UNIVERSITI PUTRA MALAYSIA

LATERAL FLOW IMMUNOASSAY FOR DETECTION OF ACTIVE TUBERCULOSIS UTILISING CFP10-ESAT6 AS BIOMARKER

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ITMA 2020 2

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

December 2019

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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December 2019

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Tuberculosis (TB) is one of the greatest health care problems in the world. Traditional diagnostic techniques based on the isolation of the tuberculosis bacillus in culture media are time consuming, and it is necessary to wait for several weeks to obtain a result. Therefore, possible biosensor that easy to use and cheap is the lateral flow immunoassay (LFIA), which is affordable, sensitive, specific, and user-friendly. LFIA has been introduced as a handheld immunoassay-based point-of-care platform for an automated detection of TB. The CFP10-ESAT6 antigen of M. tuberculosis were used as the target in early detection of TB using LFIA strip-based point of care strategy

Gold nanoparticles (AuNPs) were prepared in various shape, nanosphere, nanorod and nanostar. AuNPs in nanosphere shape were used as the colour probe for the detection of a target of interest. AuNPs were prepared through reduction of Aurum (III) Chloride with trisodium citrate. The prepared AuNPs were further conjugated with antibody (Rabbit anti *Mycobacterium Tuberculosis*). The high-resolution transmission electron microscopy (HRTEM) image and ultraviolet-visible spectrophotometer (UV-Vis) analysis confirmed that the synthesized AuNPs were appropriate for conjugation with the antibody for the immunoassay designed.

As a proof of concept, conventional enzyme-linked immunosorbent assay (ELISA) method was carried out to validate our research finding. Sputum samples were spotted onto the LFIA strips and the result were obtained after 5-10 minutes. The positive and negative sample sputum were obtained from Hospital Universiti Sains

Malaysia (HUSM) Kubang Kerian Malaysia which were confirmed by smear microscopy technique and culture technique.

12 µg/ml antibody was used for conjugation of antibody with gold nanoparticle in sphere shape. LFIA strips that were spot off with positive Tuberculosis sputum sample showed two red signals appeared on the membrane. One signal appeared on the test line and one in control line. For LFIA strips that were spot off with negative sputum sample, only one red signal appeared in membrane which is on the control line

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

IMMUNOASSAI ALIRAN LATERAL (LFIA) UNTUK MENGESAN TUBERKULOSIS MENGGUNAKAN CFP10-ESAT6 SEBAGAI BIOMARKER

Oleh

NAZIFAH BINTI ARIFFIN

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Pengerusi: Nor Azah Yusof, PhD
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Tuberkulosis (TB) adalah salah satu masalah penjagaan kesihatan terbesar di dunia. Teknik diagnostik tradisional berdasarkan pengasingan bacillus tuberculosis dalam media kultur memakan masa, dan perlu menunggu beberapa minggu untuk memperoleh hasil. Oleh itu, kemungkinan biosensor yang mudah digunakan dan murah adalah immunoassai aliran lateral (LFIA), yang berpatutan, sensitif, khusus, dan mesra pengguna. LFIA telah diperkenalkan sebagai platform point-of-care berasaskan immunoassai genggam untuk pengesanan automatik TB. Antigen CFP10-ESAT6 M. tuberculosis digunakan sebagai sasaran dalam pengesanan awal TB menggunakan strategi penjagaan titik berasaskan jalur LFIA.

Nanopartikel emas (AuNPs) disediakan dalam pelbagai bentuk, nanosfera, nanorod dan nanostar. AuNPs dalam bentuk nanosfera digunakan sebagai penyelidikan warna untuk mengesan sasaran minat. AuNPs disediakan melalui pengurangan Aurum (III) Klorida dengan trisodium sitrat. AuNPs yang disediakan telah dikaitkan dengan antibodi (Rabbit anti *Mycobacterium tuberculosis*). Imej mikroskopi elektron transmisi resolusi tinggi (HRTEM) dan analisis spektrofotometer (UV-Vis) yang dapat dilihat ultraviolet mengesahkan bahawa AuNPs yang disintesis adalah sesuai untuk konjugasi dengan antibodi untuk reka bentuk immunoassai

Sebagai bukti konsep, kaedah imunosorben berkaitan enzim yang berkaitan enzim telah dijalankan untuk mengesahkan penemuan penyelidikan kami. Contoh sputum telah dilihat pada jalur LFIA dan hasilnya diperolehi selepas 5-10 minit. Sputum sampel positif dan negatif diperoleh dari Hospital Universiti Sains Malaysia (HUSM) Kubang Kerian Malaysia yang disahkan oleh teknik mikroskopi

smear dan teknik kultur. 12 µg/ml antibodi digunakan untuk konjugasi antibodi dengan nanopartikel emas dalam bentuk sfera. Jalur LFIA yang diuji dengan sampel sputum Tuberkulosis positif menunjukkan dua isyarat merah muncul pada membran. Satu isyarat muncul pada baris ujian dan satu baris kawalan. Untuk jalur LFIA yang diuji dari sampel sputum negatif, hanya satu isyarat merah muncul dalam membran yang berada di garis kawalan

ACKNOWLEDGEMENTS

In the name of Allah, the Most Gracious, the Most Merciful

Alhamdulillah, all praises be to Allah for His Love and His Blessing, I have finally completed my research project, with the given strength, courage and guidance along my life journey.

First and foremost, I offer my sincerest gratitude to my project supervisor, Prof. Dr. Nor Azah Yusof, and my co-supervisor, Dr. Jaafar Bin Abdullah, who have supported me throughout my postgraduate journey with their patience, inspiration, expertise and financial support. I attribute my master's degree to their encouragement and continuous effort and without them this thesis would not have been completed. Along with this journey, I have gained a lot of knowledge and experience throughout the duration of this project.

Also, to my parents, for their endless support and prayers throughout my journey. This thesis is wholeheartedly dedicated to my lovely husband, Al Fikri Mohd Darbi for his patience and encouragement which make me able to achieve the success. I would also like to thank my brother and my sister for their concern about all my problems during this process. For my baby, who is still in my womb, thank you for giving me strength and I believe Allah sent you as a miracle throughout my viva voce.

Deepest gratitude also goes to all my dearest labmates, Suhainie, Fariza Aina, Umi Zulaikha, Nor Hidayat, Norzila, Ain Shahera, Fatimah Nur, Nur Hidayah, Zurmira, Izsdihar, Nur Izzah, Siti Arisya, Noremylia, Faezah, Siti Khadijah, Siti Fatimah, Nor Zida, Hamizah, Zulaiha, Syahira, Hayati, Putri Syafieqah, Nurul Asyikeen, Syahirah, Shahirah, Zarif, Zul, Chin and many more who encouraged and advised me to complete my master's degree. Next, thanks to all staff from Institute of Advanced Technolgy for their concern and help upon handling the instrumentation used in the chemistry department.

Thank you so much, you all inspired me to do my best

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

AFB	Acid Fast Bacilli
AuNPs	Gold nanoparticle
AuNRs	Gold nanorods
AuNS	Gold nanostar
BCG	Bacille Calmette-Guerin
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FESEM	Field Emission Scanning Electron Microscopy
HIV	Human Immunodeficiency Disease
HRP	Horseradish Peroxidase
LFIA	Lateral Flow Immunoassay
LTBI	Latent TB Infection
mAb	Monoclonal antibody
PAb	Polyclonal antibody
POC	Point of Care
SPR	Surface Plasmon Resonance
TEM	Transmission Electron Microscopy
TB	Tuberculosis
TST	Tuberculin Skin Test
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Background of study

Tuberculosis (TB) is one of the greatest health care problems which is the ninth leading cause of death in the world. WHO reported that in 2016, an estimated 10.4 million people fell in with TB. TB cases cause 1.3 million death among HIV negative people and 374 000 death among HIV positive people. The transmission of bacteria from one host to another is illustrated in Figure 1.1. The bacteria transmitted by people infected with tuberculosis by airborne transmission. Overall, a relatively small proportion (5–15%) of the estimated 2–3 billion people infected with M. tuberculosis will develop TB disease during their lifetime. (WHO, 2017)

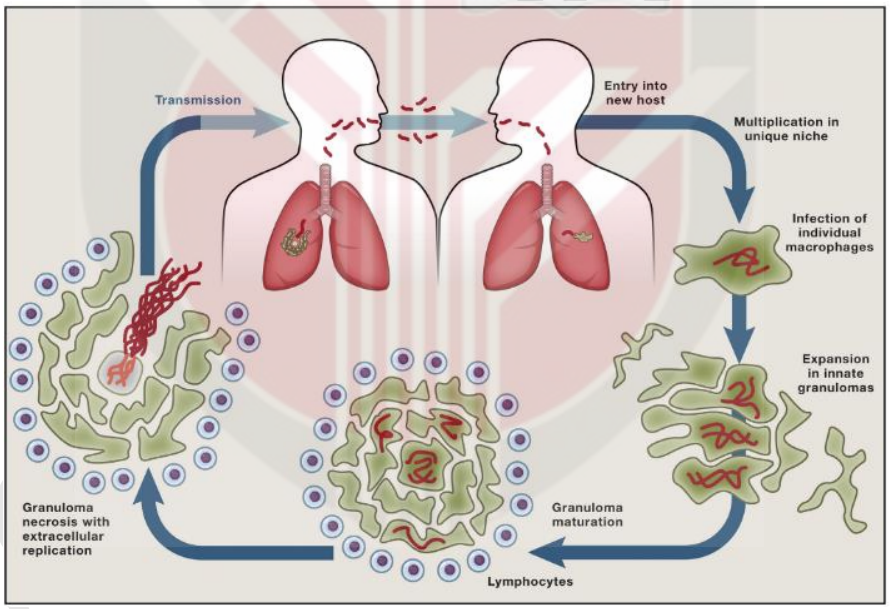

Figure 1. 1 : Transmission of Tuberculosis bacteria. (Cambier et al., 2014**)**

Tuberculosis (TB) is an infectious disease caused by the bacillus *Mycobacterium tuberculosis*. TB bacteria expel into the air for example by coughing which is spread by the people infected with pulmonary TB. The bacteria usually attack the lungs (pulmonary TB) but TB bacteria can attack any part of the body such as the kidney, spine and brain (extrapulmonary TB).

In many regions that has high case of TB, the diagnosis still rely on the standard detection of acid-fast bacilli (AFB) by using smear microscopy (Ziehl-Neelsen) and using culture media to culture *Mycobacterium tuberculosis*. (El-masry et al., 2008). In extra pulmonary cases, tuberculosis does not always detect by the classic radiological signs. This is because there are limitations especially in the sensitivity of the detection. The traditional methods used by laboratory have their limits, for example a longer time needed for cultivation of smear *Mycobacterium tuberculosis* by using culture which usually takes 6-8 weeks. Besides that, the low sensitivity of AFB smear microscopy and highly cost molecular made the detection limited.

The detection of *Mycobacterium tuberculosis* antigen has been focused for more specific Tuberculosis detection. A rapid serologic test which provide more economic and fast result give benefits rather than the traditional method. The mycobacterium antigen such as 38 kDa antigen (Zhang et al., 2009), 16 kDa (Devi et al., 2003), 45 and 96 kDa antigens (Rattan et al., 1993) used for the detection of *Mycobacterium tuberculosis* to develop biosensor detection. The detection of mycobacterium antigens using antibodies has revolutionized diagnostics for detection in antibody-based biosensors. The assays using immunosensor based on detection of antigen has shown high specificities and sensitivities. (Guo et al., 2015 ; Khurshid et al., 2017)

Recently, an advance technology based on biosensor has been focused on detection of biological factors. Optical biosensor has given a promising approach in detection of antibody-antigen based biosensor due to its rapidity, simple and high sensitivity. Biosensors with nanobiotechnology has several advantages over traditional method, such as by using enzyme-based (Grange et al., 2014), immunosensors (Shan et al., 2002), DNA biosensors (Chen et al., 2018) and optical biosensors (Yoo and Lee 2016).

Lateral flow immunoassay (LFIA) technology have become an advance diagnostic tool in POC diagnostic strategy for detecting analytes such as blood and sputum. The LFIA design is illustrated in Figure 1.2. LFIA which also known as dipstick-based biosensor are designed for detection of target analytes without laboratory equipment or trained personnel. The formats used is flow through and lateral flow membrane-based immunoassay. LFIA recognized the target analyte such as antigen by using antibodies which spotted on the nitrocellulose membrane. The interaction of antibody-antigen on the nitrocellulose membrane either in sandwich or competitive format by using nanoparticle label. (Parolo et al., 2013 ; Grant et al., 2016)

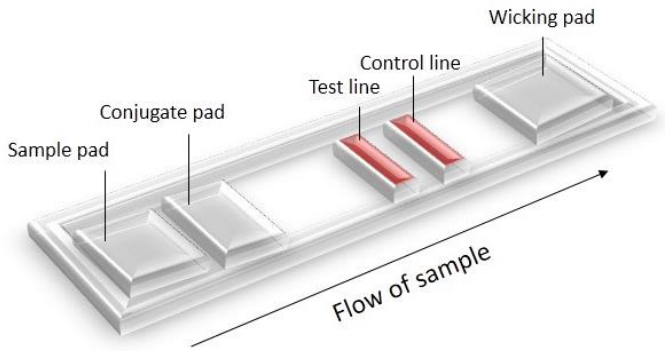

Figure 1. 2 : Lateral flow immunoassay (LFIA) which consists of sample pad, conjugate pad, test line, control line and wicking pad.

LFIA offer a rapid response of 'positive' and 'negative' result based on naked eye visual system. An interesting study was done by Kim and co-workers by using gold nanoparticle as labelled and conjugate with the antibody (Kim et al., 2016). The used of colloidal gold as labelled nanoparticle in lateral flow immunoassay is most widely used. The choice of gold nanoparticle as labelled is for its stability in producing colour on the test line and control line on the membrane of LFIA and allow an unobstructed flow of analyte through the nitrocellulose membrane. Besides that, gold nanoparticle gives an advantage when conjugate with antibodies to produce labelled antibody for LFIA. (Singh et al., 2015 ; Kashid et al., 2015)

1.2 Problem statements and research motivation

Tuberculosis (TB) detection in clinical practice is a bit difficult and challenging. The diagnosis of pulmonary TB by using chest X-ray is not specific. The important diagnosis of pulmonary TB is to reduce the spread of disease to public and as control for treating patient with TB (McCarthy and Luckman 1993 ; García-basteiro et al., 2018). Current point of care (POC) for TB diagnosis is sputum smear microscopy which using microscope to examine specimen for detection of acid-fast bacilli (AFB) staining with Ziehl-Neelsen. The current smear microscopy is inexpensive and be used in low and middle income countries. However, the sensitivity of this method is limited and very low. (Steingart, Ng, et al., 2006 ; Alnour 2018). Other traditional method for TB diagnosis is purified protein derivative (PPD) tuberculin test. As reported by Kruczak et al., the tuberculin skin test is lack of sensitivity and specificity. TST may yield a false positive result especially in BCG vaccinated patients (Goletti et al., 2014 ; Kruczak et al., 2016).

To improve the sensitivity and specificity of diagnosis, culture-based diagnostic method is used. However, this method may take several weeks up to 4-8 weeks to obtain the result. The culture of *Mycobacterium tuberculosis* using clinical sputum samples is performed by using solid media. Besides taking longer time, this method need laboratory personnel to conduct the procedure and require biosafety practices and equipment to avoid infection of the tuberculosis to the personnel. (Dorman 2010 ; Asmar and Drancourt 2015)

Recently, assays have been developed for early detection of Tuberculosis such as CFP10-ESAT6 antigen. The antigen derived from sputum sample of infected patients with tuberculosis. CFP10 and ESAT6 antigens were originally isolated from *Mycobacterium tuberculosis* culture filtrate (Pinxteren et al., 2000). Early research shows that the specific fused antigen CFP10-ESAT6 secreted by *Mycobacterium tuberculosis* showed high sensitivity compared to single antigens CFP10 and ESAT6. (Renshaw et al., 2005 ; He et al., 2016)

The naked eye detection of Tuberculosis was developed for detection of Tuberculosis by lateral flow immunoassay (LFIA). The LFIA used colloidal gold as labelled to conjugate with antibodies to form signals. The LFIA method used the transportation of targets which flow laterally on the nitrocellulose membrane and bind with the conjugated antibody. Antibodies on test line and control line of LFIA will bind with the target and produce red line signal which indicates the positive and negative signal. (Mdluli et al., 2014 ; Kolosova et al., 2008)

The advantages of LFIA represent a well technology which give results in a few minutes and easy to use without trained personnel. The early detection of Tuberculosis gives advantage in diagnosis of Tuberculosis especially in industries and clinical sectors. (Zhang et al., 2008 ; Liu et al., 2015)

1.3 Novelty of research

The lateral flow immunoassay (LFIA) was developed for detection of *Mycobacterium tuberculosis* specifically in detection of CFP10-ESAT6 antigen of Tuberculosis. The CFP10-ESAT6 antigen is the low-molecular mass fraction of culture filtrate *Mycobacterium tuberculosis* and was secreted in early culture time. Sputum samples from patients in Hospital Universiti Sains (HUSM) Kubang Kerian infected with tuberculosis was tested using LFIA to obtain positive and negative result of detection.

1.4 Research objective

The general objective in this study is to develop ultrasensitive and naked eye detection of lateral flow immunoassay (LFIA) for detection of *Mycobacterium tuberculosis*. The following specific objectives were designed to achieve the objective:

i. To synthesis and characterize different size and shape of gold nanoparticle (AuNPs)
ii. To study the interaction between the antibody with antigen of *Mycobacterium tuberculosis* using Lateral Flow Immunoassay
iii. To characterize the sensing ability of developed detectiom system lateral flow immunoassay (LFIA).
iv. To optimize the kit development of LFIA by testing on clinical sample (sputum).

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BIODATA OF STUDENT

Nazifah Binti Ariffin was born on 7th of May 1992 in Kajang Selangor. She attended her primary school at Sekolah Rendah Islam ABIM, Sungai Ramal Kajang and secondary school at SMK SAAS Kajang and SBPI Gombak. The she was offered to attend Pusat Asasi Sains UM (PASUM) in 2009. In 2010, after finishing her foundation study, she pursued her bachelor's degree (Petroleum Chemistry) at Universiti Putra Malaysia and completed her bachelor's degree with Second Class (upper) with Honours. She then pursued her master's degree (Sensor Technology) under supervision of main supervisor, Prof. Dr. Nor Azah Yusof and co-supervisor, Dr. Jaafar Bin Abdullah. During her master's degree, she was offered as Research Assistant who responsible in managing laboratory apparatus, purchasing chemicals, monitoring final year students and helping supervisor in research findings. She also experienced as a laboratory demonstrator to assist undergraduate students conducting their experiments for 2 years.

LIST OF PUBLICATIONS

Publications

Patent No: PI2018701885. Lateral Flow Immunoassay (LFIA) Strip for Detecting Mycobacterium Tuberculosis (MTB) Antigen in Biological Sample and Method Thereof

Ariffin, N., Yusof, N.A., Abdullah, J., Abd Rahman, S.F., Ahmad Raston, N.H., Kusnin, N., Suraiya, S. Lateral Flow Immunoassay for Ultrasensitive and Affordable Naked Eye Detection of Mycobacterium Tuberculosis. *Hindawi.*

Conferences

[1] Workshop on Advanced Materials and Nanotechnology 2015

[2] The 29th Malaysian Analytical Chemistry Symposium 2016

[3] Tuberculosis Disease Diagnosis Using AI, Bio-sensor and Digital Technology

[4] Symposium on Advanced Materials and Nanotechnology 2018

[5] Innovation Forum Kuala Lumpur Healthcare Business Idea Competition 2018

[6] 30th International Invention, Innovation & Technology Exhibition 2019

[7] International Workshop on Lateral Flow Assay and Biosensor Technologies 2019

List of Awards

[1] Gold Award at International Invention, Innovation & Technology Exhibition Malaysia (ITEX) 2019
[2] Third place winner at Innovation Forum Kuala Lumpur Healthcare Business Idea Competition (IFKL) 2018
[3] Best poster award at Workshop on Advanced Material and Nanotechnology Nanomedicine (WAMN) 2015

Grant Approved

This research was funded by Ministry of Energy, Science, Technology, Environment and Climate Change (MESTECC), Malaysia through the Science Fund Project [03-01-04-SF160].

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