รายงานวิจัยฉบับสมบูรณ์

โครงการ

**การศึกษาตัวบ่งชี้สภาวะปราศจากการติดเชื้อวัณโรคหลังการรักษา
ด้วยยาต้านวัณโรคในหลอดทดลอง และในมนุษย์**

(*In vitro* and *in vivo* investigations of biomarkers indicating clearance stage of *Mycobacterium tuberculosis* infection after treatment with anti-tuberculous drugs)

โดย

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(*In vitro* and *in vivo* investigations of biomarkers indicating clearance stage of *Mycobacterium tuberculosis* infection after treatment with anti-tuberculous drugs)

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มีการประมาณการว่าหนึ่งในสามของประชากรโลกติดเชื้อวัณโรคแบบแอบแฝง หรือเรียกว่า Latent Tuberculosis infection (LTBI) ปัจจุบันยังไม่มีวิธีการมาตรฐานในการตรวจวินิจฉัยและยังไม่มีตัวบ่งชี้ทางชีวภาพที่สามารถบอกถึงสภาวะปราศจากเชื้อของผู้ป่วยภายหลังการรักษาได้ คณะผู้วิจัยจึงมีวัตถุประสงค์ในการศึกษาและวิเคราะห์หาตัวชี้วัดสภาวะปราศจากเชื้อ (clearance stage) จากเซลล์ภายหลังการใช้ยาต้านเชื้อก่อวัณโรคและการตรวจวินิจฉัย โดยใช้ แมคโครฟาจที่ติดเชื้อ *Mycobacterium tuberculosis* (*M. tuberculosis*) เป็นต้นแบบและตัวอย่างซีรั่มของผู้ป่วย แล้วเปรียบเทียบโปรตีนที่ถูกสร้างขึ้นในระยะติดเชื้อกับสภาวะปราศจากเชื้อ ด้วยวิธีตัดโปรตีนด้วยเอ็นไซม์ trypsin ในเจล (in-gel tryptic digestion) ต่อด้วยการทำ liquid chromatography-tandem mass spectrometry (LC MS/MS) โดยนำโปรตีนรวมนอกเซลล์ (จากน้ำเลี้ยงเซลล์) และ โปรตีนรวมในเซลล์ (องค์ประกอบในเซลล์จากการทำให้แตกแล้ว) โดยใช้เซลล์ PBMC ที่ติดเชื้อวัณโรคสายพันธุ์ H37Rv Bj IO และ EUA ด้วยอัตราส่วนระหว่างเชื้อและเซลล์ 1:1 ซึ่งเชื้อในเซลล์ถูกฆ่าด้วยยาสัดส่วน isoniazid 3 ไมโครกรัม และ ยา rifampicin 9 ไมโครกรัม จากนั้นโปรตีนรวมจึงถูกเก็บมาวิเคราะห์ในวันที่ 1 (สภาวะติดเชื้อ) และวันที่ 5 หลังจากเติมยาแล้ว (สภาวะปราศจากเชื้อ) โดยสภาวะปราศจากเชื้อวัดจากการไม่พบ *Mtb* ในเซลล์ PBMC จากนั้นจึงเตรียมโปรตีนที่สกัดได้เพื่อวิเคราะห์ด้วย LC MS/MS ผลการศึกษาพบว่ามีชนิดของโปรตีนภายนอกเซลล์จำนวน 1073 ชนิด โดยมีโปรตีนที่พบเฉพาะสภาวะปราศจากเชื้อ (ไม่พบในสภาวะติดเชื้อ) จากภายนอกเซลล์จำนวน 2 ชนิด โดย PSTK และ FKBP8 เป็นโปรตีนที่มีแนวโน้มที่ดีในการใช้เป็นตัวบ่งชี้สภาวะปราศจากเชื้อวัณโรค จากการวิเคราะห์โปรตีนด้วย Western blot analysis จากตัวอย่างของผู้ป่วยวัณโรคแอบแฝง เปรียบเทียบกับกลุ่มผู้ป่วยวัณโรคปอดแสดงอาการ และกลุ่มควบคุมสุขภาพดี พบว่า PSTK เป็นโปรตีนที่สามารถใช้เป็นตัวชี้วัดสภาวะปราศจากเชื้อและน้ำเลือดของผู้ติดเชื้อวัณโรคภายหลังการรักษา

การศึกษา protein จากตัวอย่างโดยใช้เทคนิคการตรวจปริมาณมาก (SERS, RAMANS) พบแบบแผนโปรตีนและโมเลกุลที่ช่วยจำแนกกลุ่มผู้ป่วยวัณโรค และการตรวจวิเคราะห์ไซโตไคน์ช่วยบ่งชี้ว่า CXCL10 ช่วยในการจำแนกกลุ่มผู้ป่วยวัณโรคได้ดี ตัวบ่งชี้โปรตีนเหล่านี้อาจจะสามารถนำมาเป็นตัวชี้วัดสภาวะปราศจากเชื้อวัณโรคภายหลังจากการรักษาได้

คำสำคัญ *Mycobacterium tuberculosis*, ติดเชื้อวัณโรคแอบแฝง, comparative proteomic, LC MS/MS, clearance marker,

Abstract

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Project Title: *In vitro* and *in vivo* investigations of biomarkers indicating clearance stage of *Mycobacterium tuberculosis* infection after treatment with anti-tuberculous drugs

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One-third of the world population was infected with *Mycobacterium tuberculosis* (*M. tuberculosis)* and was defined as latent tuberculosis infection (LTBI). So far, there is no gold standard method for LTBI diagnosis and no biomarker for determining the clearance stage of *M. tuberculosis* infection after treatment. We aim to analyze the potential biomarkers for diagnosis and indicating the clearance stage of *M. tuberculosis* infection by using macrophage infection model. Comparative proteomic analysis between *M. tuberculosis* infection stage and clearance stage were performed by using in-gel tryptic digestion followed by liquid chromatography-tandem mass spectrometry (GeLC MS/MS). Extracellular (culture supernatant) proteomes from activated PBMC infected with *M. tuberculosis (*H37Rv, Bj, IO, EUA) strain (MOI = 1) and treated with 3 µg of isoniazid and 9 µg of rifampicin for 1 day (infection stage) and 5 days (clearance stage) post infection were extracted, processed for GeLC MS/MS. Clearance stage was defined as the condition with no viable *M. tuberculosis* from infected PBMC. There were overall 1073 proteins elements were found extracellular proteome. There were 4 extracellular that uniquely found in the clearance stage of all *M. tuberculosis* (H37Rv, Bj, IO, EUA) strain. PSTK and FKBP8 were promising to use as *M. tuberculosis* clearance biomarker based on LC-MS/MS data. The evaluation based on western blot of active pulmonary TB and LTBI patients compare to healthy control confirmed that PSTK is a promising clearance marker. The high throughput analysis (SERS and RAMAN) of TB patient serum revealed the protein and biomolecule pattern that can be used to differentiate the TB patients groups and chemokine analysis revealed that CXCL10 was the marker that can be used to differentiate among the TB patient groups. These proteins were the potential biomarkers for TB diagnosis and to indicate the clearance stage of MTB infection after anti-tuberculous treatment.

Keywords: *Mycobacterium tuberculosis*, latent *Mycobacterium tuberculosis* infection, LTBI, comparative proteomic, LC MS/MS, clearance marker

Executive summary

เนื่องจากปัจจุบันยังไม่มีตัวบ่งชี้ชีวภาพที่บ่งชี้ถึงสภาวะปราศจากเชื้อจากร่างกายของผู้ป่วยหลังจากการรักษาด้วยยาต้านวัณโรคเพื่อลดการเกิดวัณโรคกลับมาเป็นซ้ำและวัณโรคดื้อยา และยังไม่มีวิธีการตรวจวินิจฉัยมาตรฐานสำหรับวัณโรคแอบแฝง วัตถุประสงค์ของโครงการนี้ คือ ค้นหา biomarker เมื่อเชื้อวัณโรคถูกทำลายออกไปจากเซลล์ และประเมินผลในผู้ป่วยที่ได้รับการรักษาจริง พร้อมด้วยศึกษาการตรวจวินิจฉัยวัณโรคแอบแฝงแบบใหม่ ซึ่งสามารถนำไปใช้เป็นตัวบ่งชี้ในการตรวจติดตามการรักษาผู้ป่วยวัณโรคต่อไป

แนวความคิด คือ หากกำจัดเชื้อ *M. tuberculosis* ที่ infect ใน PBMC ด้วยยา anti-TB drugs แล้ว จะมี proteomic profile เปลี่ยนไปอย่างไร เมื่อเปรียบเทียบกับ proteomic profile ที่มาจากมาจาก PBMC อย่างเดียวที่เติมยา (ตัวควบคุม) โดยจะดูใน secreted protein (supernatants) ซึ่งสอดคล้องกับตัวบ่งชี้ที่หลังออกมาในซีรั่ม การใช้ PBMC model ทำให้แน่ใจถึงสภาวะปราศจากเชื้อจากเซลล์ ซึ่งต่างจากการศึกษาในมนุษย์ที่ไม่สามารถแน่ใจได้ว่าเชื้อได้หมดไปจากเนื้อเยื่อแล้วจริง แล้วจึงมาทำการประเมินตัวบ่งชี้ที่พบโดยการทดสอบกับตัวอย่างของผู้ป่วยที่ผ่านการรักษาด้วยยาต้านวัณโรคจนอาการหายไป

การดำเนินการวิจัยนั้น เริ่มจากการที่เลี้ยงเชื้อวัณโรคโดยใช้ *M. tuberculosis* สายพันธุ์ H37Rv ครอบคลุมถึงสายพันธุ์หลักของเชื้อวัณโรคที่พบในประเทศไทย (BJ IO EUA) ในอาหารเลี้ยงเชื้อเหลว จากนั้นนำมาทำการติดเชื้อให้กับ PBMC ที่สัดส่วน MOI = 1 (สัดส่วนที่ PBMC เซลล์ส่วนใหญ่จะถูกติดเชื้อ แต่ก็ไม่มากจนเกินกว่าจะถูกทำลายได้ด้วย anti-TB drug ภายในเวลา 5 วัน) จากนั้นเติม anti-TB drugs เพื่อฆ่าเชื้อวัณโรค (ทั้งภายนอกเซลล์และในเซลล์) จากนั้นจึงเก็บ protein จาก secreted protein (supernatants) เพื่อนำมาทำ GeLC MS/MS เพื่อวิเคราะห์หาการเพิ่มขึ้นของ protein ที่เป็นตัวบ่งชี้ พร้อมทั้งทดสอบหาโปรตีนจากตัวอย่างซีรั่มในกลุ่มผู้ป่วยโดยใช้วิธีการตรวจหาไซโตไคน์และการทดสอบที่ให้ข้อมูลจำนวนมาก (SERS และ RAMAN spectroscopy)

ผลการทดลองพบ protein ที่มีความแตกต่างอย่างมีนัยสำคัญระหว่างสภาวะที่มีเชื้อ (infection stage) และสภาวะที่ปราศจากเชื้อ (clearance stage) และไม่พบในกลุ่มควบคุม (เซลล์ไม่ติดเชื้อที่เติมยา) ที่ extracellular protein จำนวน 1073 ชนิด โดย 4 ชนิด (PSTK, FKBP8, MGMT, B-cell CLL/lymphoma 9-like protein) พบการสร้างมากขึ้น และ PSTK และ FKBP8 เป็นโปรตีนที่มีแนวโน้มที่ดีที่สุดในทุกซ้ำของการทดลอง เมื่อประเมินต่อด้วยวิธี Western blot จากตัวอย่างน้ำเลือดของผู้ป่วยวัณโรค (ATB, LTBI) ในสภาวะก่อนการรักษาและภายหลังเสร็จสิ้นการรักษา พบว่า PSTK ในน้ำเลือดของผู้ป่วยภายหลังการรักษาทั้งกลุ่ม ATB และ LTBI มีโปรตีน PSTK สูงขึ้น ($P<0.0001$) เมื่อเทียบกับตัวอย่างเลือดของผู้ป่วยวัณโรคก่อนการรักษา การทดสอบ SERS และ RAMAN ให้แบบแผนโมเลกุลทางชีวภาพที่ใช้แยกกลุ่มผู้ป่วยวัณโรคแสดงอาการและแอบแฝงจากคนปกติได้ และ CXCL10 เป็นไซโตไคน์ที่สามารถช่วยตรวจวินิจฉัยวัณโรคแอบแฝงได้ดี ตัวบ่งชี้และวิธีการเหล่านี้สามารถต่อยอดสู่การพัฒนาวิธีการทดสอบแบบใหม่เพื่อการตรวจวินิจฉัยและติดตามการรักษาผู้ป่วยวัณโรคต่อไป

<u>เนื้อหางานวิจัย</u>

1.บทนำ

Tuberculosis (TB) is one of the greatest public health problems. One-third of the global population is infected with *Mycobacterium tuberculosis* (*M. tuberculosis*), the causative agent of TB and 5-10% of the infected case developed into TB disease (1).

The standard treatment of TB takes 6 months using combined anti-tuberculous drug treatment. The long period of treatment by anti-tuberculous drugs aim to sterilize all persistent bacilli from the tissue of the patient to prevent drug resistance and TB relapse. However, various factors derived from *M. tuberculosis* (such as virulence and drug resistance) and patients (such as treatment compliance and genetics) lead to unsuccessful treatment outcome. The clearance of *M. tuberculosis* from TB patient is typically assumed by clinical and radiological improvement supplemented by sputum microscopy and/or culture. However, these conventional markers for TB treatment monitoring are not sensitive enough to clearly indicate the clearance of *M. tuberculosis* from host. The effective treatment monitoring markers that can indicate the actual clearance stage of *M. tuberculosis* infection is needed.

There are several studies try to discover the effective marker of TB treatment monitoring (2-24). Several previous studies have identified proteomic markers for LTBI and active TB (25-28). Studies exploring TB treatment outcome markers have used clinical evidence and microbiological evidence, particularly sputum microscopy and culture (29, 30). Other studies have applied molecular and serological markers for treatment monitoring of TB (13, 31-33). However, none of them determined the biomarker indicating the actual sterilization condition of *M. tuberculosis* infection, i.e., no remaining *M. tuberculosis* from host tissue after treatment. Mostly, the researchers from previous studies used human model. However, the limitation of human model study is the difficulty to confirm the *M. tuberculosis* clearance stage (such as by CFU determination) from the lungs and other tissues. The lack of effective reliable markers highlights the importance of the searching for the biomarker predicting the success treatment of TB. The more suitable model for the study (ensuring the actual clearance stage) should be performed to ensure the performance of the clearance marker. In addition, the understanding of host-pathogen interaction during anti-tuberculous treatment is important for drug development and intervention for controlling drug resistant TB. Previously, our group performed the preliminary study exploring the clearance markers using monocytic cell line (THP-1 cells). We found the potential markers indicating the clearance of *M. tuberculosis* from host cells. However, the experiment in multicelluar or actual host might provide different biological results. In this proposal, we are going to further investigate the experiments using the multicellular co-culture and validate by actual human hosts.

Diagnosis of TB status is challenging due to its diverse clinical forms and outcomes.(34, 35) The tuberculin skin test (TST), to probe for T-cell responses to purified protein derivatives, and interferon-gamma (IFN-γ) release assays (IGRAs), such as the QuantiFERON®-TB Gold In-Tube (QFT) assay, which measure responses to stimulation with specific antigens, remain the only methods useful for diagnosis of LTBI.(36) The TST suffers diminished specificity in vaccinated individuals (Bacillus Calmette–Guérin (BCG)) and in individuals infected with nontuberculous mycobacteria.(37, 38) Although IGRAs have enhanced specificity, they remain relatively insensitive especially in immunocompromised individuals.(39) Neither class of test is able to fully clarify the status of *M. tuberculosis* infection, especially ATB, LTBI and uninfected persons. Current ATB diagnosis relies on the detection of the pathogen or clinical manifestations. On the other hand, LTBI is identified when a TB-specific immunological response is detected in the absence of clinical disease or pathogen detection.(34) Screening TB-exposed individuals using QFT assays to discriminate those with LTBI and those with ATB from uninfected individuals is a questionable approach. Furthermore, there is little evidence that discrimination of *M. tuberculosis* infection status can be achieved using biomarkers.(40, 41) Recruitment of innate immune cells to the site of infection is regulated by several chemokines, especially CCL2, also known as monocyte chemoattractant protein-1, and CXCR3 ligands such as CXCL9, CXCL10 and CXCL11.(42, 43) Recent studies suggest that antigen-specific CXCL10 responses differed between individuals with ATB compared with LTBI cases.(41, 44, 45) Others have suggested that assaying responses of multiple cytokines may enhance discriminatory ability.(46, 47) It has yet to be established whether these findings are replicable in other settings, which cytokine responses are the most discriminatory and whether the responses of multiple cytokines/chemokines can be developed into an overall diagnostic decision tool.

In the proposed study, we aimed to investigate the biomarkers indicating the *M. tuberculosis* clearance stage after anti-tuberculous treatment using comparative proteomic analysis of primary immune cells via the subsequence adding of macrophages, monocyte depleted PBMC and PMN. The human blood samples of the TB patients receiving the anti-tuberculous drug treatment will be used to evaluate the obtained markers from the *in vitro* experiment. The host-pathogen interaction during anti-tuberculous drug treatment (isoniazid and rifampicin) will be also performed. The high throughput analysis (SERS and RAMAN) and cytokine/chemokine analysis of TB patient serum were performed aimed to unveil the protein and biomolecule pattern that can be used to differentiate the TB patient groups.

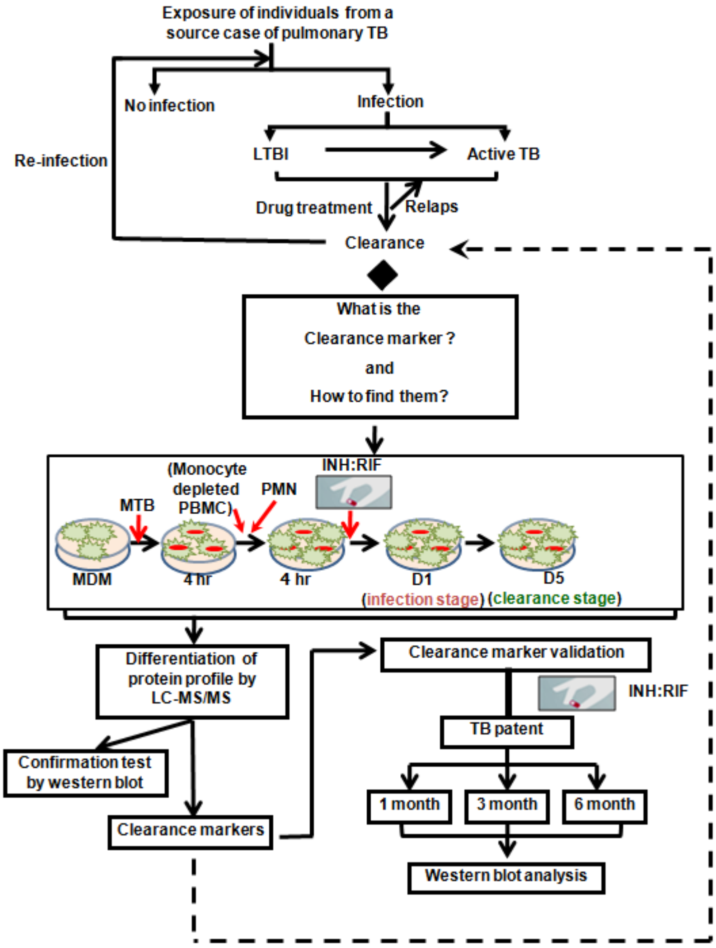
2. วัตถุประสงค์ของโครงการ

To investigate the clearance biomarkers for *M. tuberculosis* infection from host cells that can be used to predict the clearance stage of *M. tuberculosis* infection after treatment with anti-tuberculous drugs using human primary cells infection and comparative proteomic analysis and evaluate the potential markers with serum of TB patients treated with anti-tuberculous drugs. The host-pathogen interaction at proteomic level can be additionally investigated.

Additionally, the serum proteins and biomolecule was analyzed to explore for the diagnostic marker of the active TB and LTBI patients.

3. วิธีการทดลอง

The conceptual framework and experimental scheme was described (figure 1 and 2).



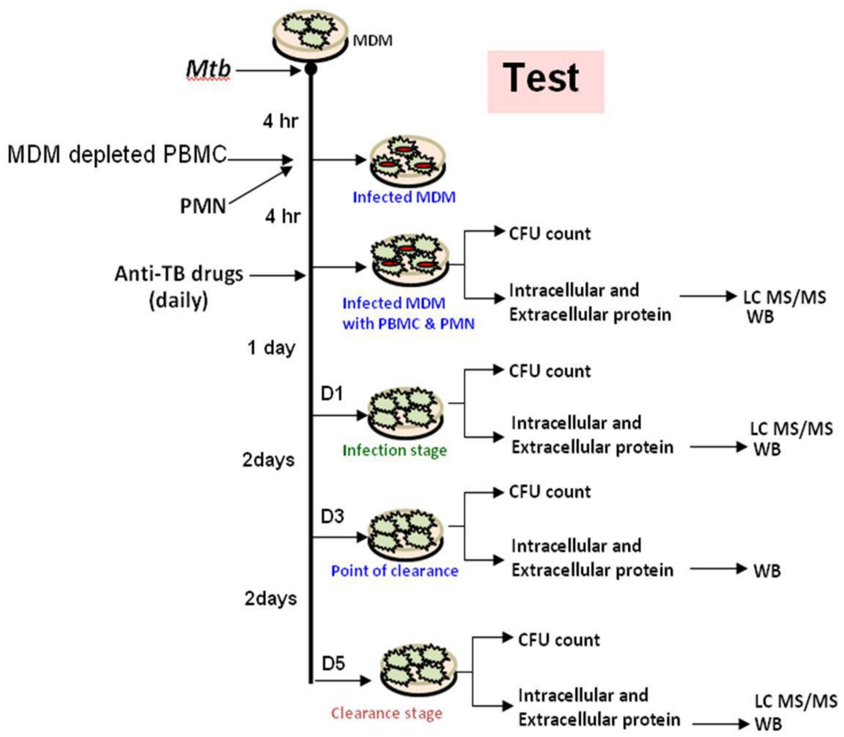
After expose to a source of pulmonary TB, the exposed individual can become infected or non-infected with *M. tuberculosis*. The infected people can develop into latent TB (LTBI) or active TB. After anti-TB treatment, *M. tuberculosis* can be sterilized, non-sterilized (can develop relapse or drug resistance) or become infected again due to re-infection. Nonetheless, there is no biomarker indicate the clearance stage of *M. tuberculosis* infection after treatment. So, in this study we aimed to search for the biomarkers indicating the clearance stage of *M. tuberculosis* by using the primary human cells co-culturing system infected with *M. tuberculosis* strains and treated with anti-tuberculous drugs. extracellular proteins are collected and analyzed by GeLC-MS/MS. The data from 3 independent experiments covering the host heterogeneity were analyzed. The in vitro clearance stages of *M. tuberculosis* infection were determined by CFU determination. The potential clearance markers were selected for validation with the serum of TB patients. The serum samples from TB patients were collected before (D1) and after drug treatment (1, 3 and 6 months). The start and the end-point of the protein marker were evaluated by western blot analysis. The serum from the patients among Active TB, LTBI and Healthy control were additionally tested with SERS/ RAMANS and cytokine analysis.

3.1 *In vitro* experiments for clearance markers

The *in vitro* experiment was perform using the human immune cells infected with *M. tuberculosis* (H37Rv, Bj, IO, EUA) to cover the genetic diversity of the pathogen. The three different patients adjusted by age and gender are included. The host were screened from the participants that never been infected with *M. tuberculosis* (no history of TB exposure, IGRA negative with BCG vaccine. This host characteristic is the common host status in the high TB endemic region. The *in vitro* model was tested using the primary cells co-culture system that were collected from venous blood to mimic the immune cell network and immunity from blood cells that concordant to the evaluated specimens from TB patients. The separate immune cell types (MDM, PMN and others) are used in the *in vitro* experiment in order to trace the immune cell that might mainly responsible for the clearance, especially PMN.

The animal model is not selected because (i) the biology of mice is difficult to reflect the immune response of humans. Hence, the protein marker that can be found from the sterilization of the *M. tuberculosis* from mice might not be found in human (ii) the sterilization of mice is also difficult to ensure. Although the multiple organs of mice such as the lungs, liver and spleen can be grinded and tested for CFU determination, the spread of the mycobacteria in the other parts of the tissues might remain.

The study experiments are separated in to two parts. The first part is the *in vitro* clearance of *M. tuberculosis* infected innate immune cell (**Figure 3**). Second part is the *in vivo* validation of TB patients treated with anti-tuberculous drugs.



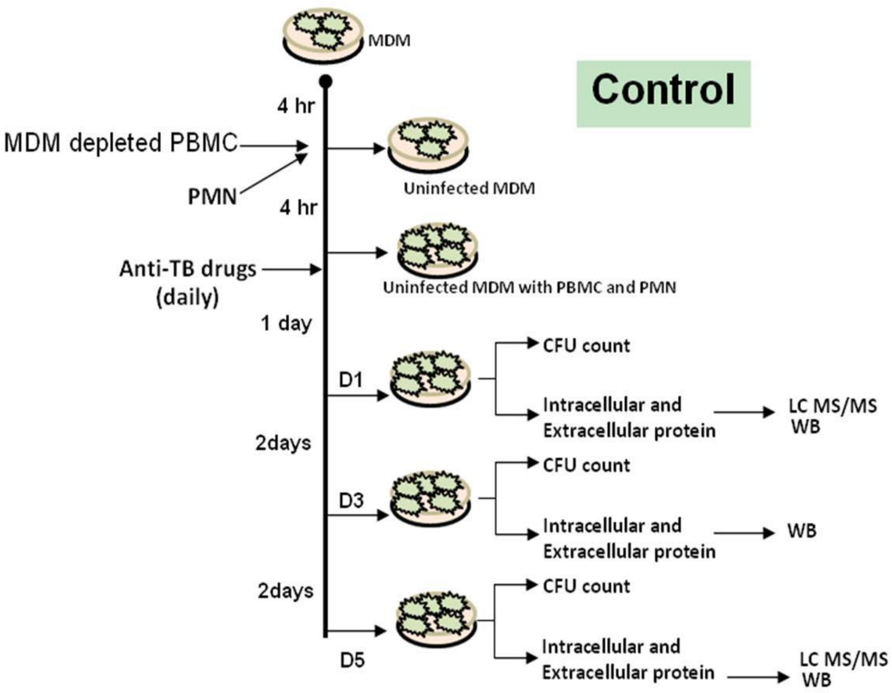


Figure 2 The *in vitro* clearance of *M. tuberculosis* infected innate immune cell (MDM, MDM depleted PBMC, PMN). The MDM are infected with *M. tuberculosis* at log-phase growth for 4 hr. After that MDM depleted PBMC and PMN were added to mimic the multi-cellular response of *M. tuberculosis* infection and incubated for 4 hr. Adding of PMN reflects the recruitment of inflammatory cells from initial infection process and effect of PMN in host-pathogen interaction can be further investigated. After 4 hr incubation, the infected cells are treated with anti-tuberculous drugs (isoniazid and rifampicin). After *M. tuberculosis* infection, the protein samples were collected before treatment and 1, 3 and 5 days after drug treatment. Uninfected cells are used as control and the intracellular and extracellular proteins are collected for GeLC-MS/MS and western blot analysis. In addition, the cells infected with *Staphylococcus aureus* (*S.aureus*) is used as non- *M. tuberculosis* infection control. The clearance of the bacilli was confirmed by plate count technique for CFU determination. Note, *Mtb* = *M. tuberculosis*, PBMC = peripheral blood mononuclear cell, PMN = polymorphonuclear cell, CFU = colony forming unit, WB = western blot, LC MS/MS = liquid chromatography tandem mass spectrometry, D1, D3, D5 = day1, day3 and day5 post treatment, respectively.

3.1.1 Isolation and culture of immune cells

Venous blood samples from participants (3-4 persons separately) were collected and the white blood cells were isolated using Percoll density gradient to isolate the MDM, MDM depleted PBMC and PMN cell (**Figure** 3). The immune cells are cultured in RPMI medium and autologous serum.

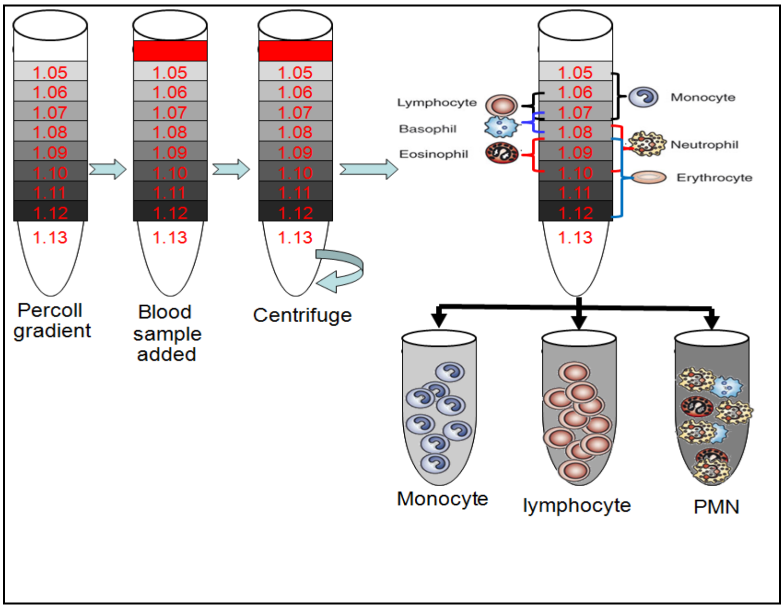


Figure 3 Blood cell isolation using Percoll density gradient. The cells were separated by the optimal density of particular cell type after centrifugation.

3.1.2 Immune cell infection

The MDMs were activated and infected with *M. tuberculosis* lineages (well characterized strains of *M. tuberculosis*, one Beijing and another non-Beijing strain) and incubated for 4 hr. In non-TB control, the host cells were infected with *S.aureus* (ATCC 25922). The extracellular bacteria are removed and the MDM depleted PBMC and PMN cells are added and incubated further for 4 hr. After 4 hr incubation, the infected cells were treated with the combination of isoniazid and rifampicin (the optimal drug concentration will be determined) for 5 days and changed the fresh drug containing medium every 24 hr. The co-culture of MDM, MDM depleted PBMC and PMN without bacteria and treated with drugs were used as control. Cells without drugs might be additionally performed.

3.1.3 Confirmation of infection

The 10 µl of culture supernatant (extracellular *M. tuberculosis*) at 24 and 120 hr after drug treatment were dropped on M7H11 agar plate to determine the CFU count.

3.1.4 Extracellular protein collection (supernatant)

After 24 hr of incubation, the 3 ml supernatant from each well are collected by pipetting all of supernatant, filtered and transferred into 10 ml conical tube. The protein-collected tubes were kept at -80 ˚C for extraction and precipitation. With the concordance to the *in vivo* study that use the serum as the testing sample (which more convenient and more practical to use as the clinical specimen for the actual application), the supernatant in the *in vitro* experiment will be a priority to be investigated.

3.1.5 Protein extraction and precipitation

The protein suspension tube was thawed at room temperature (when the protein samples are kept in the freezer). The amount of extracted proteins was measured using Lowry method and use bovine serum albumin (BSA) as a standard protein.

3.1.6 LC MS/MS analysis

The extracellular proteins adjusted the concentration at 50 µg were run on SDS-PAGE. It was found that the extracellular protein (supernatant) showed the thick bands at the MW about 45-97 kDa which was the albumin band from the media supplement (FBS). The albumin bands were the contaminated proteins which were not the target for analysis and potentially interfered the LC-MS/MS analysis. In the LC MS/MS analysis, only the proteins band lower that the albumin bands were selected to perform the test. The gel was cut into 13 pieces for each sample, and each gel plug was further cut into 1-mm and transferred into low binding 96-well plates. Tryptic digestion was performed by incubate the gel pieces with 25 mM NH_4HCO_3 for 10 min, add 200 µl acetonitrile (ACN) and incubate for 10 min with shaking. After ACN removal, 50 µl of 10 mM DTT in 10 mM NH_4HCO_3 was added and incubated at 56°C for 1 hr. Next, add 50 µl of 100 mM iodoacetamide in 10 mM NH_4HCO_3, and then, incubate the gel pieces at room temperature for 1 hr in the dark. Then, remove all liquid. Next, add 2 more cycles of 200 µl of ACN to the gel pieces and shaking for 5 min, followed by the removal of all liquid. Add 10 µl of enzyme (10 ng/µl trypsin in 10 mM NH_4HCO_3) to the gel pieces and incubating at 37°C for 3 hr. The peptide was extracted by 3 cycles of added of 50 µl of 50% ACN to the gel pieces and shaking at room temperature for 10 min. Finally, the peptide solutions are transferred into new low binding 96-well plates and dried at 40°C. The dry peptides were storage at -20°C until analysis.

3.1.7 LC-MS/MS data analysis

The LC MS/MS data were analyzed by Decyder MS 2.0 differential analysis software (Amersham biosciences, UK) and searched by Mascot database (www.ncbi.nlm.nih.gov, National Center for Biotechnology Information, U.S. National Library of Medicine, USA). The parameters for analysis were 0.1unit m/z shift tolerance and 5.0% m/z shape tolerance. Group-to-group comparisons are performed by t-test and multi-group comparisons will be performed by ANOVA. The values are normalized with BSA external intensity control. The p-values < 0.05 were considered statistically significant. The comparison of proteins between group and among group were analyzed.

The background proteins from non-infected control (but with anti-TB drugs) were subtracted from the comparison (infection vs. clearance stage of *M. tuberculosis*) to avoid confounding factors affected by anti-TB drugs.

3.1.8 Validation of clearance markers by western blot analysis

Protein samples (100 µg) were analyzed by 14% SDS-PAGE. The primary antibodies corresponded to the selected clearance markers (PSTK and FKBP8) are used to perform western blot analysis. Enhanced chemiluminescence are performed using chemiluminescent reagents.

3.2 Clinical evaluation for clearance markers

3.2.1 Setting and population

The participants from Srinagarind Hospital in Khon Kaen, Thailand, were recruited in this study. These participants include (i) patients with newly detected, active pulmonary TB (ATB group) who were positive for AFB (acid fast bacilli)(and/or culture positive) (n = 20), (ii) people in close contact (i.e., living in the same household) with TB patients or the healthcare workers (LTBI group) with positive LTBI screening test (n = 20), participant with other bacterial infectious disease rather than TB (non-TB control group) (n = 10), and the healthy control (nonpulmonary bacterial infection) (n=10) . The sample size of these participant groups is the convenient sample size. The actual number of samples from each participant will be multiplied by 3 (for non-TB control group) and 4 time-points (for ATB and LTBI group), respectively. The research protocol of this study was sent for consideration and approval by the Research Ethical Committee at Khon Kaen University, Thailand. The information of sample collection from TB patients is described in **Figure 4.**

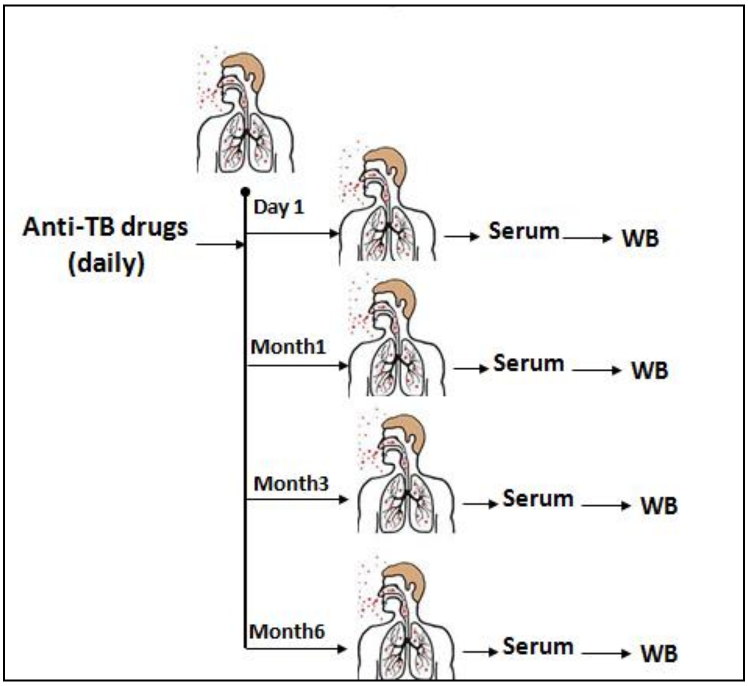


Figure 4 the clearance validation from anti-tuberculous drug treated patients. The clearance markers from *in vitro* experiment were selected to validate using serum samples from TB patients. The serum samples were collected from TB patients before (Day1) and after drug treatment daily for one, three and six month and analyzed by western blot analysis. Both active TB and LTBI patients are included into the tested population in order to evaluate the potential use of the clearance marker covering disease types.

3.2.2 inclusion and exclusion criteria

3.2.2.1 Inclusion criteria

Active tuberculosis (ATB): New cases, symptomatic, untreated, of active TB patients, 15-60 years old, who has sputum AFB positive, PCR (Xpert MTB / RIF) positive or TB culture positive and are going to be treated with anti-TB drugs for 20 cases.

Latent tuberculosis infection (LTBI): Relatives or person who take care of TB patients, 15-60 years old, who had history of TB exposure by living with a TB patient for more than two weeks with infected with tubercle bacilli based on positive for IGRA test and are going to be treated with prophylaxis treatment for LTBI for 20 cases.

Non-TB control (Bacterial infection): Participants 15-60 years old, with respiratory system infection such as patients infected with *Streptococcus pneumonia*, *Haemophilus influenza* or other bacterial pathogens, based on clinical symptoms compatible with respiratory infection such as chest X-ray or positive for bacterial culture from sputum, and are going to be treated with antibiotics for 10 cases.

Healthy control: Healthy persons who has the annual health checkup or healthy volunteer student, with age 15-60 years, for 15 participants (10 participants in the control group and 5 participants for use in primary cell culture).

3.2.2.2 Exclusion criteria

ATB: Patient with extra-pulmonary TB, patient with TB history (old case), patient who receive immunosuppressive agent, patient who HIV positive (based on patient history, clinical symptom or routine laboratory test), **or** patient who are under or used to receive ant-TB drugs or anti-HIV drugs.

LTBI: Patient with TB history, patient with pulmonary symptomatic TB or extrapulmonary TB, patient who are under or used to receive ant-TB drugs or anti-HIV drugs, patient who become HIV positive (based on patient history, clinical symptom or routine laboratory test) or patient who receive immunosuppressive agent, **or** patient who who has negative result of IGRA test.

Non-TB infection: Patient who receive antibiotics within 7 days before visiting to the hospital, patient with TB history, patients with symptomatic TB or extrapulmonary TB, patient who are under or used to receive ant-TB drugs or anti-HIV drugs, patient who become HIV positive (based on patient history, clinical symptom or routine laboratory test) or patient who receive immunosuppressive agent, **or** patient who give positive result of IGRA test or the negative sputum bacterial culture.

Healthy control: Person with evidence of TB infection (IGRA positive), person with history of close contact with TB patient or has the risk of TB infection such as being a healthcare worker with risk of TB exposure, person with chronic disease, person who are under any treatment, person with symptomatic bacterial infection, person who become symptomatic TB, person who become HIV positive (based on patient history, clinical symptom or routine laboratory test) or person who receive anti-HIV, anti-TB drugs or immunosuppressive agent.

3.2.2.3 Withdrawal criteria

ATB: TB patient treated with anti-TB drugs but symptoms does not improve e.g. severe symptoms, hemoptysis, sputum AFB is still positive one month after treatment competition or recurrent TB.

LTBI: patient develop to active TB during treatment, or become HIV positive (based on clinical symptom or routine laboratory test)

Healthy control: person who develop to active TB or become IGRA positive after inclusion.

3.2.3 Sample collection

- For 20 participants of active TB group (ATB) and 20 participants of latent TB group (LTBI), 8 ml of venous blood will be collected for 4 times, i.e. before anti-TB drug treatment and after treatment for 1, 3 and 6 months. In LTBI groups, 3 ml blood will be additionally collected in the first time (before drug treatment) for TB infection screening test (IGRA).

- For 10 bacterial infection control group: 8 ml of venous blood will be collected for 3 times, i.e. before treatment, during treatment and after the treatment (according to the treatment course). For TB infection screening test (IGRA), 3 ml blood will be additionally collected in the first time (before drug treatment).

- For 15 healthy controls, it can be divided,

- For 10 healthy control participants, 11 ml of blood will be collected in which the first 8 ml. will be used as a control group for investigating the clearance marker for TB treatment and the later 3 ml will be collected for TB infection screening test (screening for every time to ensure no infection occur during the study). The blood sample will be collected for 4 times, i.e. at the first time of registration and after the first time for 1, 3 and 6 months.

- For another 5 control participants, 20 ml of venous blood will collect 3 times for the primary cell culture.

3.3 Experiment for SERS and RAMAN spectroscopy

3.3.1 Blood serum collection

The blood collected from Active TB (ATB, n=26), Latent TB (LTBI, n=20), Early clearance (EC, n=34) and Healthy control (HC, n=38) (Total=118 samples) in clot blood tube are incubated at room temperature for 30 minutes after that centrifuge at 2,500 rpm for 10 minutes. The serum was collected and storage at -70 ̊C until analyze.

3.3.2 Proteome preparation

To increase the low MW proteins concentration, the serum protein is mixed with DI water and albumin separated by 10k Macrosep® advance centrifugal devices (Pall Life Sciences). The 10k Macrosep® containing serum are centrifuge at 3,000 g for 45 min and the filtrated protein are collected. The protein measurement by Lowry method and adjust to 0.8 ug/ul.

3.3.3 Raman spectrometry

1 ul of 0.8 ug/ul protein are dropped on aluminum foil for Raman spectrometry detection and air dry for 3 minutes. The protein fingerprint was detected by 532 nm and 785 nm laser wavelength with 16 points detections for each sample.

3.3.4 Surface-enhanced raman spectroscopy (SERs)

1 ul of 0.8 ug/ul protein are dropped on aluminum foil for silver (Ag) chip for SERs detection and air dry for 3 minutes. The protein fingerprint was detected by 785 nm laser wavelength with 16 points detections for each sample.

3.3.5 Protein fingerprint analysis

The Raman spectrometry and Surface-enhanced Raman scattering (SERs) are detected by InViaReflex from Renishaw. The protein fingerprint from Raman spectrometry spectra and SERs are processed and cleaned by WiRETM 4.2 software.

3.3.6 Bioinformatics and data analyses

Protein fingerprint analysis by R program. Sensitivity and specificity detected by randomForest package and the 10 important variables result explained by randomForestExplainer package. Confusion matrix and Statistics analysis by caret package. Boxplot analysis by ggpubr package.

3.4 Experiment for cytokine and chemokine analysis

3.4.1 Study populations and demographic data

The tested populations consisted of 48 ATB (pulmonary TB) patients, 200 healthy persons with a history of TB contact and 52 healthy individuals with no known TB exposure. All individuals were from Srinagarind Hospital, Khon Kaen Thailand (Figure 1). The participants included a subset of those from our previous study.(48) ATB was recognized based on clinical symptoms and a positive result from at least one form of microbiological evidence; staining of acid-fast bacilli, bacterial culture or a molecular test (Xpert MTB/RIF, Cepheid, Sunnyvale, CA, USA). TB exposure was defined as occurring when close contact (CC) persons shared the same house as an ATB patient for at least two weeks. Healthcare workers (HCW) at Srinagarind hospital with a high risk of TB exposure (TB wards, TB clinic, TB laboratory) for at least 6 months were also included. LTBI cases were defined as TB-exposed persons with a QFT-positive result. Individuals with putative EC were defined as TB-contact persons with a QFT-negative result.

Healthy controls (HC) were defined as apparently healthy persons with no evidence of TB exposure. Whole anti-coagulant blood samples were collected and demographic data of individuals recorded. None of the enrolled individuals was HIV-positive, had received immunosuppressive agents or had undergone a TST within three weeks prior to specimen collection. This study was approved by the Khon Kaen University Ethics Committee in Human Research (Ethics number HE551100 and HE561342).

3.4.2 QuantiFERON®-TB Gold In-Tube assay

QFT was performed according to the manufacture's protocol (Cellestis, Victoria, Australia). Briefly, 1 mL of venous blood was obtained from each participant and placed in three QFT tubes (*M. tuberculosis*-specific antigens (TBAg), negative control (Unstim) and mitogen conditions). The tubes were delivered to the laboratory and incubated at 37°C for 24 hours. The plasma was collected, IFN-γ levels measured using an enzyme-linked immunosorbent assay, and the data analyzed using QFT analysis software version 2.62 (Cellestis, Victoria, Australia). An individual was considered positive for TB when the IFN-γ level in the TBAg minus the value of the Unstim was ≥ 0.35 IU/mL and ≥ 25% of the IFN-γ concentration of the Unstim. The IFN-γ levels in IU/mL unit were converted to pg/mL unit by multiplying by a factor of 50.(49)

3.4.3 Quantification of CCL2 and CXCL10

Plasma from the Unstim and TBAg conditions of 300 participants were used to quantify chemokine levels (CCL2 and CXCL10) according to the manufacturer's instructions (Biolegend, California, USA). The optical density (OD) of chemokines was measured using a Sunrise™ absorbance reader (Tecan, Männedorf, Switzerland).

The minimum detectable limits of the assays used are 3.9 pg/mL for CCL2 and 4 pg/mL for CXCL10. When concentrations exceeded the assay range, appropriate dilutions were made and the assay repeated.

3.4.4 Data analysis

Demographic characteristics of four groups (ATB, LTBI, EC and HC) were analyzed using Student’s t-test, Mann-Whitney *U* test or chi-square tests, based on type of data. Participants returning a QFT-positive result were excluded from the HC group in analysis (Figure 1). Receiver operating characteristic (ROC) curves were used to evaluate the diagnostic performance of selected biomarkers. The area under the curves (AUC) and 95% confidence intervals (CI) were calculated for each marker. The optimum cut-off value was obtained by the calculation of maximum Youden’s index (YI) based on the formula “sensitivity + specificity -1”. McNemar’s test was used to compare the paired proportion between different diagnostic tests. These analyses were performed in SPSS version 16 (SPSS Inc., Illinois, USA). Comparisons of cytokine and chemokine levels among groups were generated and analyzed using a trial version of GraphPad Prism 5.03 (GraphPad Software, California, USA). For all analyses, p-values <0.05 were considered to indicate statistical significance.

4. แผนการดำเนินการ

แผนการดำเนินการวิจัยตามรายละเอียดวัตถุประสงค์ของการดำเนินงาน

Objectives	Months					Places for conducting experiments	ected outputs
	1st Year		2nd Year		ช่วงขยาย		
	1st - 6th	7th -12th	1st - 6th	7th -12th	1st - 6th		
1. **Establishment and prerequisite** - Ethical approval for human research - Primary cell and Mtb culture						Microb, MD, KKU [a]	- Ethical approval - Optimized protocol
2. Optimization and recruitment - condition for the *in vitro* experiment - Recruitment of TB patients and sample						BSL3, NELAC , KKU [b]	- Optimal condition for *in vitro* experiments
3. Infection and clinical samples - Infection experiment and protein extraction/ prepraration - Serum samples and protein preparation						BSL3, NELAC , KKU [b]	- Infection protocol - proteins samples - serum samples
4. LC-MS MS analysis and high throuput analysis						BIOTEC [c]	- LC MS/MS data
5. Western blot analysis: evaluation LC MS/MS data and evaluation using the serum from TB patients						Microb, MD, KKU [a]	- Western blot data
6. Data analysis and summarization						Microb, MD, KKU [a]	- Draft of manuscript (s)
7. Preparation of report and publications						Microb, MD, KKU [a]	1 report, 1-2 publications

[a] Microbiol, MD, KKU: Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Khan, Thailand

[b] NELAC, KKU: Northeastern Laboratory Animal Center, Khon Kaen University, Khon Kaen, Thailand

5. ผลการวิจัยที่ได้รับและการอภิปรายผล

5.1 Clearance biomarkers

5.1.1 Protein collection

The extracellular (supernatant) of the test and control were collected. All of protein solutions were storage at -70°C until the precipitation process.

5.1.2 Protein concentration measurement

5.1.2.1 Extracellular protein

For Extracellular protein Lowry method was used. The standard samples (n = 6) were measured for 3 times each. Then, the OD value at 750 nm of standard samples were plotted as the standard curve, by x= absorbance and y= protein concentration (ug/µl) (Figure 5). The obtained standard curve gave R-square 0.995. Then, the proteins samples from the experiments were measured and compared to the standard curve. The concentrations of the protein were calculated (Table 1).

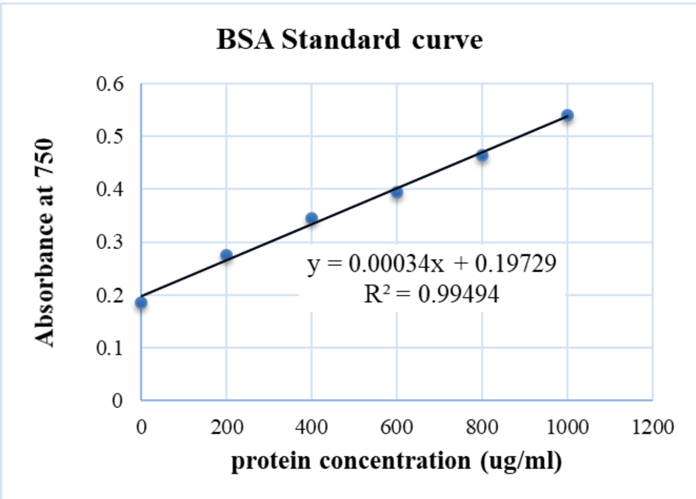


Figure 5. Standard curve of Lowry method. Standards and samples were measured 3 times and then calculated for the average before diluted to be 50µg/well for SDS PAGE test.

Table 1 Protein concentration of the samples from secreted protein.

Sample	Average (µg/µl)	Dilution factor	Protein concentration (µg/ml)
Ex1D1 *S.aureus*	405.03	10	4050.29
Ex1D1 H37Rv	235.42	10	2354.22
Ex1D1 BjDs	395.23	10	3952.25

Ex1D1 IO	246.21	10	2462.06
Ex1D1 EUA	362.87	10	3628.73
Ex1D1 Pen	375.62	10	3756.18
Ex1D1 INH/RIF	344.25	10	3442.45
Ex1D5 *S.aureus*	376.6	10	3765.98
Ex1D5 H37Rv	343.26	10	3432.65
Ex1D5 BjDs	373.66	10	3736.57
Ex1D5 IO	380.52	10	3805.2
Ex1D5 EUA	368.75	10	3687.55
Ex1D5 Pen	309.93	10	3099.31
Ex1D5 INH/RIF	321.7	10	3216.96
Ex2D1 *S.aureus*	393.26	10	3932.65
Ex2D1 H37Rv	391.3	10	3913.04
Ex2D1 BjDs	393.26	10	3932.65
Ex2D1 IO	365.81	10	3658.14
Ex2D1 EUA	421.7	10	4216.96
Ex2D1 Pen	428.56	10	4285.59
Ex2D1 INH/RIF	434.44	10	4344.41
Ex2D5 *S.aureus*	418.75	10	4187.55
Ex2D5 H37Rv	417.77	10	4177.75
Ex2D5 BjDs	409.93	10	4099.31
Ex2D5 IO	399.15	10	3991.47
Ex2D5 EUA	407.97	10	4079.71
Ex2D5 Pen	430.53	10	4305.26
Ex2D5 INH/RIF	278.77	10	2787.72
Ex3D1 *S.aureus*	421.75	10	4217.54
Ex3D1 H37Rv	288.42	10	2884.21
Ex3D1 BjDs	392.81	10	3928.07
Ex3D1 IO	404.21	10	4042.11
Ex3D1 EUA	376.14	10	3761.4
Ex3D1 Pen	405.09	10	4050.88
Ex3D1 INH/RIF	375.26	10	3752.63
Ex3D5 *S.aureus*	402.46	10	4024.56
Ex3D5 H37Rv	408.6	10	4085.96
Ex3D5 BjDs	398.07	10	3980.7
Ex3D5 IO	345.44	10	3454.39
Ex3D5 EUA	355.96	10	3559.65
Ex3D5 Pen	420	10	4200
Ex3D5 INH/RIF	418.25	10	4182.46

Note: Ex=experiment, Number (1,2,3) = number of experiment, D=day of experiment, Number (1,5)= day of samples, *S.aureus*= *S.aureus* infected cells with penicillin treatment, H37Rv = *M.*

tuberculosis H37Rv infected cells with anti TB drugs, Bj= *M. tuberculosis* Bj infected cells with anti TB drugs, IO= *M. tuberculosis* IO infected cells with anti TB drugs. EUA = *M. tuberculosis* EUA infected cells with anti TB drugs. Pen= uninfected cell with penicillin treatment, INH/RIF= uninfected cell with anti TB drugs.

5.1.2.3 Bradford dye binding assay

For serum protein Bradford dye binding protein assay was used. The standard samples (n = 6) were measured for 3 times each. Then, the OD value at 540 nm of standard samples were plotted as the standard curve, by x= absorbance and y= protein concentration (ug/ml) (Figure 6). The obtained standard curve gave R-square 0.995. Then, the proteins samples from the experiments were measured and compared to the standard curve. The concentrations of the protein were calculated (Table 2).

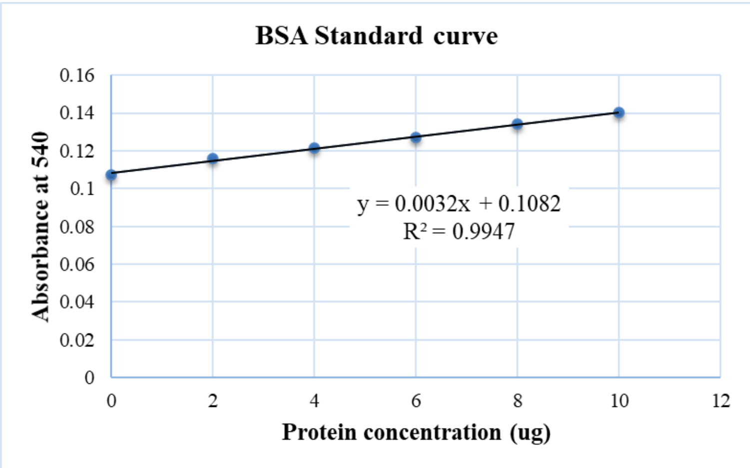


Figure 6. Standard curve of Bradford dye binding protein assay. Standards and samples were measured 3 times and then calculated for the average before diluted to be 100µg/well for SDS PAGE test.

Table 2. Protein concentration of the samples from serum protein.

Sample	Average (µg/µl)	Dilution factor	Protein concentration (µg/ul)
ATB1S	1.57	50	78.54
ATB2S	1.74	50	86.88
ATB3S	1.95	50	97.29

ATB1E	2.07	50	103.54
ATB2E	2.03	50	101.46
ATB3E	1.99	50	99.38
LTBI1S	2.18	50	108.75
LTBI2S	2.13	50	106.67
LTBI3S	2.09	50	104.58
LTBI1E	1.97	50	98.33
LTBI2E	1.88	50	94.17
LTBI3E	1.86	50	93.13
HC1S	1.78	50	88.96
HC2S	1.93	50	96.25
HC3S	1.82	50	91.04
HC1E	1.76	50	87.92
HC2E	1.97	50	98.33
HC3E	2.01	50	100.42

Note: ATB= Active TB serum, LTBI= Latent TB serum, HC= Healthy control, number (1,2,3) = Patient number, S= Sample at start point, E= Sample at end point.

5.1.3 1D SDS-PAGE

The protein concentration was measure by Lowry method and the protein concentration were calculated and adjusted to 50 μg/well. The protein solutions were run by SDS-PAGE analysis and the results were show in figure 7.

The extracellular proteins adjusted the concentration at 50 μg were run on SDS-PAGE. It was found that the extracellular protein (supernatant) showed the thick bands at the MW about 45-97 kDa which was the albumin band from the media supplement (FBS). The albumin bands were the contaminated proteins which were not the target for analysis and potentially interfered the LC-MS/MS analysis. In the LC MS/MS analysis, only the proteins band lower that the albumin bands were selected to perform the test. The gel was cut into 13 pieces for each sample, and each gel plug was further cut into 1-mm and transferred into low binding 96-well plates. Tryptic digestion was performed by incubate the gel pieces with 25 mM NH4HCO3 for 10 min, add 200 μl acetonitrile (ACN) and incubate for 10 min with shaking. After ACN removal, 50 μl of 10 mM DTT in 10 mM NH4HCO3 was added and incubated at 56°C for 1 hr. Next, add 50 μl of 100 mM iodoacetamide in 10 mM NH4HCO3, and then, incubate the gel pieces at room temperature for 1 hr in the dark. Then, remove all liquid. Next, add 2 more cycles of 200 µl of ACN to the gel pieces and shaking for 5 min, followed by the removal of all liquid. Add 10 µl of enzyme (10 ng/µl trypsin in 10 mM NH4HCO3) to the gel pieces and incubating at 37°C for 3 hr. The peptide was extracted by 3 cycles of added of 50 µl of 50% ACN to the gel pieces and shaking at room temperature for 10 min. Finally, the peptide solutions are transferred into new low binding 96-well plates and dried at 40°C. The dry peptides were storage at -20°C until analysis.

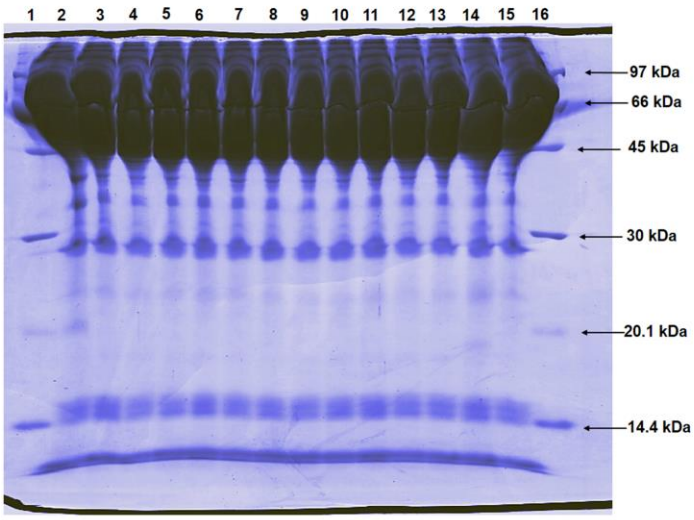


Figure 7. SDS-PAGE of 50 µg extracellular protein from PBMC infected with *M. tuberculosis* (H37Rv, Bj, IO, EUA), *S.aureus* and treated with anti-tuberculosis drugs.
Lane1=Protein markers, Lane2= D1 *S.aureus*, Lane3= D1 H37Rv, Lane4= D1 BjDs, Lane5= D1 IO, Lane6= D1 EUA, Lane7= D1 Pen, Lane8= D1 INH/RIF, Lane9= D5 *S.aureus*, Lane10= D5 H37Rv, Lane11= D5 BjDs, Lane12= D5 IO, Lane13= D5 EUA, Lane14= D5 Pen, Lane15= D5 INH/RIF, Lane16=Protein markers. D = day of experiment.

5.1.4 Proteomic profile of infection and clearance stage of MTB infected PBMC after anti tuberculous treatment.

After LC MS/MS analysis, 1073 unique elements from extracellular proteome differentially compared between infection and clearance stage and not found in control were revealed.

5.1.4.1 Linage response specific proteins

There were 63 extracellular proteins elements that were uniquely found in the infection stage (Figure 8). There were 25 proteins that consistently found in all type of *M. tuberculosis* used in this experiment but not found in uninfected control condition. There were three proteins uniquely found in uninfected condition. There were three, one and five proteins are found in H37Rv, IO and EUA infected condition respectively.

There were 77 extracellular proteins elements that were uniquely found in the clearance stage when compare to uninfected condition (Figure 14). 31 proteins all type of *M. tuberculosis* used in this experiment but not found in uninfected control condition. The protein that uniquely found in H37Rv, Bj, IO and EUA was four, one, three and one element.

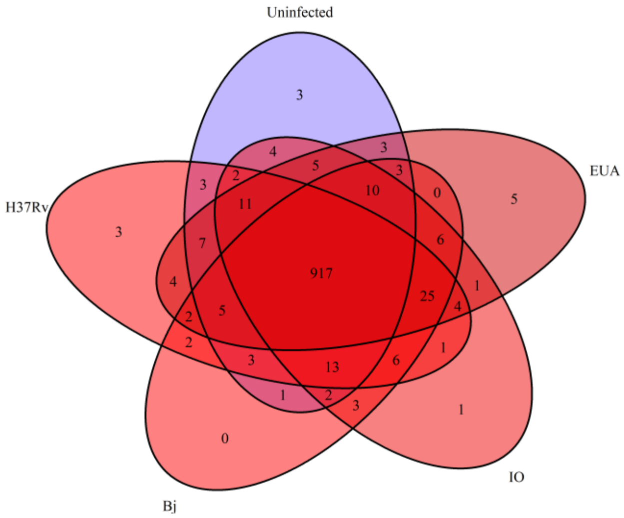


Figure 8. Venn's diagram of 5 samples extracellular proteome analysis. Numbers show the number of protein elements found in each condition. Uninfected referred to control (uninfected cells with anti TB drugs) in day 1. H37Rv referred to test (*M. tuberculosis* H37Rv infected cells with anti TB drugs) in day 1. Bj referred to test (*M. tuberculosis* Bj infected cells with anti TB drugs). IO referred to test (*M. tuberculosis* IO infected cells with anti TB drugs) in day 1. EUA referred to test (*M. tuberculosis* EUA infected cells with anti TB drugs) in day 1.

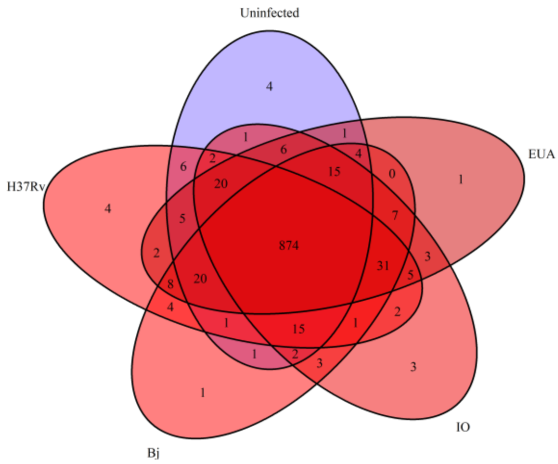


Figure 9. Venn's diagram of 5 samples extracellular proteome analysis. Numbers show the number of protein elements found in each condition. Uninfected referred to control (uninfected cells with

anti TB drugs) in day 5. H37Rv referred to test (*M. tuberculosis* H37Rv infected cells with anti TB drugs) in day 5. Bj referred to test (*M. tuberculosis* Bj infected cells with anti TB drugs) in day 5. IO referred to test (*M. tuberculosis* IO infected cells with anti TB drugs) in day 5. EUA referred to test (*M. tuberculosis* EUA infected cells with anti TB drugs) in day 5.

5.1.4.2 Differential proteins specific to infection and clearance stage

The protein response in infection stage and clearance stage of differences *M. tuberculosis* (H37Rv, Bj, IO, EUA) infection were shown in figure 15 and figure 10)

There were 10 proteins uniquely found in clearance stage of *M. tuberculosis* H37Rv and 10 proteins were sustained found in both infection and clearance stage (figure15A and figure16A). For *M. tuberculosis* Bj linage infections, were found 1, 8 and 10 proteins in infection stage, clearance stage and both stages respectively (figure15B and figure16B). For *M. tuberculosis* IO linage infections, were found 4, 5 and 9 proteins in infection stage, clearance stage and both stages respectively (figure15C and figure16C). For *M. tuberculosis* EUA linage infections, were found 3, 6 and 12 proteins in infection stage, clearance stage and both stages respectively (figure15D and figure16D).

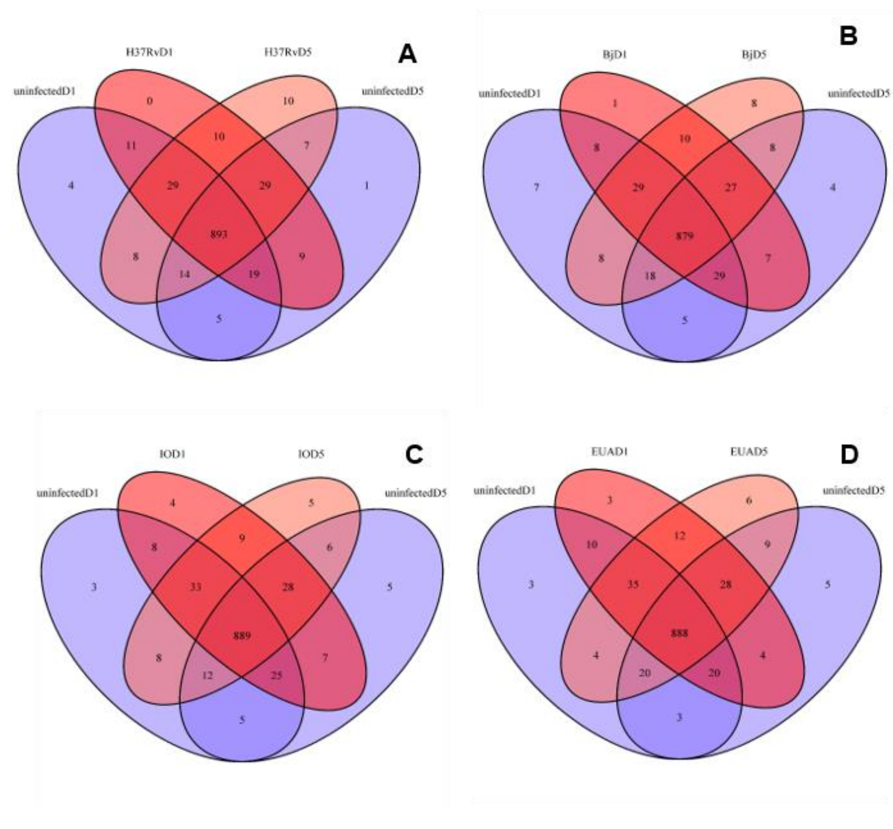


Figure 10. Venn's diagram of *M. tuberculosis* (H37Rv, Bj, IO, EUA) infection stage and clearance stage. Numbers show the number of protein elements found in each condition. Uninfected

referred to control (uninfected cells with anti TB drugs). H37Rv referred to test (*M. tuberculosis* H37Rv infected cells with anti TB drugs). Bj referred to test (*M. tuberculosis* Bj infected cells with anti TB drugs). IO referred to test (*M. tuberculosis* IO infected cells with anti TB drugs). EUA referred to test (*M. tuberculosis* EUA infected cells with anti TB drugs). D follow by number referred to day of experiment (1=infection stage, 5=clearance stage).

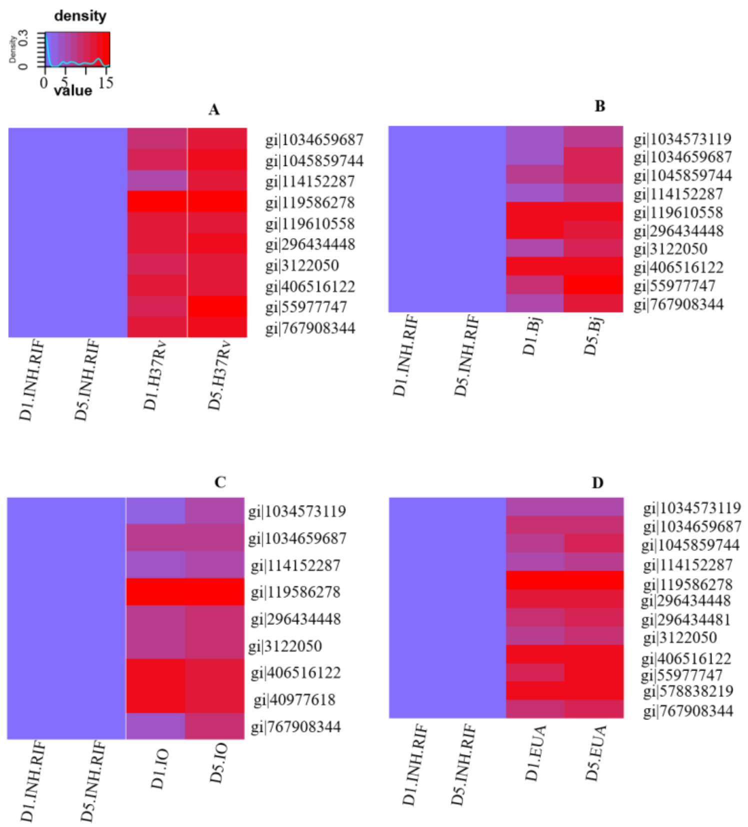


Figure 11. Heat map show the protein that express in each *M. tuberculosis* (H37Rv, Bj, IO, EUA) condition at day 1 and 5 but have a significant difference in their level from 1 and 5 day of test. Color from blue to red color represent the protein intensity from 0-15, The gi number referred to the protein gi number, D1.INH.RIF referred to control (uninfected cells with anti TB drugs at day1), D5.INH.RIF referred to control (uninfected cells with anti TB drugs at day5)
, H37Rv referred to test (*M. tuberculosis* H37Rv infected cells with anti TB drugs), Bj referred to test (*M. tuberculosis* Bj infected cells with anti TB drugs), IO referred to test (*M. tuberculosis* IO infected cells with anti TB drugs), EUA referred to test (*M. tuberculosis* EUA infected cells with anti TB drugs), D follow by number referred to day of experiment (1=infection stage, 5=clearance stage).

5.1.4.3 Clearance stage specific proteins

There were four (L-seryl-tRNA(Sec) kinase (PSTK), FK506-binding protein 8 (FKBP8), O-6-methylguanine-DNA methyltransferase (MGMT), B-cell CLL/lymphoma 9-like protein) extracellular protein elements that were uniquely found in the clearance stage of all *M. tuberculosis* (H37Rv, Bj, IO, EUA) infection condition but not found in other bacterial infection *(S.aureus)* as show in figure 12. The relative intensity of protein detected by LC-MS/MS of PSTK, FKBP8, MGMT and B-cell CLL/lymphoma 9-like protein were show in figure 13.

PSTK, FKBP8 and MGMT were found in *M. tuberculosis* (H37Rv, Bj, IO, EUA) clearance condition but not in other (control and *S.aureus*) condition. For B-cell CLL/lymphoma 9-like protein were found in infection stage (Bj, IO, EUA) and clearance stage (H37Rv, Bj, IO, EUA).

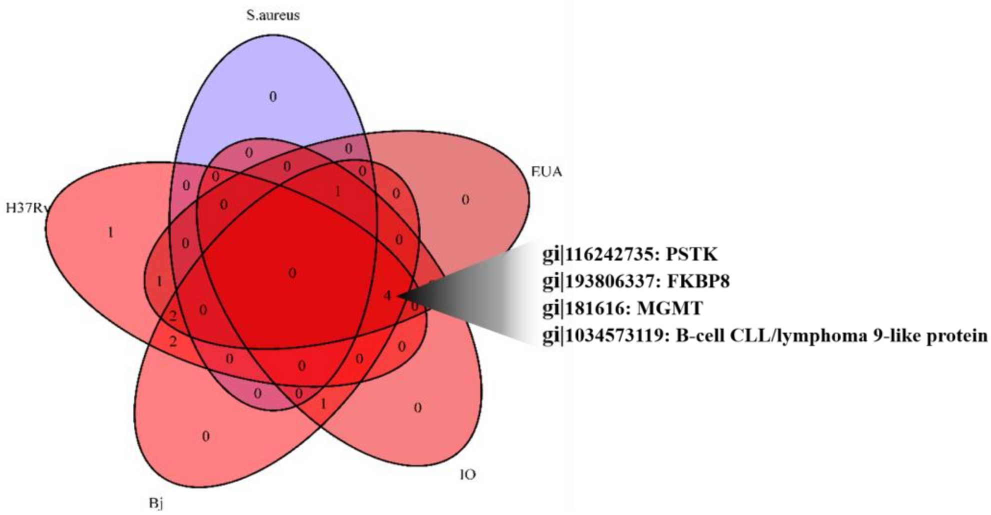


Figure 12. Venn's diagram of *M. tuberculosis* (H37Rv, Bj, IO, EUA) in clearance stage compared with *S.aureus*. Numbers show the number of protein elements found in each condition. Uninfected referred to control (uninfected cells with anti TB drugs). H37Rv referred to test (*M. tuberculosis* H37Rv infected cells with anti TB drugs). Bj referred to test (*M. tuberculosis* Bj infected cells with anti TB drugs). IO referred to test (*M. tuberculosis* IO infected cells with anti TB drugs). EUA referred to test (*M. tuberculosis* EUA infected cells with anti TB drugs). *S.aureus* referred to test (*S.aureus* infected cells with penicillin treatment).

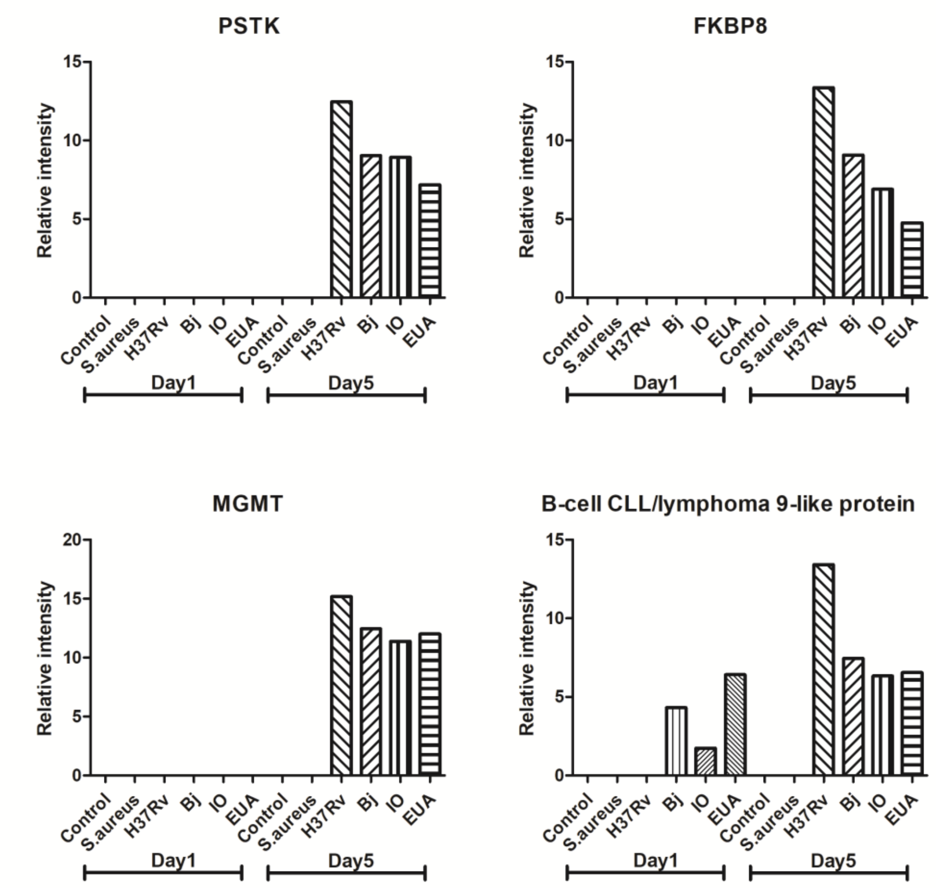


Figure 13. Relative intensity of PSTK, FKBP8, MGMT, B-cell CLL/lymphoma 9-like protein at infection stage and clearance stage detected by LC-MS/MS. H37Rv referred to test (*M. tuberculosis* H37Rv infected cells with anti TB drugs). Bj referred to test (*M. tuberculosis* Bj infected cells with anti TB drugs). IO referred to test (*M. tuberculosis* IO infected cells with anti TB drugs). EUA referred to test (*M. tuberculosis* EUA infected cells with anti TB drugs). *S.aureus* referred to test (*S.aureus* infected cells with penicillin treatment), Uninfected referred to control (uninfected cells with anti TB drugs), day1 referred to infection stage, day5 referred to clearance stage.

5.1.5 Biomarkers validation

Protein samples (50 μg) are analyzed by 14% SDS-PAGE. The proteins are transferred onto nitrocellulose membranes (0.2 μm, Bio-Rad, USA) by a blotting machine (Trans-Blot SD semi-dry transfer cell, Bio-Rad, USA). Protein signal detected by ECL reagent (GE Healthcare Life Sciences) and signal collected by imageQuant 600 (GE Healthcare Life Sciences). Signal analysis by ImageJ program. The target protein signal is normalized by transferrin signal.

5.1.5.1 Secreted protein

5.1.5.1.1 Total protein signal in SDS PAGE

Protein samples (100 μg) from cell culture supernatant are analyzed by 14% SDS-PAGE. Signal collected by imageQuant 500 (GE Healthcare Life Sciences. The triplicate running of SDS PAGE was used as total protein control for secreted protein detection (figure14). Total protein signal analysis by ImageJ program and plot by Prism5 program. The 100 μg secreted protein from each sample were not significate difference in SDS-PAGE (figure15).

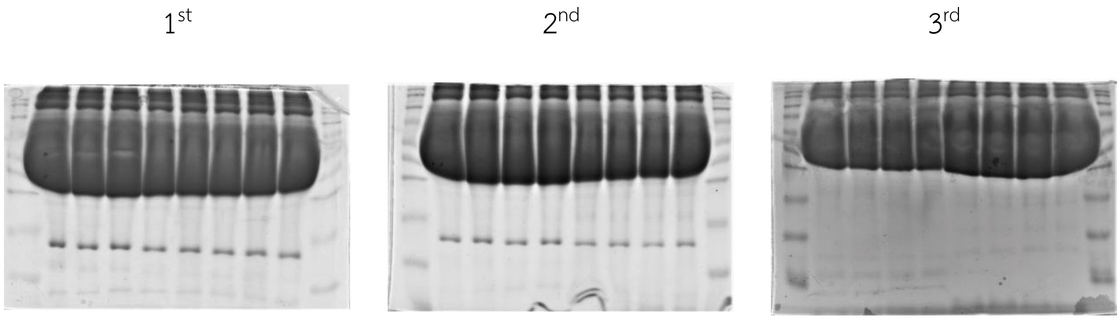


Figure 14. SDS-PAGE of 50 μg extracellular protein from PBMC infected with *M. tuberculosis* (H37Rv, Bj, IO, EUA*)* at day 1 (infection stage) and day5 (clearance stage). Lane1=Protein markers, Lane2= D1 H37Rv, Lane3= D1 BjDs, Lane4= D1 IO, Lane5= D1 EUA, Lane6= D5 H37Rv, Lane7= D5 BjDs, Lane8= D5 IO, Lane9= D5 EUA, Lane10=Protein markers. D = day of experiment.

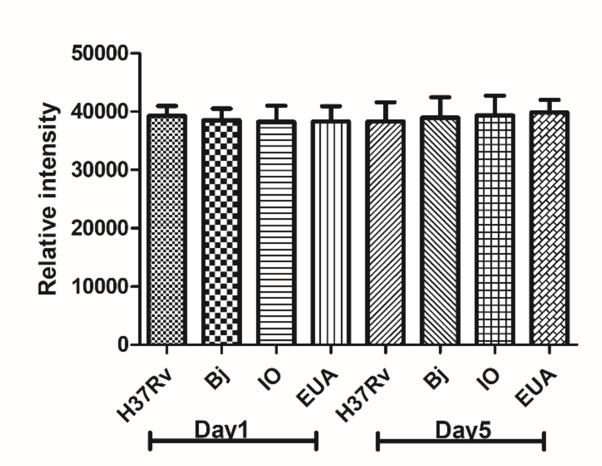


Figure 15. Relative intensity of total protein from SDS-PAGE. H37Rv referred to test (*M. tuberculosis* H37Rv infected cells with anti TB drugs). Bj referred to test (*M. tuberculosis* Bj infected cells with anti TB drugs). IO referred to test (*M. tuberculosis* IO infected cells with anti TB drugs).

EUA referred to test (*M. tuberculosis* EUA infected cells with anti TB drugs). day1 referred to infection stage, day5 referred to clearance stage.

5.1.5.1.2 Western blot analysis of FKBP8 protein

FKBP8 protein analysis by western blot using 1:100 ul of anti FKBP8. The signal developed by anti-muse igG-HRP antibody (1:2,000) and signal detected by ECL reagent (GE Healthcare Life Sciences). The protein signal collected by imageQuant 500 (GE Healthcare Life Sciences) and analysis by ImageJ program (figure16). Western blot signal of FKBP8 were show in figure 17 and the signal analysis were show in figure 22.

FKBP8 from secreted protein were significant difference when compare between infection stage with clearance stage of H37Rv ($P<0.01$), IO ($P<0.05$) and EUA ($P<0.05$) but not significant difference in Bj linage.

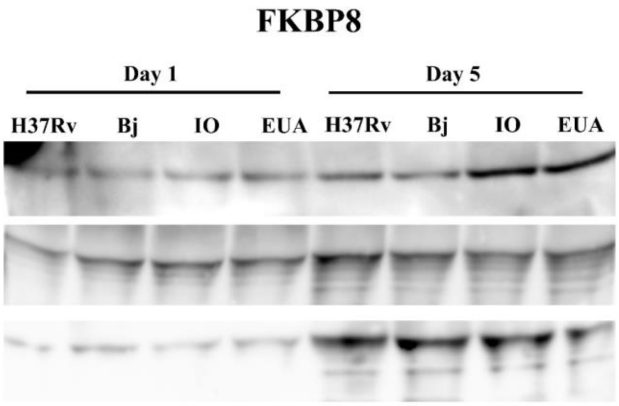


Figure 16. Western blot analysis of FKBP8 protein. H37Rv referred to test (*M. tuberculosis* H37Rv infected cells with anti TB drugs). Bj referred to test (*M. tuberculosis* Bj infected cells with anti TB drugs). IO referred to test (*M. tuberculosis* IO infected cells with anti TB drugs). EUA referred to test (*M. tuberculosis* EUA infected cells with anti TB drugs). day1 referred to infection stage, day5 referred to clearance stage.

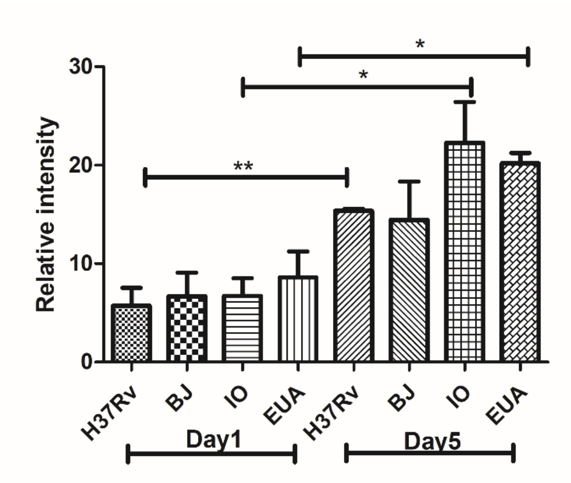


Figure 17. Relative intensity of FKBP8 form secreted protein analysis by western blot. H37Rv referred to test (*M. tuberculosis* H37Rv infected cells with anti TB drugs). Bj referred to test (*M. tuberculosis* Bj infected cells with anti TB drugs). IO referred to test (*M. tuberculosis* IO infected cells with anti TB drugs). EUA referred to test (*M. tuberculosis* EUA infected cells with anti TB drugs). Uninfected referred to control (uninfected cells with anti TB drugs), day1 referred to infection stage, day5 referred to clearance stage, * referred to P value<0.05, ** referred to P value<0.01.

5.1.5.1.3 Western blot analysis of PSTK protein

PSTK protein analysis by western blot using 1:100 ul of anti FKBP8. The signal developed by anti-muse igG-HRP antibody (1:2,000) and signal detected by ECL reagent (GE Healthcare Life Sciences). The protein signal collected by imageQuant 500 (GE Healthcare Life Sciences) and analysis by ImageJ program. Western blot signal of FKBP8 were show in figure 18 and the signal analysis were show in figure 19.

PSTK from secreted protein were show signal expression in clearance stage of *M. tuberculosis* (H37Rv, Bj, IO, EUA) but not in infection stage.

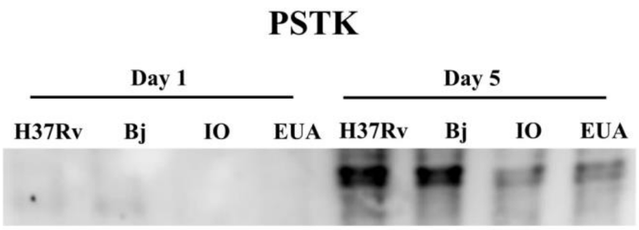


Figure 18. Western blot analysis of PSTK protein. H37Rv referred to test (*M. tuberculosis* H37Rv infected cells with anti TB drugs). Bj referred to test (*M. tuberculosis* Bj infected cells with anti TB drugs). IO referred to test (*M. tuberculosis* IO infected cells with anti TB drugs). EUA referred to test (*M. tuberculosis* EUA infected cells with anti TB drugs). day1 referred to infection stage, day5 referred to clearance stage.

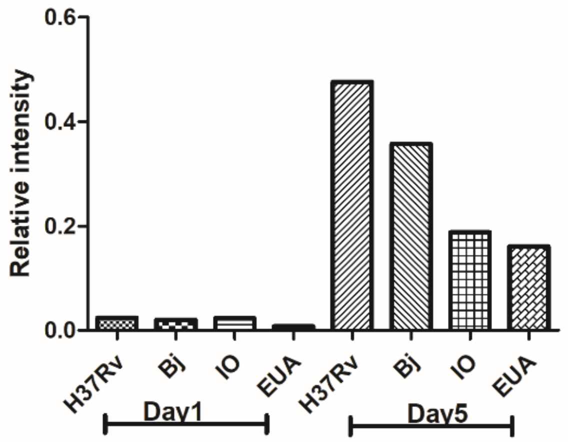


Figure 19. Relative intensity of PSTK form secreted protein analysis by western blot. H37Rv referred to test (*M. tuberculosis* H37Rv infected cells with anti TB drugs). Bj referred to test (*M. tuberculosis* Bj infected cells with anti TB drugs). IO referred to test (*M. tuberculosis* IO infected cells with anti TB drugs). EUA referred to test (*M. tuberculosis* EUA infected cells with anti TB drugs). Uninfected referred to control (uninfected cells with anti TB drugs), day1 referred to infection stage, day5 referred to clearance stage.

5.1.6 Serum protein evaluation

Serum protein samples (100 µg) are analyzed by 14% SDS-PAGE. The proteins are transferred onto nitrocellulose membranes (0.2 µm, Bio-Rad, USA) by a blotting machine (Trans-Blot SD semi-dry transfer cell, Bio-Rad, USA). The PSTK proteins was detected by specific antibody (1:100). The target protein signal is controlled by anti-transferrin (1:2,000). Protein intensity from start point (D0) and end point (completed) after drug treatment was shown in figure 20. The signal intensity analysis by ImageJ program and plot by Prism program (figure 21).

PSTK signal from serum protein show significant different between before drug treatment with completed drug treatment from 3 LTBI ($P<0.0001$), 3ATB ($P<0.05$). The protein signal was difference when compare between healthy control with LTBI ($P<0.0001$) and compare between healthy control with ATB (P<0.0001). PSTK signal from healthy control compared with LTBI before treatment show significant difference with p value < 0.05 but not significant when compared between healthy control with ATB before treatment.

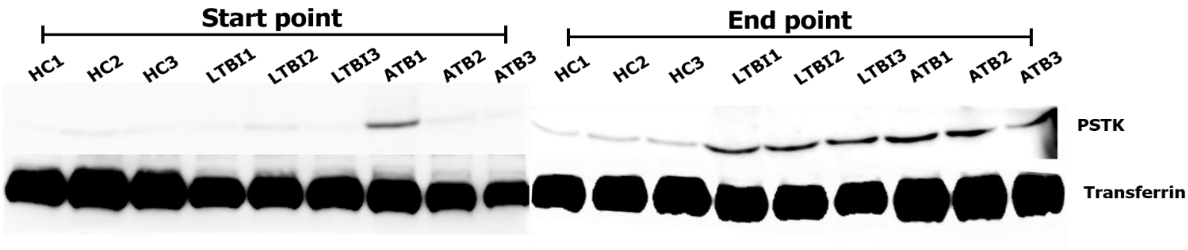


Figure 20. Western blot analysis of PSTK protein from serum sample. HC referred to healthy person, LTBI referred to latent TB patient, ATB referred to Active TB patient, number referred to patient number, start point referred to before drug treatment, end point referred to completed treatment (9 month).

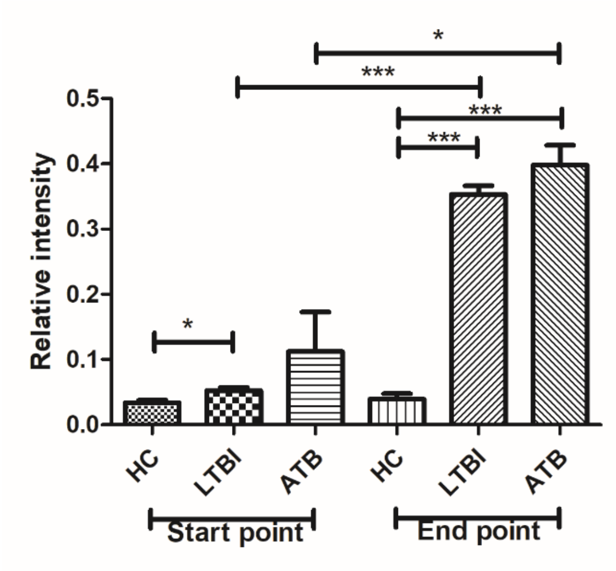


Figure 21. Relative intensity of PSTK form 100ug serum protein analysis by western blot. HC referred to healthy person, LTBI referred to latent TB patient, ATB referred to Active TB patient, number referred to patient number, start point referred to before drug treatment, end point referred to completed treatment (9 month). * referred to P value <0.05, ** referred to P value <0.01, *** referred to P value <0.001.

5.1.7 Discussion for clearance markers

The biomarker indicating the clearance stage of *M. tuberculosis* infection could facilitate the treatment monitoring both active and LTBI. Previously, there were several studies related to proteomic markers for indicating LTBI and active TB (25-28). Previously, the proteomic studies for exploring the biomarker for monitoring TB outcome were based on the clinical evidence and microbiological evidences, especially sputum AFB and culture (29, 30). Others applied molecular and serological marker for TB treatment monitoring (31-33). Resistin was found as a promising biomarker for TB treatment monitoring in TB patients (13). Animal study is the promising models for studies the *M. tuberculosis* clearance because the *M. tuberculosis* sterilization can be ensured from all tissue organs. However, these studies did not ensure the *M. tuberculosis* clearance stage. Our previous study exploring the clearance markers using monocytic cell line (THP-1 cells). We found the potential markers indicating the clearance of *M. tuberculosis* from host cells. However, the experiment in multicellular or actual host might provide different biological results. To response for this gap, we used the PBMC infected with *M. tuberculosis* and GeLC-MS/MS for screening the biomarkers indicating the clearance stage of *M. tuberculosis* infection. We found several potential proteins that might be used as the biomarkers to indicate the clearance of MTB after anti-tuberculous treatment.

There were 1073 protein element from extracellular (culture supernatants). Compare between the infection stage and clearance stage, there were proteins infection stage and clearance stage specific proteins. There were 63 extracellular proteins elements that were uniquely found in the infection stage, 25 proteins that consistently found in all type of *M. tuberculosis* used in this experiment but not found in uninfected control condition. There were three proteins absent after *M. tuberculosis* infection. There were three, one and five proteins are found in H37Rv, IO and EUA infected condition, respectively. There were 77 extracellular proteins elements that were uniquely found in the clearance stage when compare to uninfected condition, 31 proteins found all type of *M. tuberculosis* used in this experiment but not found in uninfected control condition. The protein that uniquely found in H37Rv, Bj, IO and EUA was four, one, three and one proteins element. There were 10 proteins uniquely found in clearance stage of H37Rv and 10 proteins were sustained found in both infection and clearance stage. For Bj linage infections, were found 1, 8 and 10 proteins in infection stage, clearance stage and both stages respectively. For IO linage infections, were found 4, 5 and 9 proteins in infection stage, clearance stage and both stages respectively. For EUA linage infections, were found 3, 6 and 12 proteins in infection stage, clearance stage and both stages respectively.

There were 4 proteins uniquely found in the clearance stage. PSTK, FKBP8 and MGMT are uniquely found in clearance stage of all *M. tuberculosis* (H37Rv, Bj, IO, EUA) strain due to LC-MS/MS data. PSTK and FKBP8 are selected to validated in secreted protein. Our experiment found PSTK presence with high quantity and the associated function to *M. tuberculosis* clearance stage from western blot analysis of secreted protein and from serum protein of ATB and LTBI after completed drug treatment (P value <0.001) compared to untreated condition. FKBP8 had a function as prevent the accumulation of misfolded proteins, prevent endoplasmic reticulum-associated apoptosis and protects the heart from hemodynamic stress (50). FKBP8 had a function as prevent apoptosis during mycophagy (51) , antiviral signaling regulation (52). FKBP8 might play role in anti-tissue damage and protect hemodynamic stress after clearance of *M. tuberculosis* of lung tuberculosis patient. PSTK had a function associated with cell proliferation (53) and increased in lung fibrosis caused by paraquat poisoning (54). PSTK might play role in cell multiplication after clearance of *M. tuberculosis.*

Based on high throughput screening approach, LC MS/MS, in the PBMC infection model, we showed that there were several proteins that can potentially use as the biomarkers for TB treatment monitoring. There were 4 extracellular uniquely found in the clearance stage. Two proteins that were promising to use as MTB clearance biomarker after anti-tuberculous treatment were PSTK and FKBP8. PSTK show high quantity and associated with MTB clearance stage from western blot analysis of secreted protein. PSTK protein show possibility to use as treatment monitoring marker of LTBI and ATB after drug treatment.

5.2 Diagnostic markers for LTBI and active TB

5.2.1 SERS and RAMAN Spectroscopy

5.2.1.1 Protein concentration optimization for Raman spectroscopy

1 ul of serial diluted protein from 0, 0.4, 0.8 and 1.0 ug/ul are drop on on aluminum foil and air dry for 3 minutes. The dry protein was detected by Raman spectrometry at 785 nm. 0.8 ug/ul protein show highest signal when compared to other protein concentration (Figure 22).

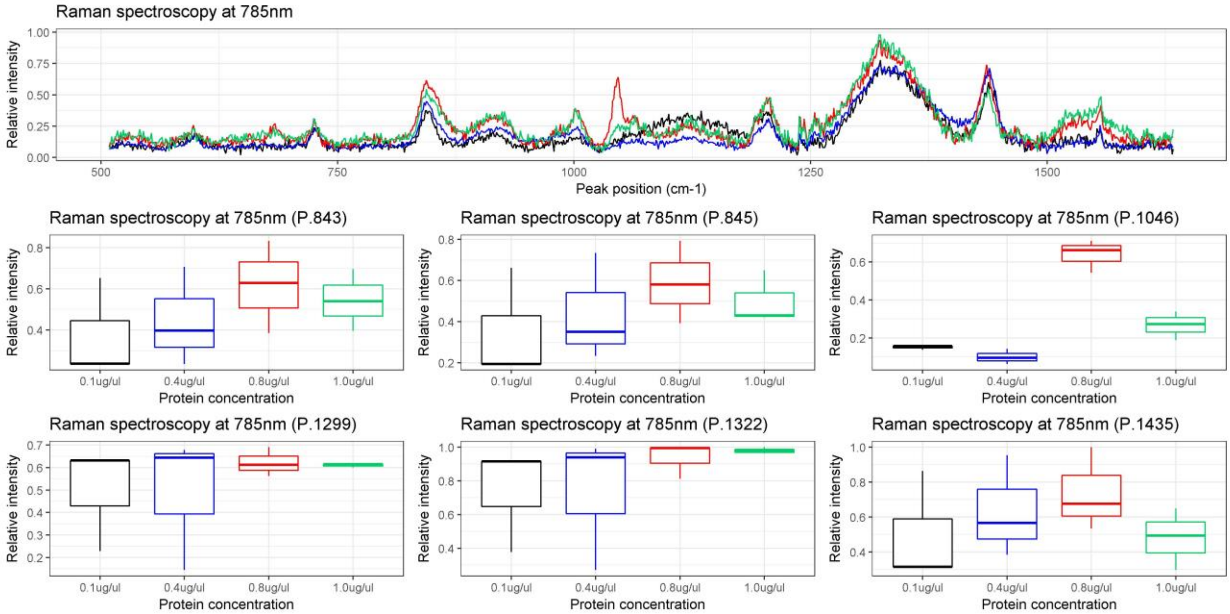


Figure 22. Protein concentration optimization for Raman spectroscopy detection. Line plot are referred to average of protein fingerprint detected**;** Black line referred to protein fingerprint at 0.1 ug/ul, blue line referred to protein fingerprint at 0.4 ug/ul, red line referred to protein fingerprint at 0.8 ug/ul, green line referred to protein fingerprint at 1.0 ug/ul. Boxplot present relative intensity comparison of protein concentration 0.1, 0.4, 0.8,1.0 ug/ul form peak position at 843, 845, 1046, 1299, 1322 and 1435 cm^{-1}.

5.2.1.2 Protein condition optimization for Raman spectroscopy

1 ul of 0.8 ug/ul protein in soluble condition and dry condition are detected by Raman spectrometry at 532 nm and Raman spectrometry at 785 nm. Protein in solid condition was higher in relative intensity signal when compared to soluble condition (Figure 23).

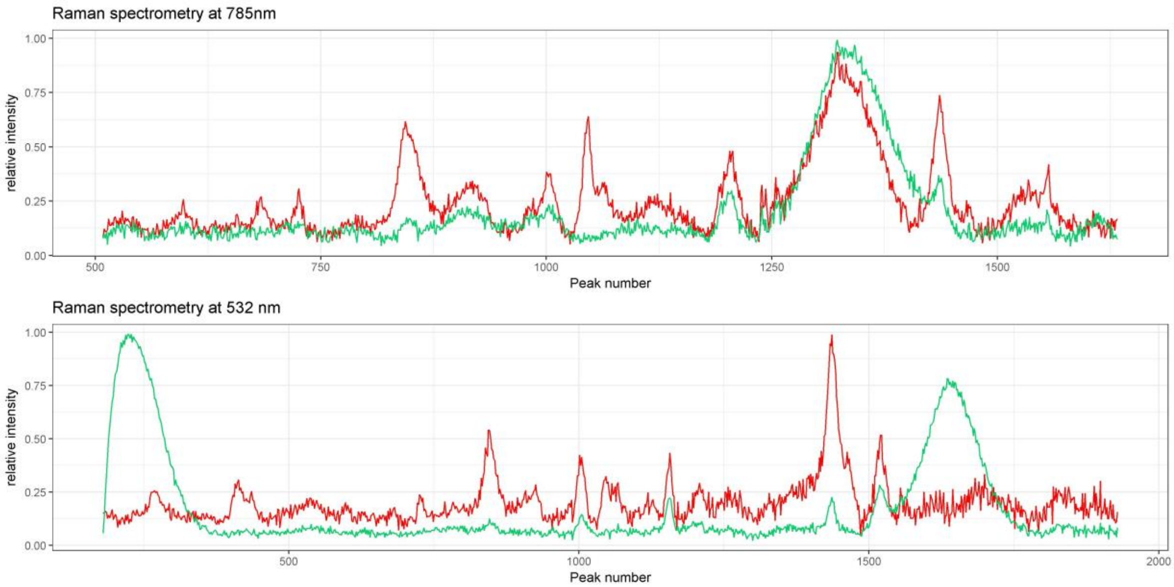


Figure 23. Protein condition optimization for Raman spectroscopy at 532 nm and 785 nm detection. Line plot are referred to average of protein fingerprint detected; red line referred to protein fingerprint in solid condition, green line referred to protein fingerprint in solution condition.

5.2.1.3 Sensitivity and specificity of Raman spectroscopy and Surface-enhanced raman spectroscopy analysis.

The protein fingerprints of Active TB (ATB), Latent TB (LTBI), Early clearance (EC) and Healthy control (HC) are compared and analysis by Raman spectroscopy at 532 nm, Raman spectroscopy at 785 nm and SERs. The 60% of all data are used training the data to for build random forest tree with 50,000 trees by randomForest package and used the result to predict sensitivity, specificity and accuracy of the 40% data (test set). The result of sensitivity and specificity from protein fingerprint analysis were show in table 3.

Table 3. Sensitivity and specificity of Raman spectroscopy and SERs to diagnostic of Active TB (ATB), Latent TB (LTBI), Early clearance (EC) and Healthy control (HC) serum sample.

Groups comparison	Raman spectroscopy (532 nm)		Raman spectroscopy (785 nm)		Surface-enhanced Raman spectroscopy	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
ATB, LTBI, EC, HC	ATB=68.90 EC=64.43 HC=83.33 LTBI=46.281	ATB=86.85 EC=88.95 HC=85.18 LTBI=96.154	ATB=63.19 EC=60.27 HC=65.88 LTBI=57.72	ATB=90.12 EC=84.29 HC=76.44 LTBI=96.389	ATB=100.00 EC=90.00 HC=96.25 LTBI=97.50	ATB=96.67 EC=98.75 HC=99.58 LTBI=99.00
ATB, LTBI, HC	ATB=84.76 HC=85.00 LTBI=51.24	ATB=86.98 HC=82.11 LTBI=94.55	ATB=66.26 HC=92.28 LTBI= 54.07	ATB=94.49 HC=64.77 LTBI=97.56	ATB=89.47 HC=91.18 LTBI=100.00	ATB=97.06 HC=94.44 LTBI=98.61
EC, LTBI, HC	EC=85.86 HC=86.67 LTBI=46.67	EC=86.94 HC=82.96 LTBI=94.66	EC=70.94 HC=81.25 LTBI=67.23	EC=84.40 HC=76.09 LTBI=97.74	EC=76.67 HC=90.62 LTBI=87.10	EC=90.48 HC=9180 LTBI=95.16
ATB, LTBI	92.00	77.21	80.00	91.07	100.00	87.50
ATB, EC	72.33	74.75	71.98	87.62	90.91	86.49
LTBI, EC	91.13	71.19	92.75	66.67	93.33	75.00
ATB, HC	87.80	91.25	66.26	91.37	90.00	93.75
EC, HC	87.43	90.83	71.36	70.98	91.89	100
LTBI, HC	90.83	59.52	94.12	58.91	100.00	96.67

Note: ATB referred to active TB, LTBI referred to latent TB. EC referred to early clearance TB, HC referred to healthy person.

5.2.1.4 ATB, LTBI and HC diagnosis

Protein sample form ATB, LTBI and HC were subjected to Raman spectroscopy at 532 nm, Raman spectroscopy at 785 nm and SERs. The highest sensitivity and specificity analysis was SERs method with sensitivity test were 89.47%, 100.00% and 91.18% for ATB, LTBI and HC diagnosis, respectively. Specificity of SERs detection were 97.06%, 98.61% and 94.44% for ATB, LTBI and HC diagnosis, respectively (Table3).

Protein sample form LTBI and HC were subjected to Raman spectroscopy at 532 nm, Raman spectroscopy at 785 nm and SERs. The protein fingerprint was analysis by randomForest package. The highest sensitivity and specificity analysis was SERs method with sensitivity and specificity were 100% and 96.67%, respectively (Table3).

Protein sample form LTBI and HC were subjected to Raman spectroscopy at 532 nm, Raman spectroscopy at 785 nm and SERs. The protein fingerprint was analysis by RandomForest package. The highest sensitivity and specificity analysis was SERs method with sensitivity and specificity were 100% and 96.67%, respectively (table3).

Discussion for SERS and Raman Spectroscopy analysis

Active TB (ATB) diagnosis is currently relied on of symptom of TB disease and laboratory diagnosis such as finding of *Mtb* from sputum of TB patient. *Mtb* nucleic acid amplification test (NAAT) (55-58) became a primary mean for ATB diagnosis. For LTBI diagnosis, indirect approach includes measurement of cellular immunity via skin tests (Tuberculin skin test; TST) (59, 60), blood test (Interferon gamma releasing assays; IGRAs)(41, 61) were commonly used. However, none of them can differentiate between ATB and LTBI. The laboratory diagnosis of both ATB and LTBI has limitations mainly due to the delayed turnaround time. The rapid diagnosis of *Mtb* infection is necessary for TB treatment and control. Raman spectroscopy and SERs have been used for study of disease diagnosis such as cancer (62-66). The examples of tuberculosis analysis by Raman spectroscopy and SERs such as tuberculous meningitis diagnosis (67), *Mtb* stain analysis by mycolic acid profiles (68) and detection of CFP-10 antigen (69), but none were systematically applied for diagnosis from human serum samples.

We use serum samples from Active TB (ATB, n=26), Latent TB (LTBI, n=20), Early clearance (EC, n=34) and Healthy control (HC, n=38) (Total=118 samples) analyzed by Raman spectroscopy and SERs. Advance statistical analysis were performed.

The optimization of the serum sample preparation for SERS and RAMAN spectroscopy TB diagnosis have never been discussed elsewhere. We found that 1 ul of 0.8 ug/ul protein in solid stage was proper to use in Raman spectroscopy analysis. Coefficient of determination (r^2) was measure. The average R^2 of data from Raman spectroscopy at 532 nm, Raman spectroscopy at 785 nm and SERs analysis were 0.915, 0.972 and 0.988, respectively. SERs have highest sensitivity and specificity analysis compared to Raman spectroscopy at 532 nm, Raman spectroscopy at 785 nm. Sensitivity and specificity of SERs in diagnosis of ATB, LTBI and HC serum sample was better than Raman spectrometry at 532 nm and Raman spectrometry at 785 nm. Sensitivity of SERs detection were 89.47%, 100.00% and 91.18% for ATB, LTBI and HC diagnosis, respectively. Specificity of SERs detection were 97.06%, 98.61% and 94.44% for ATB, LTBI and HC diagnosis, respectively. The sensitivity and specificity of LTBI and HC diagnosis by SERs were 100% and 96.67%. The sensitivity and specificity of LTBI and ATB diagnosis by SERs were 100% and 87.5%. The sensitivity and specificity of HC and ATB diagnosis by SERs were 90% and 93.75%. This is the first study to demonstrate the optimization protocol and parameters that should be concerned for Raman spectroscopy based TB diagnosis from serum samples.

Raman spectroscopy (532nm, 785nm, SERs) had faster turnaround time for ATB, LTBI and HC diagnosis compared to with standard diagnosis. The simplicity by just adding the sample and analyzed directly by the machine with a few minutes required to obtain the result is the facinatign characteristic of this technique for clinical laboratory. However, the analysis of the peak patterns are challenging. The sensitivity and specificity of Raman spectroscopy (532nm, 785nm, SERs) could be improved by using an optimized algorithm in Machine learning analysis. Raman spectroscopy (532nm, 785nm, SERs) data from the diagnosis process can be use as the library data to improve sensitivity and specificity of diagnosis in clinical laboratory. With larger dataset, the performance of these tool for diagnosis and classification of the disease possibilities could be also improved. Our study was the first to demonstrate the application of SERS and RAMAN for TB diagnosis.

In summary, SERs showed highest sensitivity and specificity of SERs to differentiate ATB, LTBI and HC groups when compared to Raman spectroscopy at 532 nm and Raman spectroscopy at 785 nm.

5.2.2 Cytokine/ chemokine analysis

5.2.2.1 Demographic characteristics of participants

The tested populations consisted of 48 ATB (pulmonary TB) patients, 200 healthy persons with a history of TB contact and 52 healthy individuals with no known TB exposure. All individuals were from Srinagarind Hospital, Khon Kaen Thailand (Figure 1).

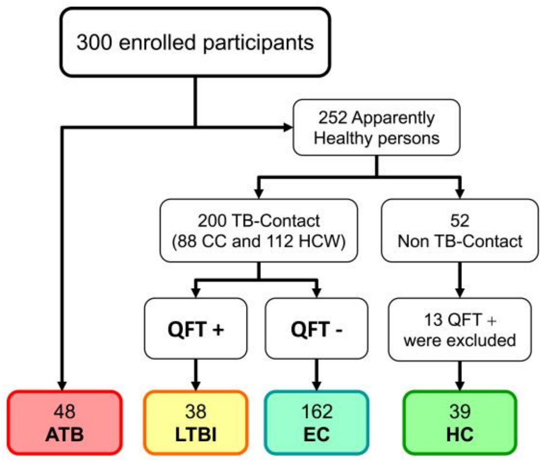


Figure 1. Study population and classification of the study groups.

Active TB = active tuberculosis patients (symptomatic patients positive for acid-fast bacilli, molecular test or culture for TB); CC = close contact persons; HCW = health care workers; LTBI = latent tuberculosis infections (TB exposed persons with QFT positive results); EC = early clearance (TB exposed persons with QFT negative results); HC = healthy controls (healthy persons with no known risk of TB exposure); QFT = QuantiFERON-TB Gold in-tube.

Most enrolled participants in each group were female, except for the ATB group in which there was a significantly greater proportion of males (Table 1). The ATB group was significantly greater in age and had significantly lower BMI values when compared to other groups. Age and BMI values of participants in the LTBI group were also significant higher than in the EC group. The proportion of BCG-vaccinated persons in the ATB group was significantly lower than in the EC and HC groups. Similarly, the proportion vaccinated in the EC group was significantly lower than in the HC group (Table 1).

Table 1

Demographic characteristics of study groups.

Characteristics	ATB (n=48)	LTBI (n=38)	EC (n=162)	HC (n=39)	P-values					
					a	b	c	d	e	f
Gender male, n (%)	31 (64.6)	7 (18.4)	55 (34.0)	7 (17.9)	<0.001	<0.001	<0.001	0.062	0.957	0.052
Age (yr.) mean±SD	52±15	45±12	37±16	40±14	0.023	<0.001	<0.001	0.002	0.067	0.329
BMI (kg/m^2) mean±SD	20.6±3.2	24.4±4.7	22.4±4.4	22.3±3.3	<0.001	0.009	0.018	0.016	0.03	0.867
BCG vaccination*, n (%)	36 (75)	26 (89.7)	122 (89.7)	39 (100)	0.116	0.012	0.001	1	0.073	0.042

Note: a (ATB versus LTBI), b (ATB versus EC), c (ATB versus HC), d (LTBI versus EC), e (LTBI versus HC), f (EC versus HC). * Missing information in 9 LTBI and 26 EC. Active TB = active tuberculosis patients; LTBI = latent tuberculosis infections; EC = early clearance; HC = healthy controls; QFT = QuantiFERON-TB Gold in-tube; BMI= Body Mass Index.

5.2.2.2 Diagnostic performance of single chemokines as TB diagnostic biomarkers

CCL2 and CXCL10 from three conditions (TBAg, Unstim and TBAg minus Unstim) were evaluated as biomarkers to differentiate among ATB, LTBI and HC (Table 2 and Supplementary Figure 1). CXCL10 performed well in differentiating among groups (AUC values > 0.85). CXCL10 Unstim condition was the best marker to differentiate between ATB and HC (95.8% sensitivity and 94.9% specificity) and between ATB and LTBI (87.5% sensitivity and 78.9% specificity). To differentiate between LTBI and HC, CXCL10 levels in the TBAg condition showed the highest performance (89.5% sensitivity and 92.3% specificity). The CCL2 marker showed poorer diagnostic performance with AUC values approximately 0.5. The best performance of CCL2 was in the discrimination between ATB and HC using TBAg minus Unstim condition (70.8% sensitivity and 74.4% specificity) (Table 2 and Supplementary Figure 1).

Table 2

Diagnostic performance of CCL2 and CXCL10 as individual biomarkers for ATB and LTBI diagnosis.

Markers	AUC	Std. Error	95% CI	P-values	Cut-off (pg/mL)	Sensitivity (%)	Specificity (%)	YI
ATB versus HC								
CCL2								
TBAg	0.513	0.063	0.390-0.636	0.838	12,468.00	31.2	82.1	0.133
Unstim	0.288	0.059	0.174-0.403	0.001	37,827.00	4.2	100	0.042
TBAg-Unstim	0.723	0.055	0.615-0.830	<0.001	169.5	70.8	74.4	0.452

CXCL10								
TBAg	0.883	0.036	0.813-0.953	<0.001	6,319.00	79.2	87.2	0.664
Unstim	0.969	0.021	0.928-1.010	<0.001	2,372.00	95.8	94.9	0.907
TBAg-Unstim	0.728	0.057	0.616-0.839	<0.001	6,964.00	58.3	97.4	0.557
ATB versus LTBI								
CCL2								
TBAg	0.54	0.064	0.415-0.665	0.526	4,020.50	79.2	34.2	0.134
Unstim	0.479	0.064	0.353-0.605	0.741	4,295.50	62.5	55.3	0.178
TBAg-Unstim	0.55	0.063	0.426-0.674	0.429	295	68.8	44.7	0.135
CXCL10								
TBAg	0.453	0.062	0.331-0.576	0.46	27,699.00	41.7	71.1	0.128
Unstim	0.866	0.042	0.785-0.948	<0.001	2,812.50	87.5	78.9	0.664
TBAg-Unstim	0.394	0.061	0.274-0.514	0.092	27,820.50	33.3	73.7	0.07
LTBI versus HC								
CCL2								
TBAg	0.475	0.067	0.343-0.607	0.706	13,193.50	26.3	84.6	0.109
Unstim	0.32	0.062	0.199-0.441	0.007	874.5	100	2.6	0.026
TBAg-Unstim	0.661	0.062	0.538-0.783	0.015	179	57.9	74.4	0.323
CXCL10								
TBAg	0.961	0.019	0.923-0.999	<0.001	6,947.50	89.5	92.3	0.818
Unstim	0.723	0.058	0.610-0.837	0.001	1,354.00	55.3	82.1	0.374
TBAg-Unstim	0.955	0.021	0.913-0.997	<0.001	6,163.00	84.2	94.9	0.791

Active TB = active tuberculosis patients; LTBI = latent tuberculosis infections; EC = early clearance; HC = healthy controls; QFT = QuantiFERON-TB Gold in-tube; TBAg = presence of antigen; Unstim = absence of antigen; TBAg-Unstim = condition specific to the presence of antigen (TBAg minus Unstim).

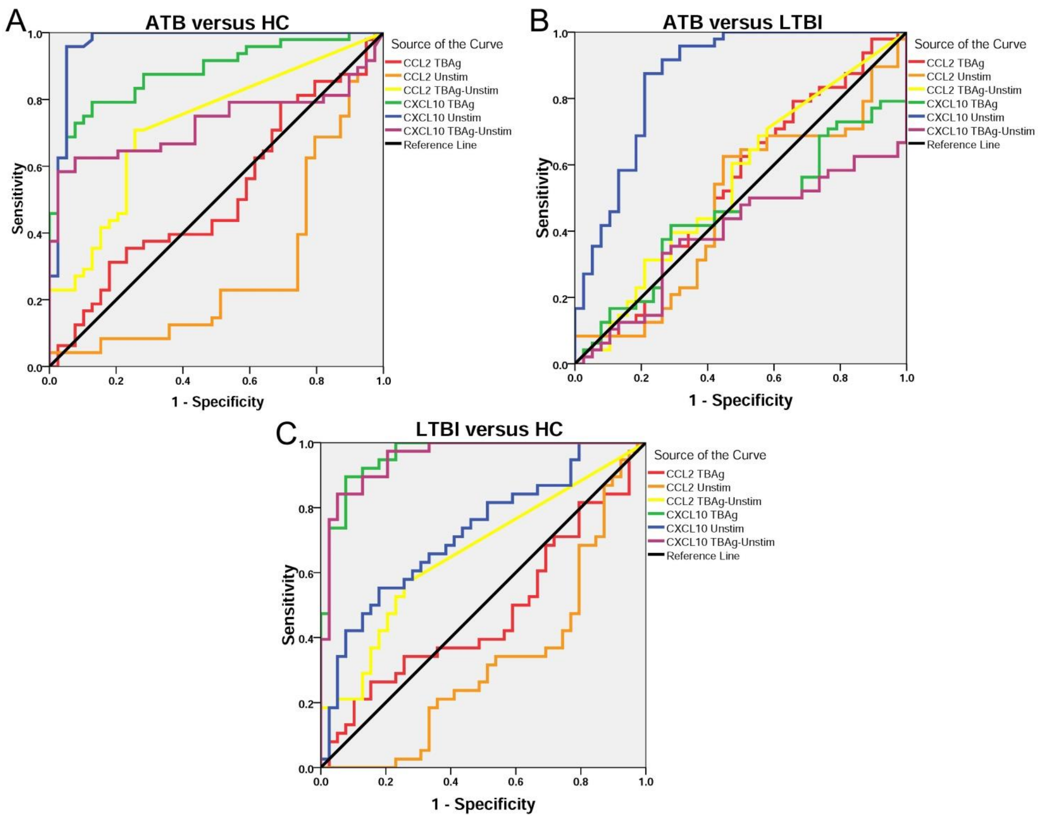


Figure 1. Receiver operator characteristics (ROC) analysis of chemokines with various conditions consisting of CCL2 TBAg; red line, CCL2 Unstim; orange line, CCL2 TBAg-Unstim; yellow line, CXCL10 TBAg; green line, CXCL10 Unstim; blue line and CXCL10 TBAg-Unstim; purple line) for ATB and LTBI diagnosis. (A) ROC analysis of ATB versus HC. (B) ROC analysis of ATB versus LTBI. (C) ROC analysis of LTBI versus HC. The reference line is black.

The QFT test, which is based on IFN-γ alone, was poor at identifying ATB (32 QFT-positive, 15 QFT-negative and 1 indeterminate). IFN-γ alone showed low sensitivity for ATB (72.9%) and LTBI (16.7%) diagnosis (Table 3 and Supplementary Table 1).

5.2.2.3 Improved diagnostic performance of the IFN-γ release assay in conjunction with CXCL10 and CCL2 for ATB and LTBI diagnosis, and improved differentiation of ATB and LTBI.

The combination of "IFN-γ or CXCL10" provided 97.9% sensitivity and 94.9% specificity for discrimination of ATB from HC, which is significantly higher than using IFN-γ alone ($p<0.001$). Similarly, "IFN-γ or CXCL10" provided the highest sensitivity (89.6%) and specificity (71.1%) to discriminate ATB from LTBI, which is significantly higher than using IFN-γ alone ($p<0.001$) (Table 3).

5.2.2.4 CCL2 level is associated with the early clearance phenotype

The EC group showed a highly significantly lower CCL2 level ($p <0.0001$) than did the HC group in the TBAg condition (Figure 2D) and the Unstim condition (Figure 2E). In TBAg minus Unstim, there was no significant difference in CCL2 levels between the EC and HC groups because the TBAg condition cannot elicit CCL2 levels compared to the Unstim condition. Unlike CCL2, levels of CXCL10 were comparable between EC and HC groups (Figure 2G and 2H). IFN-γ levels in the Unstim condition were also significantly lower in the EC group relative to the HC group (Figure 2B).

Table 3. Diagnostic performance of IFN-γ assays in combination with additional chemokines for ATB and LTBI diagnosis

Marker	Sensitivity (%)	Specificity (%)	P-values
ATB versus HC group			
IFN-g (11.8 pg/mL*)	72.9	100	
IFN-g or CXCL10	**97.9**	**94.9**	**<0.001**
IFN-g and CXCL10	70.8	100	1
ATB versus LTBI group			

IFN-g (194.8 pg/mL*)	16.7	86.8	
IFN-g or CXCL10	**89.6**	**71.1**	**<0.001**
IFN-g and CXCL10	14.6	94.7	0.125
LTBI versus HC group			
IFN-g (17.8 pg/mL*)	100	100	
IFN-g or CXCL10	100	92.3	0.25
IFN-g and CXCL10	89.5	100	0.125

CXCL10 Unstim condition was used as an additional biomarker to IFN-γ (TBAg minus Unstim condition) for ATB and LTBI diagnosis (cut-off = 2,372 pg/mL and 2,812.5 pg/mL in the comparisons between ATB versus HC and ATB versus LTBI, respectively). CXCL10 TBAg condition (cut-off = 6,947.5 pg/mL) was used as an additional biomarker to IFN-γ (TBAg minus Unstim condition) for LTBI versus HC setting *Cut-off values obtained from ROC analysis of IFN-γ.

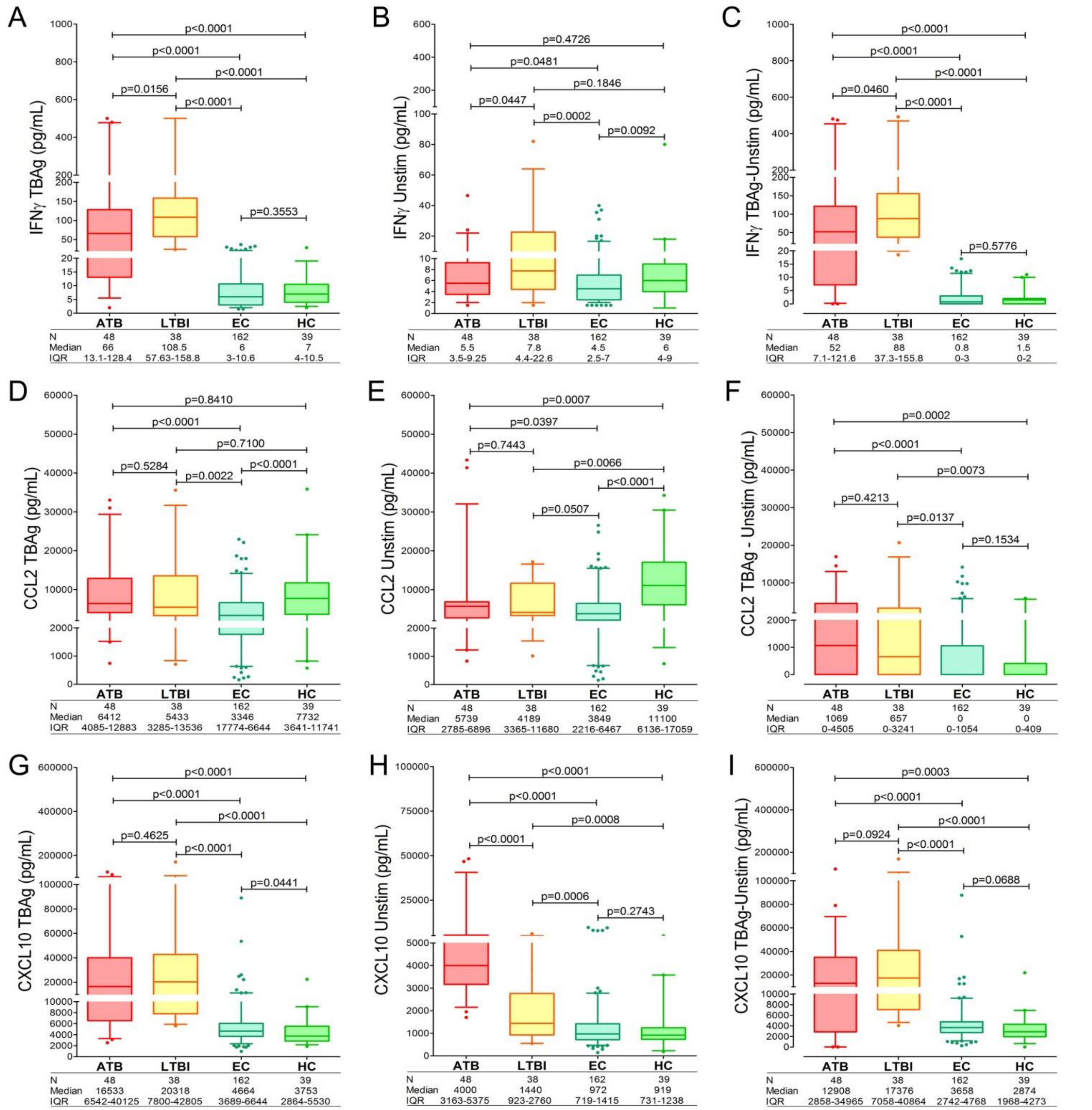


Figure 2. Comparison of cytokine and chemokine levels in TBAg, Unstim and TBAg-Unstim (TBAg minus Unstim) conditions among different groups. (A, B and C) Comparison of IFNγ levels. (D, E and F) Comparison of CCL2 levels. (G, H and I) Comparison of CXCL10 levels. Boxes denote median and interquartile range, and whiskers extend to median±1.5 IQR.

Supplementary Table 1. Diagnostic performance of IFN-γ for ATB and LTBI diagnosis

Markers	AUC	Std. Error	95% CI	P-values	Cut-off (pg/mL)	Sensitivity (%)	Specificity (%)	YI
ATB versus HC								
IFN-g								
TBAg	0.882	0.036	0.811-0.953	<0.001	20	68.8	97.4	0.662
Unstim	0.455	0.062	0.333-0.577	0.471	9.3	25	82.1	0.071
TBAg-Unstim	0.911	0.032	0.848-0.974	<0.001	11.8	72.9	100	0.729
ATB versus LTBI								
IFN-g								
TBAg	0.347	0.059	0.232-0.462	0.015	197	18.8	84.2	0.03
Unstim	0.373	0.062	0.252-0.495	0.045	1.8	97.9	2.6	0.005
TBAg-Unstim	0.374	0.06	0.256-0.491	0.045	194.8	16.7	86.8	0.035
LTBI versus HC								
IFN-g								
TBAg	0.997	0.002	0.994-1.001	<0.001	23	100	96.3	0.963
Unstim	0.697	0.049	0.6-0.794	<0.001	7.8	50	80.2	0.302
TBAg-Unstim	1	0	1-Jan	<0.001	17.8	100	100	1

Active TB = active tuberculosis patients; LTBI = latent tuberculosis infections; EC = early clearance; HC = healthy controls; QFT = QuantiFERON-TB Gold in-tube; TBAg = presence of antigen; Unstim = absence of antigen; TBAg-Unstim = condition specific to the presence of antigen (TBAg minus Unstim).

5.2.2.5 Discussion for cytokine chemokine markers

Diagnosis of the complete range of *M. tuberculosis* infection possibilities is challenging in endemic settings. We evaluated the diagnostic efficacy of single and multiple cytokines and chemokines among Thai adults differing in infection status. In our study, infected, symptomatic individuals (ATB), infected asymptomatic individuals who had experienced TB exposure (LTBI), uninfected individuals who had experienced TB exposure (EC), and healthy persons without TB exposure (HC) could be differentiated based on the semi-overlapping signatures of chemokine secretion in the presence of antigen (TBAg), the absence of antigen (Unstim) and the difference between these (TBAg minus Unstim). We evaluated and built a diagnostic model including unstimulated and antigen-specific IFN-γ, CCL2, and CXCL10 responses that significantly improved on the diagnostic accuracy of using IFN-γ alone. Because of the relative simplicity of chemokine measurement, we believe this approach warrants validation in additional populations.

Use of a single biomarker may limit diagnostic performance for ATB and LTBI. IFN-γ is the most promising marker for LTBI diagnosis but it is a poor marker to differentiate between ATB and LTBI. We evaluated the abilities of CCL2 and CXCL10 to discriminate between ATB and LTBI. CCL2 is a potent chemotactic factor inducing migration of monocytes and lymphocytes to the infected site.(70) Although some studies have reported that CCL2 is a potential marker for LTBI diagnosis,(46, 71) others have noted that CCL2 levels did not differ between ATB and LTBI groups or control groups.(71-73) In our study, CCL2 levels showed insufficient sensitivity and specificity to discriminate between LTBI and HC and between ATB and LTBI, but were adequate (>70% sensitivity and specificity) to discriminate between ATB and HC groups. CXCL10 is a T-cell chemoattractant involved in T-cell trafficking and is strongly induced by IFN-γ.(74) Previous studies showed that combination of CXCL10 with other cytokines and chemokines increased the sensitivity for ATB(75) and LTBI diagnosis.(71) In some studies, CXCL10 was additionally used to differentiate between ATB and LTBI and the control group(41, 45, 76, 77), but validation of this in other settings was unsuccessful.(73, 77) We found CXCL10 to be a good biomarker for ATB and LTBI diagnosis and provide 95% sensitivity and specificity using HC as the control group. CXCL10 also provided the highest sensitivity and specificity to differentiate between ATB and LTBI compared to IFN-γ and CCL2. In QFT, the

IFN-γ level in the TBAg-stimulated condition minus the baseline condition (Unstim) is calculated. As with CXCL10, this approach is applicable because IFN-γ is responsive to TBAg stimulation. However, we found that CCL2 seemed to be unresponsive to TBAg, yielding levels comparable to those in the Unstim condition. Hence the "TBAg minus Unstim" value was not applicable for the CCL2 marker. Therefore, CCL2 levels from either the TBAg or the Unstim condition could be used as the biomarker.

Previous studies suggested variable performance of multiple cytokines and chemokines for ATB and LTBI diagnosis.(71, 73, 75) Such combinations have the potential to improve diagnosis of ATB and LTBI. CXCL10 alone(78), or in combination with EGF, MIP-1β, sCD40L and IL-1α(79), CCL2 and IL-5(46), IL-1Ra, CXCL-10 and VEGF(80), and other cytokines, showed potential diagnostic value. Given that CXCL10 showed the highest AUC and high sensitivity and specificity, we evaluated the ability of this chemokine additional to IFN-γ to diagnose and discriminate between ATB and LTBI. This combination markedly improved sensitivity and specificity for ATB and LTBI diagnosis compared to using IFN-γ alone.

To evaluate the performance of cytokines and chemokines for diagnosis of ATB and LTBI, we regarded the EC group as inappropriate for use as the control group. Instead, we used healthy controls with no known TB exposure. The use of QFT as a standard test for LTBI diagnosis in our study means that IFN-γ (TBAg minus Unstim) obviously showed the best discriminatory power with 100% sensitivity and 100% specificity when discriminating between the LTBI and HC groups. Therefore, we did not analyze the combination of multiple biomarkers in this comparison as it did not improve the diagnostic performance.

EC is a postulated phenomenon in which TB-exposed persons show no evidence of infection based on IGRA/TST, indicating TB resistance.(35) If such individuals can be identified, study of their mechanisms of resistance may lead to discovery of protective immune factors and narrow down the target population for LTBI prophylaxis treatment. In EC, pathogen eradication seems to occur without adaptive immune development. However, evidence for this is lacking: EC and HC conditions may be the same. Although we found that CCL2 was a relatively poor marker for differential diagnosis of ATB and LTBI, this chemokine showed some association with the putative TB resistance phenotype. CCL2 levels were lower in the EC group than in the HC and LTBI groups. Previously, we have reported that persons with the GG genotype (susceptible) had significant lower CCL2 levels (TBAg minus Unstim value).(48) However, this association was apparent only within the LTBI group as the CCL2 level cannot be measured in HCs, which have no memory response, or in ATB cases, which normally suffer from an immunompromised condition. Furthermore, other studies have reported that CCL2 mutants showed either increased CCL2 levels in the ATB group(81), or decreased levels in the LTBI group compared to wild type.(82) Here, we found that the EC group (putatively TB-resistant) had very significantly lower CCL2 levels compared to the HC or LTBI (TB susceptible) groups in both TBAg and Unstim conditions. Analysis of the role of CCL2 in TB pathogenesis is needed. This is the first biological evidence to support the existence of an EC phenotype of *M. tuberculosis* infection.

Previous studies (40, 46, 79) have suffered three limitations: the control groups have been heterogeneous mixtures of individuals with and without TB contact, antigen-specific responses have been inconsistently studied, and little attempt at building diagnostic tools has been made following observation of differential expression. Few studies(40, 41) have evaluated the cytokine and chemokine levels in ATB, LTBI and EC categories. In those studies, few subjects were confirmed to have a history of contact with ATB patients(40) and no healthy controls were included in the studied population. In this study we provide many novel findings; i) the evaluation of chemokines additional to IFN-γ for diagnosis based on the complete range of *M. tuberculosis* infection possibilities in the Thai population, ii) the demonstration of EC persons that, although clinically resembling HC persons, can be differentiated based on CCL2 levels, iii) proposing the model combining IFN-γ and CXCL10 for discrimination of ATB, LTBI and HC. The well-defined study population, covering the spectrum of TB infection possibilities, and especially the inclusion of a group with carefully ascertained TB exposure, is a major strength of our study. The main limitation of our study is there is no gold standard for LTBI diagnosis. As we worked in a TB-endemic area, and in order to minimize false positives due to BCG vaccination, QFT was used to define LTBI cases in our setting. Furthermore, cross reactions with some non-tuberculous mycobacteria in the QFT test might have led to some false positives.

In conclusion, we found that chemokine levels differ according to the disease spectrum, i.e. ATB, LTBI and HC, and may therefore be exploited for improved diagnosis of these various disease possibilities. Our results support the additive value of CXCL10 with IFN-γ for diagnosis of ATB and LTBI and for differentiation between the two disease types. CCL2 levels provided evidence of EC and can be used to differentiate EC from HC and LTBI.

ข้อเสนอแนะในการทำงานวิจัยในอนาคต

- ควรสนับสนุนในการศึกษาต่อยอดในการนำ Biomarker (เช่น PSKT serum marker) เพื่อพัฒนาต่อจนเป็นชุดทดสอบเชิงพาณิชย์ ชุดทดสอบตรวจติดตามการรักษาวัณโรคจากเลือดผู้ป่วย หารตรวจวินิจฉัยแยกโรควัณโรค และภาวะติดเชื้อวัณโรคแอบแฝง

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Output ที่ได้จากโครงการ

1. ตัวบ่งชี้ต้นแบบ

   1.1 PSTK serum biomarker for treatment monitoring of LTBI and active TB

   1.2 New Method of aplication of SERS and RAMAN spectroscopy for TB and LTBI diagnosis

   1.3 CXCL10 adjunct to IFN-gamma for LTBI diagnosis

2. ผลงานวิจัยตีพิมพ์ในวารสารนานาชาติ

   2.1 Published article: Nonghanphithak D, Reechaipichitkul W, Namwat W, Naranbhai V, Faksri K*. Chemokines additional to IFN-gamma can be used to differentiate among Mycobacterium tuberculosis infection possibilities and provide evidence of an early clearance phenotype. Tuberculosis 2017, 105:28-34.

   2.2 Manuscript: Serum biomarker indicating the clareance of *Mycobacterium tuberculosis* infection after antituberculous treatment: in-vito and in-vivo study
   อยู่ในช่วงการพัฒนาร่างต้นฉบับเพื่อส่งตีพิมพ์ในวารสาร ISI Q1-2

   2.3 Manuscript: Application of SERS and RAMAN Spectroscopy for diagnosis of active tuberculosis and latent tuberculosis infection
   อยู่ในช่วงการพัฒนาร่างต้นฉบับเพื่อส่งตีพิมพ์ในวารสาร ISI Q1-2

3. ได้ผู้ช่วยวิจัยและบัณฑิต

   3.2 บัณฑิตระดับปริญญาโท 1 คน (นายดิษฐวัฒน์ หนองหารพิทักษ์)

   3.1 บัณฑิตระดับปริญญาเอก 1 คน (นางสาวเบญจวรรณ แก้วสีขาว กำลังอยู่ในช่วงการเตรียมวิทยานิพนธ์เพื่อสอบป้องกันวิทยานิพนธ์)

<u>**ภาคผนวกโครงการ**</u>

- **บทความสำหรับเผยแพร่:** 1 เรื่อง (ISI/Scopus Q2) และร่างต้นฉบับอีก 2 เรื่อง (Clearance marker และ SERS/RAMANS for TB diagnosis)
- **กิจกรรมที่เกี่ยวข้อง:**
  1. การนำเสนอผลงานแบบ Oral presentation เรื่อง “Proteomic analysis of diagnostic and treatment monitoring markers for tuberculosis” ในการประชุมประจำปี “นักวิจัยรุ่นใหม่...พบ...เมธีวิจัยอาวุโส สกว.” ครั้งที่ 17 ในวันที่ 10-12 มกราคม 2561 –ณ โรงแรมเดอะรีเจนท์ ชะอำ บีช รีสอร์ท จังหวัดเพชรบุรี
  2. Oral presentation งานประชุม ระดับนานาชาติ เรื่อง Proteomic and genomic analysis for anti-tuberculosis drug resistant, diagnostic and treatment monitoring markers ในงานประชุมวิชาการนานาชาติ 10th Anniversary of Protein & Peptide Conference (PepCon-2017) ณ Fukuoka, Japan

Abstract: Oral presentation ในการประชุมประจำปี "นักวิจัยรุ่นใหม่...พบ...เมธีวิจัยอาวุโส สกว." ครั้งที่ 17 ในวันที่ 10-12 มกราคม 2561 –ณ โรงแรมเดอะรีเจนท์ ชะอำ บีช รีสอร์ท จังหวัดเพชรบุรี

Proteomic analysis of diagnostic and treatment monitoring markers for tuberculosis

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Abstract

Tuberculosis is the leading infectious disease with 1.8 million deaths annually. Rapid and effective diagnosis for active and latent tuberculosis is challenging. The effective treatment monitoring marker for tuberculosis is lacking. Our research group applied the proteomic analysis to discover the markers for treatment monitoring and diagnostic markers for active and latent tuberculosis. In this lecture, the studies regarding the applications of proteomic analysis for tuberculosis will be presented. Using the human monocytic cell line infected with H37Rv strains of *Mycobacterium tuberculosis*, analyzed with GeLC MS/MS and compared before and after intracellular clearance revealed treatment monitoring markers in which SSFA2 is one of the candidates. The subsequent *in vitro* on going experiment using the primary cell line with the assessment of the serum samples collected from tuberculosis patients will be presented. Finally, the proteomic LC MS/MS analysis with SERS, Raman spectrometry and LC MS/MS of blood samples collected from active and latent tuberculosis patients revealed the candidate diagnostic markers for active and latent tuberculosis. In conclusion, proteomic analysis is the high-throughput method that can be used to discover the diagnostic and treatment monitoring markers for effective control of tuberculosis.

Keywords: Clearance biomarker; Latent tuberculosis; *Mycobacterium tuberculosis*; SERS; Ranman Spectrometry; LC MS/MS

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