the most promising candidates for incorporation into the biopolyester nanobead platform (Nguyen *et al.*,2014; Zvi *et al.*, 2008).

1.5 Research Hypothesis

MTB-antigen-coated biopolyester nanobeads (BMTB) can be optimised as a potential novel diagnostic platform to discriminate between different stages of TB infection i.e., PTB, TSTp and TSTn, by evaluating individual cytokine responses or selected cytokine combinations. While this study focused on currently available antigens, the integration of immunoinformatically shortlisted MTB antigens may further enhance the diagnostic performance of the BMTB platform in future applications, particularly for improved detection of latent TB infection (LTBI).

1.6 Research Aim and Objectives

To evaluate optimal experimental setups for the BMTB, compare its performance to the existing QFT, and further elucidate cytokine profiles for active TB, LTBI, and non-MTB groups as a viable alternative to current diagnostic methods for discriminating TB infection among the three groups. As the BMTB platform is versatile and allows the incorporation of multiple additional antigens without increasing cost, a bioinformatics analysis was conducted to shortlist potential MTB antigens for future BMTB application.

1.6.1 Specific Objectives

a) To optimise the amount of loaded biopolyester nanobeads for discrimination of false positive detection of TB infection by plain biopolyester nanobeads.

b) To compare, the discriminitive power of BMTB and QFT, using IFN-γ, for differentiation of TB infections in TSTn, TSTp, or PTB stages.

c) To analyse the responses of other cytokines individually i.e., IL-2, TNF-α, IP-10, CCL-3 and CCL-11, and their combinations, which could serve as better alternatives to IFN-γ for improving discrimination of TB infections in these three groups.

d) To screening for potential antigens using immunoinformatics approaches to enhance the currently used antigens, i.e., ESAT-6, CFP-10 and TB7.7.

1.7 Research Framework

This section provides a comprehensive summary and scope of the research conducted in this thesis. The research began with laboratory based approach, which involved a feasibility study of the BMTB platform, adapting the concept of IGRA for use as a TB diagnostic method (refer to Figure 1.1). This phase aimed to explore alternatives to the high costs of commercially available IGRA tests by comparing the performance of the BMTB platform with the QFT. Additionally, further assessments were conducted to evaluate the potential of individual cytokines or cytokine combinations as biomarkers to improve the differentiation of TB stages. This approach includes Subchapters 2.1 to 2.4 for the literature review, Subchapters 3.1 to 3.5 for materials and methods, and Subchapters 4.1 to 4.5 for results and discussion.

Immunoinformatics approach was conducted following the feasibility study on the BMTB platform. This phase aimed to find potential antigens to further increase BMTB diagnostic method sensitivity by shortlisting potential MTB antigens using immunoinformatic techniques. This includes Subchapters 2.5 for the literature review, Subchapters 3.6 for materials and methods, and Subchapters 4.6 for results and discussion.

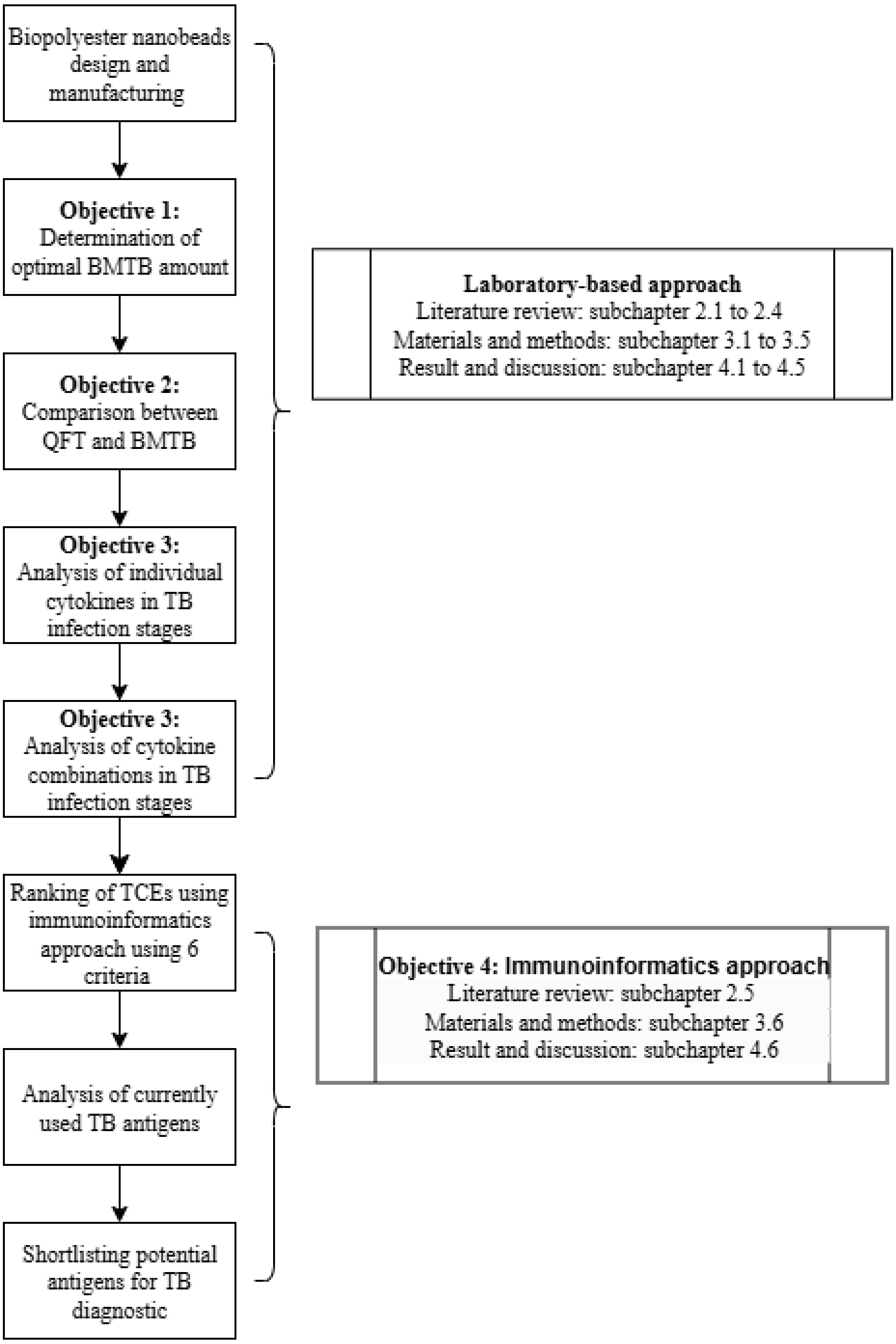

Figure 1.1: The research framework and flowchart of the research.

CHAPTER 2

LITERATURE REVIEW

2.1 Tuberculosis (TB)

2.1.1 Pathogenesis

TB is one of the leading global health threats and remains one of the top ten causes of mortality from infectious diseases. Prior to the emergence of COVID-19, TB posed a major global public health threat and was among the leading causes of death from infectious diseases (Aguilera *et al.*, 2020; WHO, 2023). The WHO report estimated that 1.3 million people worldwide died from TB in 2022, with a 50% mortality rate among those with untreated active TB. TB prevalence varies between regions, and accurate numbers are difficult to obtain. However, the 5% probability of LTBI progressing into active TB suggests that millions could develop the disease (Houben and Dodd, 2016; WHO, 2023).

The decrease in newly diagnosed active TB cases from 2020 to 2021 was due to the increase in undiagnosed and untreated cases, resulting from the significant focus placed on managing the COVID-19 pandemic. The reallocation of resources towards COVID-19 diagnosis and treatment contributed to this trend (WHO, 2023). However, WHO (2023) suggested the trend has rebounded to pre-COVID-19 levels due to improving conditions of healthcare availability in the post-pandemic period. The report also highlighted the rise of drug-resistant (DR), multiple drug-resistant (MDR), and rifampicin-resistant (RR) TB cases, with 3.1 million cases reported compared to 10.6 million new TB cases overall. Along with the growing non-resistant TB strain, DR-TB has been increasing for 2022, i.e., 450,000 newly reported RR-TB cases.

TB is a chronic infectious disease caused by MTB. It was first discovered by Robert Koch in 1882 (Sakula, 1982; WHO, 2023). The mechanisms of TB pathogenesis are complex, involving a dynamic interplay between the host immune system and the bacterium's survival strategies (refer to Figure 2.1) (Prasanna *et al.*, 2025; Zhou *et al.*, 2022). The pathogenesis of TB involves intricate interactions between MTB and the host immune system. During infection, MTB enters the body through inhalation and infects alveolar macrophages in the lungs, resulting in the formation of granulomas (Kumar, 2021). These infected macrophages play a crucial role in controlling the infection during the early phases, although the mechanisms determining whether the infection progresses to active disease or remains latent are not fully understood (Peng *et al.*, 2022).

Although only a small percentage (i.e., approximately 5% to 10%) of individuals with LTBI actually develop active TB during their lifetime, addressing this issue is a crucial aspect to eradicate TB completely (Tang *et al.*, 2022; Yuan *et al.*, 2023). Individuals with LTBI, which remains asymptomatic and non-infectious, are sustained by the robust host immune response that inhibits the replication of the bacteria and prevents the spread of the infection (Sakula, 1982).

This emphasises the significance of studying the immune responses of individuals with LTBI in comparison to those with active or no TB infection. Understanding the disparities in immune responses between these two conditions is essential to create effective new TB diagnostic biomarkers and tools.

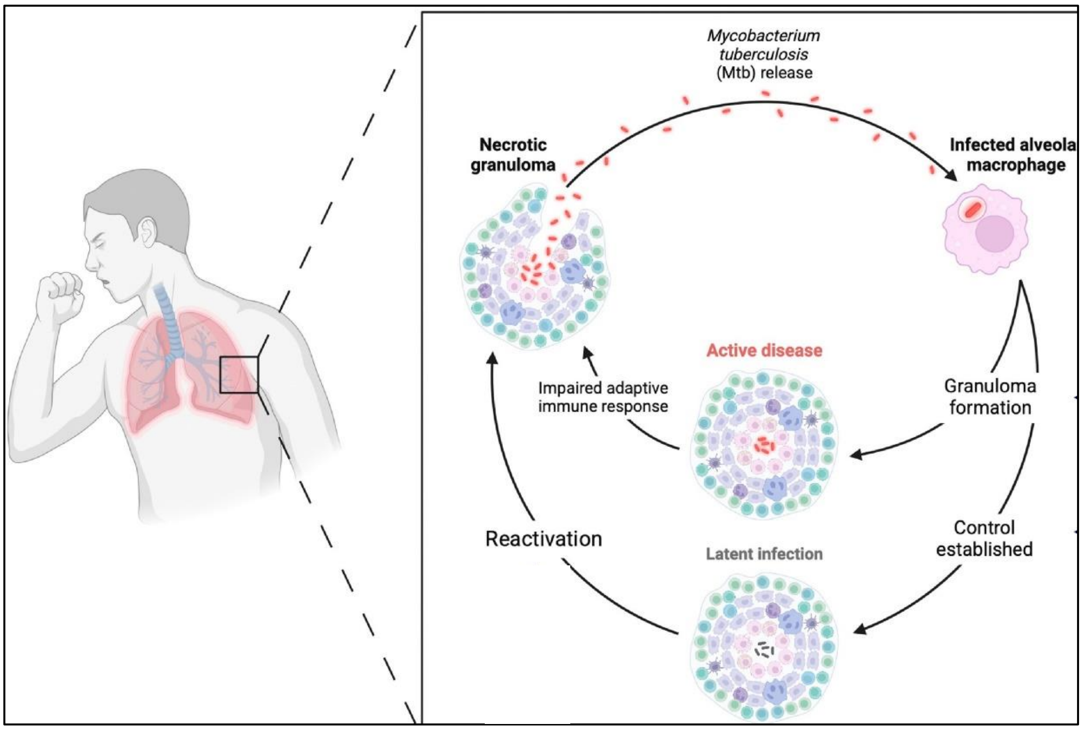

Figure 2.1:. The pathogenesis of tuberculosis (TB) in humans. The intricate interaction between MTB and the host immune system leads to the formation of granulomas, which may result in either active TB or latent TB infection (LTBI). This figure is adapted from (Prasanna *et al.*, 2025).

2.1.2 The Efficacy of Bacille Calmette-Guérin (BCG) Vaccine and Current Therapeutics

The BCG vaccine was named after its developers, Albert Calmette and Camille Guérin, and has been used for decades as a preventative measure against TB (Bourel, 2024; Calmette *et al.*, 1926). Its effectiveness has been widely studied, and while BCG vaccination may not prevent all cases of TB, it has been shown to be effective against severe forms of the disease in children. Some studies have shown the BCG vaccine generally provides only around 50% efficacy in preventing TB and offers limited protection against adult pulmonary TB (Jaffet *et al.*, 2022; Y. Li *et al.*, 2022). Despite the effectiveness of the BCG vaccine in preventing severe forms of TB, there is a need for more effective vaccines, particularly for adult populations who are at a higher risk of developing active TB.

Numerous efforts have been undertaken to develop new or improved TB vaccines, including those utilising multi-epitopes and recombinant proteins (Jiang *et al.*, 2023; Ohara *et al.*, 2018). Recent studies have tried to address the importance of triggering a broad range of immune cells (e.g., $CD4^+$ and $CD8^+$ T cells, B cells, and NK cells), aiming to provide lifetime protection against TB (Zhou and Zhang, 2023). BCG was proven to primarily trigger $CD4^+$ cells, but limited research has been done on its effect on $CD8^+$ cells, where its role remains unclear. The immunity provided by $CD4^+$ cells triggered by the BCG vaccine, as explained by Zhou and Zhang (2023), can be characterised into four categories: a) central memory T cells (T_{CM}), which are predominantly located in the lymphoid organs and maintain a high capacity for proliferation; b) upon subsequent exposure to antigens, T effector memory cells (T_{EM}) form from T_{CM} cells and are mainly located in the circulation and peripheral sites; c) tissue-resident memory cells (T_{RM}), where a portion of T_{EM} cells persists in the lungs; and d) newly recruited T effector cells (T_{EFF}), which arrive following infection.

The mortality rate for untreated active TB is 50% (WHO, 2022). The current recommended treatments, as outlined in the report, include rifampicin (R), isoniazid (H), ethambutol (E), and pyrazinamide (Z) in a 4-6 month regimen, which has an 85% success rate. A recent recommendation has been made to include rifapentine (P) and moxifloxacin (M) for those over 12 years old. The report also emphasised that the treatment success rate for rifampicin-resistant (RR) or multidrug-resistant (MDR) TB is significantly lower, at only around 60%. Preventive treatment also involves the use of the same medications but in different regimens (refer to Table 2.1). The medication regimens for active TB are notably longer and more complex compared to the preventive treatment regimens. It is even more complex for RR, MDR, or extremely drug-resistant (XDR) TB strains (WHO, 2022). This underscores the urgent need for improved diagnostic tools, especially for LTBI, to utilise the preventive treatment options, as all of the medications have their own side effects (e.g., unexplained diminished appetite, nausea, persistent burnings, limb numbness, fatigue, and change of vision) depending on the regimens usage (CDC, 2016). An effective LTBI diagnosis is important, as a shorter preventive medication regimen reduces medication side effects and patient non-compliance.

MDR-TB is defined as a strain that is resistant to rifampicin and isoniazid, and XDR-TB is defined as a strain that is resistant to rifampicin, fluoroquinolone, and either bedaquiline or linezolid. Both strains pose significant challenges to the effectiveness of the current treatment regimens. These resistant strains can arise from many factors, primarily driven by the misuse or inappropriate use of TB drugs, including incomplete treatment courses, medication non-compliance, and incorrect dosages. In order to effectively combat DR-TB, it is crucial to prioritise the appropriate use of TB drugs, improve treatment compliance, and develop new drugs that are effective against resistant strains (Linderman and Kirschner, 2015). The treatments recommended by WHO (2023) are as follows:

Table 2.1: Standard drug regimens are used to treat active TB patients for both pulmonary and extrapulmonary LTBI. This list does not include those with MDR or XDR-TB strains. The acronyme used reflected the name of the drugs and its treatment period e.g. Isoniazid (H), rifapentine (P), pyrazinamide (Z), mozifloxacin (M) for 4 omnths as 4HPZM.

Treatment options	Active TB treatment regimen	LTBI regimen
1	Isoniazid, rifapentine, pyrazinamide, mozifloxacin (for patients 12 years old or older) (4HPZM) for 4 months.	Isoniazid and pyrazinamide (1HZ-daily dose) for a month.
2	2 months of isoniazid, rifampicin, and pyrazinamide (or replacing pyrazinamide with ethambutol), followed by isoniazid and rifampicin for another 2 months. (for patients 3 months to 16 years old with no drug resistant strains or severe TB) (2HRZ & 2HR).	Isoniazid and pyrazinamide for 3 months long (3HZ-weekly dose).
3	All 4 drugs (isoniazid, rifampicin, ethambutol, and pyrazinamide) in 4 months, followed by isoniazid and rifampicin for another 2 months or more (4HREZ & 2HR).	Isoniazid and rifampicin for 3 months long (3HR-daily dose).
4		Rifampicin (4R-daily dose) for 4 months long.
5		Isoniazid for 6 months or more (6H-daily dose).

Currently, the progression of resistance is faster than the success of developing new effective medication. However, researchers have made efforts to combine routine TB medications with cycloserine in MDR-TB patients, which has shown good prognosis when administered early (Zhao *et al.*, 2021).

In the post-COVID-19 era, WHO, (2023) predicted the rise of TB incidences starting from 2025. Effective and correct treatment, especially for those with RR, MDR, and XDR-TB, is crucial to prevent further spread of TB and reduce the burden of the disease. Based on a study by Zürcher *et al.* (2021), 12% of tuberculosis (TB) patients died during treatment due to various factors, including the use of inappropriate antimicrobial therapies in 15% of cases, which significantly contributed to poor treatment outcomes. Effective TB diagnosis is essential for reducing the MTB reservoir by enabling the prescription of preventive treatments and providing faster interventions for active TB, thereby reducing bacterial spread.

2.1.3 Factors Related to TB Infection and Severity

Factors related to the severity and infection of TB include the individual's immune response, host genetics, bacterial strain virulence, environmental factors, and comorbidities. Understanding the complexity of these factors and their interplay is crucial for developing targeted interventions and strategies to effectively control TB and improve patient outcomes.

Tobacco smoking (including second-hand smokers) and alcohol consumption, which are often contemporaneous, have also been identified as risk factors for developing severe TB and exacerbating the disease progression (Burusie *et al.*, 2020; Kasim, 2022; Komolafe and Pacurari, 2022). Smokers also increase the risk of getting TB, as the habit has negative effects on the lungs, increases blood pressure, and weakens the immune system (Kasim, 2022; Thapa *et al.*, 2021). Moreover, Burusie *et al.* (2020) and Kasim (2022) suggest both habits can contribute to the development of MDR-TB strains. Excessive alcohol consumption is also recognised as a risk factor for early illness and death, although some argue that there is no safe level of alcohol consumption (Park and Kim, 2020). Park and Kim (2020) also stated that alcohol contributes 19.6% (approximately 250,000) to TB deaths globally. Africa and South-East Asia have the highest rates of alcohol-attributable deaths from infectious diseases, including TB, accounting for 33% and 14% of such deaths in these regions, respectively. This is due to the fact that alcohol impairs liver functions, lowers immune responses, and alters the pharmacokinetics of the drug used in TB treatments (Fairbairn *et al.*, 2016; Singh *et al.*, 2017).

Research has highlighted that immunocompromised individuals (e.g., those with HIV infection) or immunosuppressed patients, i.e., those undergoing chemotherapy or organ transplant recipients, are more susceptible to severe TB infections (Berra *et al.*, 2022; Namuganga *et al.*, 2021). TB has a significant impact on HIV patients by delaying the recovery of $CD4^+$ T cells, which accelerates the progression from HIV to AIDS.

Moreover, individuals with comorbidity or chronic illness (e.g., diabetes mellitus, COVID-19, renal disease) also have a higher risk of contracting TB due to their lower immunity (Tang *et al.*, 2022). Patients with diabetes are at two to three times greater risk of contracting TB compared to individuals without diabetes, and standard TB treatments may be less effective for them (Thapa *et al.*, 2021). Although the mechanism of COVID-19 co-infection with TB remains unclear, Thapa *et al.* (2021) suggested that the systemic inflammatory response, e.g., interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-10 (IL-10), interferon gamma-induced protein10 (IP-10), and tumour necrosis factor alpha (TNF-α), can trigger the activation of TB. In some cases, steroids, e.g., prednisolone or corticosteroid, may be used to reduce cytokine storms, but their use should be carefully considered and monitored due to the potential risk of reactivating LTBI (Rahman *et al.*, 2022).

Another factor influencing immunity is age. Older individuals and children under the age of five, who are still developing their immune systems, tend to have weaker immune responses and are more susceptible to TB (Ma *et al.*, 2022). This effect is particularly significant for older adults, who already experience a decline in physical strength. Active TB infection can further reduce their quality of life if not properly treated (Sofiana and Nugraheni, 2022).

Access to preventive TB-BCG vaccination and healthcare also plays a huge role in the severity and outcomes of TB infections (Iyer *et al.*, 2020; Osei-Yeboah *et al.*, 2021). Despite its limitations in preventing adult pulmonary TB infection, the BCG vaccine is effective in preventing TB, particularly in children. Regular screening, especially for high-risk individuals (e.g., exposed to high-prevalence TB, people living with HIV (PLWHIV), or pregnant mothers), can have preventive treatment in a timely manner, resulting in a better prognosis (Jose-Raul *et al.*, 2023; Rahman *et al.*, 2022). Limited access to healthcare (i.e., due to geographical location, confinement, and cost) can delay the diagnosis and treatment, and poor living conditions contribute to the spread of TB (Y. Li *et al.*, 2022). This was evident during the COVID-19 pandemic, where access to healthcare for routine screening and TB diagnosis was limited, which contributed to further local transmission and delays in treatment (Rahman *et al.*, 2022). Even individuals with a normal immune system can be susceptible to sepsis from undiagnosed and untreated widespread TB, highlighting the importance of early detection and proper healthcare access for all individuals (Jaffet *et al.*, 2022; Jose-Raul *et al.*, 2023).

Exposure to environmental factors and poor hygiene practices, such as overcrowding and inadequate ventilation (e.g., in prison environments), as well as contact with TB patients (e.g., in hospital settings, living with TB patients, or in areas with high TB prevalence) and exposure to silica dust, contribute to the transmission and severity of TB infections (Li *et al.*, 2022b). Overcrowding, which can reduce hygiene standards, may lead to resistance to pathogens, including MTB (Al-Tawfiq and Memish, 2021). Low humidity due to climate change (e.g., drought) is also a factor contributing to antibiotic-resistant TB strains (Burnham, 2021).

Poverty is one of the directly related factors contributing to the increase in TB cases and worsening the TB mortality rate among TB patients (WHO, 2023). Munisankar *et al.* (2022) observed that individuals living in impoverished conditions are more likely to suffer from malnutrition, which affects approximately a quarter of the global population. This malnutrition weakens the immune system and increases susceptibility to severe forms of TB. Undernutrition, particularly protein deficiency, is a direct consequence of poverty and contributes to increased susceptibility to severe forms of TB infection (Baek *et al.*, 2019). A low body mass index (i.e., less than 18.5 kg/m^2) can increase the risk of contracting a TB infection up to 10-fold. Malnutrition, particularly undernutrition, can lead to metabolic issues (e.g., a predisposition to obesity, diabetes mellitus, and high blood pressure), which indirectly contribute to the development and progression of TB infections (Munisankar *et al.*, 2022).

Understanding the risk factors associated with the transmission and severity of TB, such as exposure to environmental factors, poor hygiene practices, and poverty, is crucial in implementing effective strategies for preventing and managing the disease.

2.2 Active and Latent Tuberculosis Diagnostic Methods

2.2.1 Active TB Infection Diagnostic Methods

Active TB infection is a condition where the patient commonly shows the respiratory symptoms of TB, e.g., prolonged cough with or without blood in the sputum, fever, loss of appetite, and weight loss (Thapa *et al.*, 2021). LTBI occurs when an individual is infected with the TB bacteria but does not exhibit any symptoms. There are a few different diagnostic methods available to detect active TB and LTBI.

The differences in the diagnostic methods are due to the aim of the methods; active TB diagnosis is to treat the infection and reduce the spread, but LTBI is often targeted for preventive measures to decrease the likelihood of advancing to an active state of the disease as the latent dissemination acts as a reservoir for the pathogen (Al-Zamali *et al.*, 2022). Currently, no viable method exists to discriminate between active TB, LTBI, and non-infected individuals at a low cost (Korma *et al.*, 2020b). Thus, both TB categories need to be diagnosed separately using different methods depending on various factors, such as the individual's symptoms, risk factors, and availability of resources (Alnakli *et al.*, 2022; Camacho *et al.*, 2020; Donkeng-Donfack *et al.*, 2022; Zhao *et al.*, 2021).

Diagnosis methods for active TB infection are sputum smears, bacterial culture, chest X-rays, CT scans, and molecular techniques such as polymerase chain reaction (GeneXpertMTB) and TB loop-mediated isothermal amplification (LAMP) (Cheng *et al.*, 2022; Donkeng-Donfack *et al.*, 2022; Lu *et al.*, 2021; Zhao *et al.*, 2021). Given MTB's ability to infect various parts of the body, including the lymph nodes and eyes, a comprehensive diagnostic approach is essential despite the obvious symptoms mentioned above (Cheng and Chen, 2021; Daniel *et al.*, 2022; Ludi *et al.*, 2023).

Although all of the diagnostic techniques mentioned are valuable, each has its shortcomings. Sputum smears and chest x-rays have limited sensitivity, particularly in extrapulmonary and miliary TB, and sometimes do not provide a conclusive result (Donkeng-Donfack *et al.*, 2022). Donkeng-Donfack *et al.* (2022) also highlighted the loop-mediated isothermal amplification (LAMP) technique, which reduces the waiting time by more than 70% when compared to the GeneXpertMTB method following the negative sputum smear, but its accuracy still needs to be improved. GeneXpert MTB is a nucleic acid amplification technique that uses polymerase chain reaction (PCR) to analyse sputum samples (CDC, 2024). GeneXpertMTB is a highly sensitive rapid molecular technique, but it is expensive for deployment in resource-limited settings, which require significant laboratory infrastructure and trained personnels.

2.2.2 Latent TB Infection Diagnostic Methods

Current practices for detecting LTBI include the use of the more than century-old Mantoux Tuberculin Skin Test (TST) and Interferon Gamma Release Assay (IGRA), such as QuantiFeron Gold-In-Tube (QFT-IT; Cellestis/Qiagen, CA, USA) and the PBMC/ELISPOT-based T-SPOT®.TB test (Oxford Immunotec, Abingdon, UK) (Park *et al.*, 2022). Currently, there is no gold standard for determining LTBI. Both methods can detect immune responses (i.e., memory T cells) that reflect previous MTB infection, with surrogate markers suggesting LTBI. However, IGRA is considered superior to TST and has been recommended by WHO (2023).

The TST is performed by injecting 0.1 mL of tuberculin-purified protein derivative (PPD) intradermally (i.e., usually on the forearm inner surface) (CDC, 2020; Pollock *et al.*, 2013). The induration reaction is measured within 48 to 72 hours after injection. Induration is caused by the processing of tuberculin antigens by antigen-presenting cells (APC) in the skin and their interaction with T cells, a delayed-type memory response occurs at the injection site. This indicates that the host's immune system has been sensitised to the injected antigens prior to this occurrence. The skin reaction is due to the presence of sensitised T cells and the release of signals (i.e., lymphokines), leading to induration through vasodilation, swelling, and the recruitment of more pro-inflammatory cells and cytokines in that area. However, the sensitised reaction will happen (i.e., indicate a positive result) whether an individual currently has TB, was previously exposed to MTB, or has received Bacillus Calmette-Guérin (BCG) (Pollock *et al.*, 2013).

Despite its many drawbacks, this method continues to be used due to the lack of a better low-cost alternative. It remains cost-effective and useful to screen populations for TB infection. The primary drawback of this technique is its variable specificity, which ranges from 40 to 60% (Lu *et al.*, 2022). Other limitations of this technique include the need to visit the clinic twice, the subjective interpretations of results, and its potential for false positive or false negative results (Ludi *et al.*, 2023; Madan *et al.*, 2021). It is important to note that the TST result can be affected by BCG, which is widely used in many countries. This may lead to a false positive result due to the similarity between the TB-causing pathogen and the protein derivatives used in the test, as well as the test's inability to distinguish between LTBI and active TB infection in some cases (Asemahagn *et al.*, 2020; Sorsa, 2020).

The blood test, which measures the release of interferon-gamma (IGRA) in response to selected specific MTB antigens, is an alternative to the TST. Interferon gamma (IFN-γ) is a cytokine released by T cells (Pollock *et al.*, 2013). The antigens used by T.SPOT.TB (Oxford Immunotech, UK) and QuantiFeron Gold-In-Tube (Qiagen, US) are 10 kDa culture filtrate antigen (CFP-10/Rv3874), 6 kDa early secretory antigenic target (ESAT-6/Rv3874), and an additional possible PhiRv2 prophage protein (TB7.7/Rv2654c) in the QuantiFERON kit (Oxford Immunotech,US). The mechanism of IGRA (Pollock *et al.,* 2013), is illustrated in Figure 2.2. The APCs (e.g., macrophages, dendritic cells) in the blood samples will process the MTB antigens, and present them to the specific T cells. The reaction causes the sensitised specific MTB-activated T cells to produce IFN-γ which acts as signalling between cells, thus producing a higher concentration of IFN-γ (refer to Figure 2.2) (Pollock *et al.,* 2013).

The IGRA technique is currently one of the best diagnostic methods and is recommended by the WHO. The result can be obtained within 24 hours, compared to the delayed reaction time of the TST. Examples of this technique include quantitative methods (e.g., QuantiFERON) and qualitative methods (e.g., T-SPOT.TB). Although it has limitations, IGRA remains widely used. A major drawback of these methods is that they have a significantly higher cost than TST, especially considering that the majority of TB cases are found in developing countries.

Therefore, there is an urgent need to develop affordable, accessible, and accurate diagnostic tools for both active TB infection and LTBI at the point of care. In conclusion, the study of immune responses in individuals with LTBI compared to those with active or no TB infection is crucial for developing effective diagnostic tools and treatment strategies for TB.

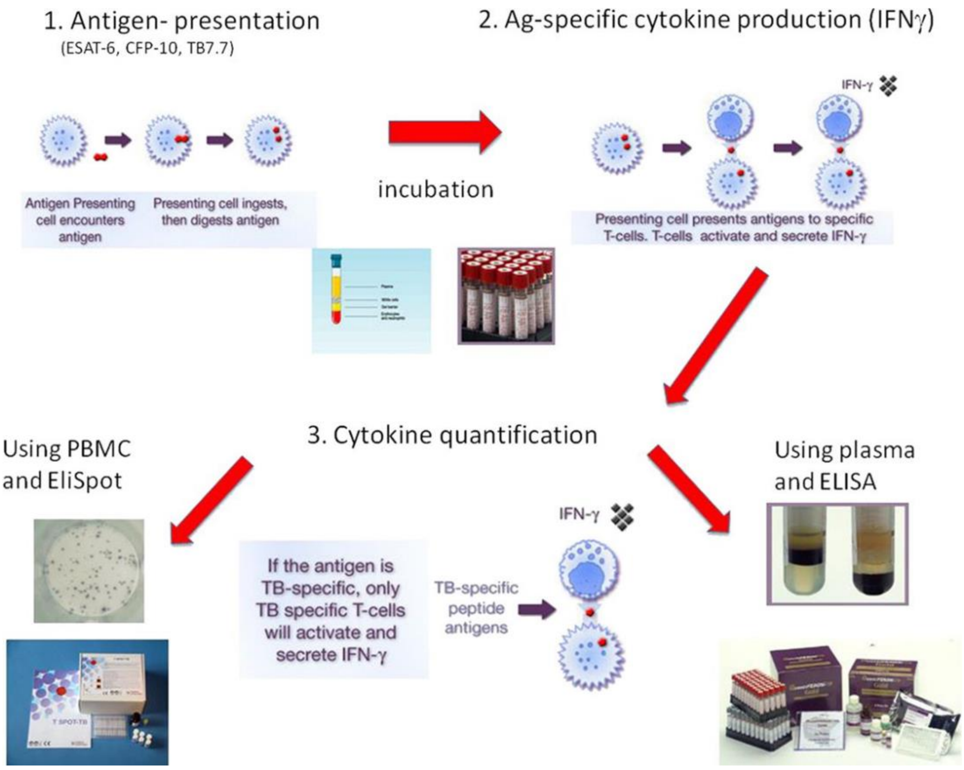

Figure 2.2: The procedure and process involved in the QuantiFERON assay. This figure is adapted from Pollock *et al.* (2013).