DETECTION OF HUMORAL IMMUNE RESPONSE IN MICE IMMUNISED WITH PURIFIED PROTEIN OF MILK EXPRESSING SELECTED MULTI-TUBERCULOSIS EPITOPES

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DETECTION OF HUMORAL IMMUNE RESPONSE IN MICE IMMUNISED WITH PURIFIED PROTEIN OF MILK EXPRESSING SELECTED MULTI-TUBERCULOSIS EPITOPES

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Bachelor of Health Science (Honours)

(Biomedicine)

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CERTIFICATE

This is to certify that the dissertation entitled “Detection of Humoral Immune Response in Mice Immunised with Purified Protein of Milk Expressing Selected Multiple Tuberculosis Epitopes” is bona fide record of research work done by Miss. Dharshiga A/P Ravi from August 2024 to January 2025 under my supervision. I have read this dissertation and in my opinion, it confirms to be acceptable standards of society presentation and is fully adequate, in scope and quality, as a dissertation to be submitted in partial fulfilment for the degree of Bachelor of Health Science (Honours) (Biomedicine).

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……..……

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research, and promotional purposes.

…….

(DHARSHIGA A/P RAVI)

Date: 27 January 2025

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"காலத்தி னாற்செய்த நன்றி சிறிதெனினும்

ஞாலத்தின் மாணப் பெரிது"

A timely help, though small it may appear, is in fact greater than the world itself in its true value. This profound truth from Thirukkural eloquently captures the essence of gratitude for the timely guidance and support received during my academic journey, making it particularly fitting for a thesis acknowledgement.

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	Degree Celsius
%	Percentage
μL	Microliter
mL	Milliliter
μg/mL	Microgram per milliliter
Acr	Alpha crystallin protein
Ag85B	Antigen 85B protein
APCS	Antigen-presenting cells
BCG	Bacillus Calmette-Guérin
BAL	Bronchoalveolar lavage
DOTS	Directly Observed Therapy, Short-course
DR-TB	Drug-resistant tuberculosis
ELISA	Enzyme-Linked Immunosorbent Assay
XDR	Extensively drug-resistant
HRP	Horseradish peroxidase
HIV	Human immunodeficiency virus
IRIS	Immune reconstitution inflammatory syndrome
IgA	Immunoglobulin A
IgG	Immunoglobulin G

IFN-γ	Interferon-gamma
IL-17	Interleukin-17
INH	Isoniazid
LTBI	Latent TB infection
MHC	Major Histocompatibility Complex
MDR	Multidrug-resistant
MTB	*Mycobacterium tuberculosis*
OD	Optical density
PBS	Phosphate buffer saline
RpfE	Resuscitation-promoting factor E
SIgA	Secretory Immunoglobulin A
H2SO4	Sulphuric acid
TMB	3,3’,5,5’- tetramethylbenzidine
Th	T-helper
TB	Tuberculosis
TNF-α	Tumor necrosis factor-alpha
WHO	World Health Organization

PENGESANAN GERAK BALAS IMUN HUMORAL DALAM MENCIT YANG DIIMUNISASI DENGAN PROTEIN TULEN SUSU YANG MENGEKSPRESKAN EPITOP-EPITOP TUBERKULOSIS TERPILIH

ABSTRAK

Walaupun terdapat pelbagai usaha global untuk mengurangkan jangkitan yang disebabkan oleh *Mycobacterium tuberculosis* serta pelaksanaan program imunisasi, tuberkulosis (TB) kekal sebagai salah satu penyebab utama kematian di seluruh dunia. Para penyelidik telah berusaha mencari strategi pencegahan alternatif untuk mengurangkan insiden TB dan menangani kelemahan kaedah vaksinasi Bacillus Calmette-Guérin (BCG) yang sedia ada. Salah satu kelemahan utama vaksin BCG adalah ketidakmampuannya untuk memberikan imuniti mukosa yang mencukupi, yang menekankan keperluan mendesak untuk pembangunan vaksin mukosa yang lebih berkesan dalam mencegah jangkitan invasif, terutamanya kerana MTB menjangkiti sistem pernafasan. Pasukan penyelidikan kami telah membangunkan vaksin mukosa dengan menggunakan epitope TB tertentu, iaitu Ag85B, Acr, dan RpfE. Vaksin ini telah diberikan melalui proses transfeksi kelenjar susu dari seekor kambing hamil, dan susu yang dihasilkan telah dikumpulkan setiap hari selama 21 hari sebelum dimurnikan. Objektif kajian ini adalah untuk menilai keberkesanan protein susu yang dimurnikan dalam merangsang tindak balas imun humoral di dalam serum dan sistem mukosa tikus Balb/C yang menerima calon vaksin tersebut. Keberkesanan protein yang dimurnikan dan dinilai dengan mengukur tahap antibodi yang dihasilkan pada plat antigen melalui teknik ELISA. Setelah menginkubasi serum, air liur, dan sampel bronchoalveolar lavage (BAL) daripada tikus yang telah diimunisasikan, kepadatan optik (OD) dibaca menggunakan pembaca ELISA. Hasil kajian menunjukkan bahawa kedua-dua protein yang dimurnikan sahaja dan gabungan BCG dengan protein yang dimurnikan menghasilkan peningkatan tahap antibodi IgG dalam sampel serum,

serta peningkatan tahap antibodi IgA dalam air liur dan sampel BAL. Kesimpulannya, penemuan ini menunjukkan bahawa protein yang dimurnikan dari susu, yang mengandungi epitope TB terpilih, mampu merangsang pengeluaran antibodi, sama ada diberikan secara berasingan atau bersama BCG. Ini menunjukkan potensinya sebagai calon vaksin yang berdaya maju bagi menghadapi TB.

DETECTION OF HUMORAL IMMUNE RESPONSE IN MICE IMMUNISED WITH PURIFIED PROTEIN OF MILK EXPRESSING SELECTED MULTI-TUBERCULOSIS EPITOPE

ABSTRACT

Despite numerous global efforts to reduce infections caused by *Mycobacterium tuberculosis* and the implementation of immunization programs, tuberculosis (TB) remains one of the leading causes of mortality worldwide. Researchers have sought alternative preventive strategies to decrease the incidence of TB and address the limitations of existing Bacillus Calmette-Guérin (BCG) vaccination methods. A significant drawback of BCG vaccinations is their inability to provide adequate mucosal immunity, highlighting the urgent need for the development of more effective mucosal vaccines that can prevent invasive infections, particularly as MTB primarily targets the respiratory system. Our research team has developed a mucosal vaccine utilizing specific TB epitopes, namely Ag85B, Acr, and RpfE. The vaccine was administered by transfecting a pregnant goat's mammary gland, and the resulting milk was collected daily for 21 days and subsequently purified. The objective of this study was to evaluate the efficacy of the purified milk protein in stimulating humoral immune responses in the serum and mucosal systems of Balb/C mice that received the vaccine candidate intranasally. The effectiveness of the purified protein was assessed by measuring the levels of antibodies generated on antigen-coated plates using the ELISA technique. After incubating the serum, saliva, and bronchoalveolar lavage (BAL) samples from the immunized mice, optical densities (OD) were read using an ELISA reader. The study's results indicated that both the purified protein alone and the combination of BCG with purified protein resulted in elevated levels of IgG antibodies in serum samples, as well as increased levels of IgA antibodies in saliva and BAL samples. In conclusion, the findings demonstrate that the

purified protein derived from milk, which contains selected TB epitopes, can induce antibody production, whether administered alone or in conjunction with BCG. This suggests its potential as a viable vaccine candidate against TB.

CHAPTER ONE: INTRODUCTION

1.1 Research Background

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis*. Primarily affecting the lungs, TB can also manifest in extrapulmonary forms that impact other organs. The disease is transmitted through airborne droplets when an infected person coughs, sneezes, or talks, making it highly contagious in crowded settings (Tobin & Tristram, 2024). Despite advances in vaccination and treatment, TB remains a formidable global health threat, with an estimated 10.4 million new cases and 1.8 million deaths reported annually (Sterling et al., 2020). TB manifests in two distinct forms, which are active TB and latent TB. Approximately one-third of the population is estimated to be infected with MTB, yet only about 10% of individuals progress to active TB disease during their lifetime. The remaining individuals often harbour the bacterium in a latent state, which can be reactivated under certain conditions, such as immune suppression or co-infection with HIV (Escalante et al., 2021).

The Bacillus Calmette-Guérin (BCG) vaccine is the most widely used current preventive against TB. Administered intradermally, typically shortly after birth, the BCG vaccine helps stimulate immune responses that protect against severe manifestations of TB, such as TB meningitis and disseminated TB in children (Lai et al., 2023). While the BCG vaccine has been instrumental in reducing TB incidence in many countries, its efficacy does not guarantee protection against pulmonary TB in adults. Despite the protective benefits offered by the BCG vaccine, challenges remain due to a lack of strong immunity against the reactivation of latent infections and the rising incidence of drug-resistant TB strains, indicating the necessity for more effective vaccination strategies (Scriba et al., 2020).

Mucosal immunity refers to the immune responses at mucosal surfaces, including the gastrointestinal, respiratory, and urogenital tracts. This specialized branch of the immune system comprises various cells and lymphoid tissues that work together to protect against pathogens while maintaining tolerance to non-harmful antigens (Choi et al., 2023). The mucosal immune system plays a crucial role in protecting against *Mycobacterium tuberculosis* infections, as it is responsible for producing secretory Immunoglobulin A (IgA) antibodies and facilitating local T-cell responses. Understanding the mechanisms of mucosal immunity is essential for TB vaccine development, as a robust mucosal response can significantly enhance vaccine efficacy by providing targeted protection at the sites of infection (McGill, 2019). Moreover, advancing research into mucosal immunity may improve vaccination strategies that better prevent TB and other respiratory infections by utilizing mucosal routes for vaccine delivery, ultimately enhancing global health outcomes.

This study focuses on developing a mucosal vaccine against TB by utilizing purified milk protein expressing selected TB epitopes (Ag85B, Acr, and RpfE). The study aims to investigate the humoral immune response induced in Balb/c mice after immunization with the purified protein, in comparison to the traditional BCG vaccine. Given TB's high prevalence and the limitations of current vaccines, this approach explores an alternative vaccination strategy to enhance mucosal immunity

1.2 Problem statement

The Bacillus Calmette-Guérin (BCG) vaccine, commonly used to prevent tuberculosis (TB), provides limited protection against pulmonary diseases. While it can stimulate a systemic immune response, it does not effectively promote strong mucosal immunity in the respiratory tract. This is significant because TB primarily infects the lungs, highlighting the need for immune defenses that target mucosal surfaces to prevent initial infections and disease progression.

Additionally, the BCG vaccine is insufficient for combating the full range of TB infections, including active, latent, and reactivatable forms. Although it offers some protection against severe TB in children, its effectiveness in preventing pulmonary TB in adults is low. Studies show that vaccinated individuals can still develop latent infections or experience reactivation of dormant TB, indicating a need for more comprehensive vaccination strategies that focus on both systemic and mucosal immunity.

Given these challenges, mucosal immunization strategies are more desirable for pathogens that enter through the respiratory mucosa. These vaccines can induce localized immune responses and produce secretory immunoglobulin A (sIgA), which is crucial for neutralizing pathogens at entry sites. By focusing on mucosal immunity, new vaccine formulations could enhance protection against TB infections and improve the effectiveness of vaccination strategies beyond what the current BCG vaccine offers.

1.3 Rationale of Study

The rationale for this study is based on the premise that vaccines must correspond to the route of infection to effectively establish first-line defenses at the sites where pathogens enter the body. In the case of TB, which primarily enters through the respiratory mucosa, it is crucial to develop vaccination strategies that stimulate mucosal immunity. Traditional vaccination methods, such as the BCG vaccine, mainly induce systemic immune responses and often lack the necessary mucosal protection. By focusing on mucosal vaccination, this study seeks to improve the effectiveness of immune defenses against TB, thereby preventing initial infections and reducing disease progression.

Moreover, the creation of a mucosal vaccine that incorporates multiple TB epitopes presents an opportunity to provide comprehensive protection against the entire spectrum of TB infections, including inactive, active, and reactivatable forms. Existing vaccination strategies often fall short in addressing the complexities of TB pathology, particularly regarding latent and reactivatable infections. A multi-epitope approach has the potential to generate robust immune responses that not only enhance protection against active disease but also effectively mitigate the risks associated with latent TB infections. This strategy is especially significant given that a large proportion of the global population is affected by latent TB, which poses a serious risk for reactivation.

Additionally, the use of purified milk proteins for vaccine production offers considerable advantages in terms of cost, ease of production, and stability. Milk-derived vaccines can be produced on a larger scale, making them more accessible and affordable for mass immunization efforts. Utilizing transgenic animals facilitates the efficient expression of recombinant proteins, ensuring high yields of vaccine antigens that remain stable and effective. This aspect is critical for public health initiatives, especially in resource-limited

settings where cost-effective vaccination strategies are essential for combating the increasing threat of tuberculosis. By integrating these components, the study aims to contribute to the development of a more effective and sustainable vaccine solution for TB prevention.

1.4 Objectives

1.4.1 General Objectives

To investigate the levels of humoral immune response in mice immunized with mIgAm purified protein.

1.4.2 Specific objectives

I. To determine the levels of IgG in Balb/c mice serum when immunized with mIgAm purified protein.

II. To determine the levels of IgA in Balb/c mice saliva when immunized with mIgAm purified protein.

III. To determine the levels of IgA in Balb/c mice bronchoalveolar lavage (BAL) when immunized with mIgAm purified protein.

1.5 Hypothesis

1.5.1 Null hypothesis (H_0)

There will be no increase in the level of humoral and mucosal immune response in Balb/c mice immunized, with purified protein expressed in milk containing multi-epitope TB.

1.5.2 Alternative hypothesis (H_A)

There will be an increase in the level of humoral and mucosal immune response in Balb/c mice immunized, with purified protein expressed in milk containing multi-epitope TB.

CHAPTER TWO: LITERATURE REVIEW

2.1 Tuberculosis

Tuberculosis (TB) is a bacterial infectious disease that is highly transmissible from person to person. The infectious nature of TB is due to airborne particles released when an infected person coughs, sneezes or speaks. It is caused by the *Mycobacterium tuberculosis* (MTB), which belongs to the Mycobacteriaceae family. This aerobic, rod-shaped bacterium is notable for its unique, waxy cell wall, which consists of mycolic acid, a characteristic that provides enhanced resistance to environmental factors and contributes to its virulence. MTB primarily targets the lungs, although it can disseminate to other organs within the body through the bloodstream. The bacterium has a slow growth rate, typically requiring several weeks for cultivation in a laboratory setting. This slow proliferation and the bacterium's characteristics complicate its identification using standard Gram staining techniques. When inhaled, the bacteria are engulfed by alveolar macrophages, but instead of being destroyed, they persist within these immune cells, leading to latent infections that may reactivate later under conditions of immune compromise (Tobin & Tristram, 2024).

Tuberculosis is classified into two primary categories which are latent TB infection (LTBI) and active TB disease. LTBI occurs when a person is infected with the *Mycobacterium tuberculosis* (MTB) bacterium but does not exhibit symptoms and is not contagious. In this dormant state, the bacteria remain inactive within the body, potentially becoming active if the immune system weakens due to various factors such as illness or stress. Active TB disease, on the other hand, represents the stage where the bacteria multiply and cause symptoms, which can be classified further into two categories. Firstly, pulmonary TB is the most common manifestation, involving the lungs and is characterized by cough, fever, and weight loss. Secondly, extrapulmonary TB refers to

infections that spread beyond the lungs, affecting other areas like the lymphatic system, bones, and central nervous system. This includes specialized forms such as TB lymphadenitis, skeletal TB, TB meningitis, and miliary TB, each presenting distinct clinical symptoms according to the affected organs (Coussens et al., 2024). Understanding these classifications is essential for effective diagnosis, treatment, and public health strategies to manage TB effectively.

2.2 Epidemiology of Tuberculosis

2.2.1 Global Prevalence of Tuberculosis

TB continues to pose a significant public health challenge worldwide, with the World Health Organization (WHO) estimating that approximately 10.6 million people fell ill with TB in 2022. The disease remains the second leading cause of infectious disease-related death globally after COVID-19, responsible for 1.6 million fatalities. The burden of TB is not evenly distributed across the globe, with certain regions experiencing disproportionately high rates (Bai & Ameyaw, 2024). The epidemic affects all regions but predominantly impacts low- and middle-income countries. In Southeast Asia, the region reported the highest burden, accounting for approximately 46% of new TB cases. Countries like India and Indonesia contribute significantly to these statistics. Africa follows closely, contributing 23% of new cases, with several countries facing high rates of co-infection with Human immunodeficiency virus (HIV), further complicating TB management. The Western Pacific region also reports substantial cases, with countries such as the Philippines and Vietnam significantly impacted (Ghebreyesus et al., 2023). The global incidence of TB has shown fluctuations, with a notable decline between 2015 and 2019, where the reported incidence rate dropped from 137 to 127 cases per 100,000 population. However, this downward trend was jeopardized by the COVID-19 pandemic, which led to disruptions

in TB services, resulting in an estimated 4.1 million missing TB cases in 2020 and 2021 combined (Marco et al., 2024).

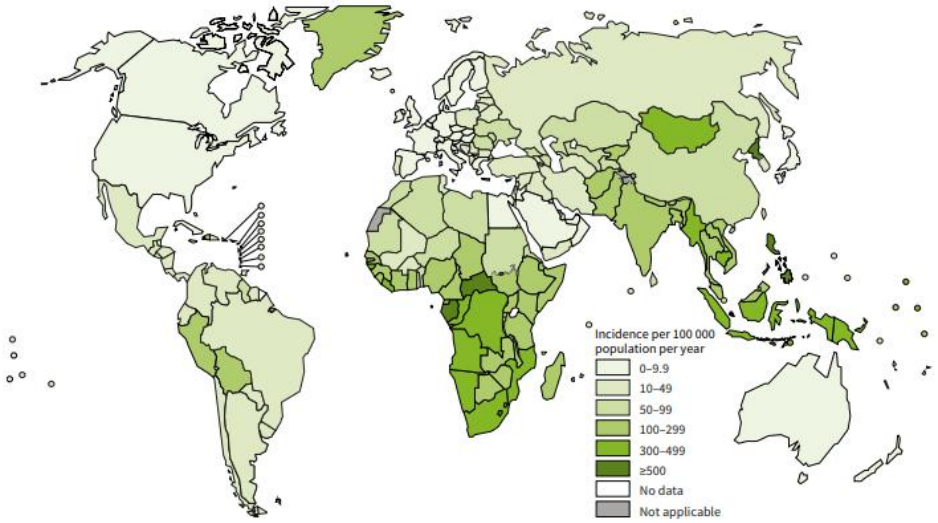

Figure 2. 1: Estimated TB incidence rates, 2022 (Ghebreyesus et al., 2023).

2.2.2 Prevalence of Tuberculosis in Malaysia

The trends in the regional prevalence of TB in Malaysia over the past 20 years have been characterized by fluctuations in incidence rates and varying impacts due to public health initiatives and global health challenges. From 2000 to 2019, Malaysia experienced an increase in reported TB cases, with notifications rising from approximately 10,000 cases in the early 2000s to around 14,578 cases in 2019, translating to a national incidence rate of about 44 cases per 100,000 population (Zhang et al., 2024). However, reports from 2012 to 2018 indicated a prevalence of about 128 cases per 100,000 in Sabah, with a concerning rise in multidrug-resistant TB detected at around 0.3% (Goroh et al., 2020). The distribution of TB cases has not been uniform across regions where the urban areas, particularly states like Selangor and Kuala Lumpur, have consistently reported higher incidence rates due to factors such as population density, urbanization, and socioeconomic conditions. These regions

are characterized by greater movement and interaction, facilitating the spread of TB. Conversely, rural areas exhibit lower prevalence rates but often face issues such as limited access to healthcare services, contributing to undiagnosed cases or a rise in multi-drug resistance. The current landscape highlights ongoing challenges, particularly with drug-resistant TB and the need for continued public health interventions to manage and reduce TB prevalence in Malaysia effectively.

2.3 Pathogenesis of Tuberculosis

2.3.1 Mechanism of Infection

The mechanism of infection in TB begins when MTB is inhaled into the lungs. It then encounters alveolar macrophages. These immune cells can infect themselves while attempting to engulf and destroy the bacteria. This will allow the bacteria to replicate within them. This process triggers an immune response characterized by the formation of granulomas, and clusters of immune cells, including macrophages, T lymphocytes, and other immune components that contain the infection. If the immune system effectively manages to encapsulate the bacteria within these granulomas, the individual may enter a latent phase where the infection remains dormant as shown in figure 2.2. However, if the immune response is inadequate, the bacteria can multiply uncontrollably, leading to active TB, which is marked by symptoms such as chronic cough, fever, and weight loss (Heemskerk et al., 2015).