QUANTIFERON-TB GOLD IN-TUBE PERFORMANCE FOR DIAGNOSING ACTIVE AND LATENT TUBERCULOSIS IN MALAYSIAN POPULATION

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Bachelor of Health Sciences (Biomedicine)

May 2013

ACKNOWLEDGEMENT

I would like to express my deepest gratitude to The Most Gracious and The Most Merciful Allah S.W.T for giving me the strength, patience, knowledge and passion to finish this report properly. It is with His grace and His will that I have been able to gain experience, knowledge and along with the moral values throughout this research.

Besides that, I would like to express my gratitude to the LRGS Grant Tropical Tuberculosis lead by Prof. Norazmi Mohd Nor for providing me the financial support for my Final Year Project. I would also like to send my heartiest thanks to my wonderful supervisor Dr. Aziah Ismail for giving me guidance, support and for teaching me about the research and also giving me life lessons to gain from. It has been pleasure learning under your supervision.

Special thanks to Dr. Wan Amir for your guidance and support in helping me to complete this thesis. I would have truly faced a lot of difficulties if it was not for your help. Besides that, I cannot forget my fellow colleagues in INFORMM especially Miss Irna Zulikha and Mr. Faizul for your friendship, and your continuous support for me throughout this project.

My extended thanks to my fellow course mates especially Miss Tengku Farah Adilah, Miss Siti Hajar and Miss Wan Nor Izzati for supporting me during the easy and hard times. Your support has given me strength to complete and finish this thesis and your friendship has cheered me up during hardships.

Finally, I cannot leave out my dear family for their endless love, care and support in any situation. Your affection and understanding has made my journey doing these project surely an easier one and for that I cannot thank all of you enough.

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LIST OF ABBREVIATIONS

TB	Tuberculosis
ZN	Ziehl Neelson
LTBI	Latent Tuberculosis Infection
IGRA	Interferon Gamma Release Assays
TST	Tuberculin Skin Test
IFN□	Interferon Gamma
CDC	Control Disease Centre
WHO	World Health Organization
HIV	Human Immunodeficiency Virus
QFT-GIT	QuantiFERON TB Gold In-Tube
PCR	Polymerase Chain Reaction
FDA	Food Drug Administration
PPD	Protein Purified Derivative
ELISA	Enzyme linked Immunoabsorbent Assay
QFT	QuantiFERON
BCG	Bacillus Calmette-Guérin
HLA	Human Leukocyte Antigen

MHC	Major Histocompatibility Complex
PBS	Phosphate Buffered Saline
RCF	Rotation Centrifugal Force
SS	Sungai Siput
HC/CT	Healthy Contact
OA	Orang Asli
HG	Hospital Gombak
PH	Pahang
HKB	Hospital Kota Bharu
OT	Others (normal)
USM	Universiti Sains Malaysia
OR	Odd Ratio
CI	Confidence Interval

ABSTRACT

Halting the progression of active tuberculosis (TB) involves the detection of TB from a latent stage. Currently, in-vitro interferon-gamma assays (IGRAs) are being used to detect for active TB as well as latent TB besides Tuberculin Skin Test (TST). One of IGRA used to diagnose for Latent Tuberculosis Infection (LTBI) is the QuantiFERON-TB (QFT) Gold assay which measures the levels of interferon gamma stimulated by QFT peptides (CFP-10, ESAT 6 and TB7.7) in patient plasma. However, the information regarding the performance of this kit especially in Malaysian population is limited. Therefore this study aims to test for the sensitivity and specificity of the QFT kit in Malaysian population. Whole blood samples were collected from 47 confirmed TB patients, 28 high-risk TB individuals (healthy contacts) and 24 BCG vaccinated low-risk TB individuals (non-TB) and stimulated with TB antigens prior to ELISA for QFT. Specificity and sensitivity of the QFT was analysed based on known TB patients and non-TB individuals. The specificity of QFT was determined as 79.16 % among low-risk patients and the sensitivity of QFT for Malaysian population was 68.08% among confirmed TB patients. Meanwhile, the percentage of positivity for latent TB detected among high-risk TB patients was 46.42%. Our study showed comparable results to the studies conducted at other neighbouring regions such as Singapore and India. Our findings revealed that the QFT is also suitable to be used as a diagnostic kit for the detection of TB and latent TB.

ABSTRAK

Menghalang perkembangan jangkitan batuk kering peringkat aktif memerlukan diagnosis untuk jangkitan batuk kering pada peringkat pendam. Kini selain kaedah ujian kulit tuberculin, kaedah in-vitro interferon-gamma sedang digunakan untuk mengesan jangkitan batuk kering bagi peringkat aktif dan peringkat pendam. Antara IGRA yang digunakan untuk mengesan jangkitan batuk kering yang terpendam adalah QuantiFERON-TB (QFT) Gold dimana ia mengukur paras interferon gamma yang rangsang oleh peptide QFT (CFP-10, ESAT 6 and TB7.7) di dalam plasma pesakit. Walau bagaimanapun informasi mengenai prestasi set ujian ini terutamanya bagi populasi Malaysia adalah sedikit. Oleh itu, penyelidikan ini bertujuan untuk mengenal pasti prestasi dan tahap sensitiviti dan spesifisiti kit QFT di dalam populasi Malaysia. Sampel darah diambil daripada 47 pesakit batuk kering yang dikenal pasti, 24 pesakit yang mempunyai risiko tinggi untuk mendapat jangkitan batuk kering dan 24 individu yang telah diberi vaksin BCG yang dikenal pasti sebagai kumpulan yang mempunyai risiko rendah untuk mendapat jankitan batuk kering. Sampel-sampel ini di rangsang dengan antigen TB sebelum diuji dengan kaedah ELISA untuk QFT. kadar spesifisiti dan sensitiviti telah di analisis berdasarkan individu yang dikenal pasti mempunyai jangkitan batuk kering dan daripada para individu yang dikenal pasti untuk tidak mempunyai jangkitan batuk kering. Kadar spesifisiti untuk QFT adalah 79.16% dalam kalangan individu yang tidak mempunyai jangkitan batuk kering dan kadar sensitiviti ujain QFT untuk populasi Malaysia adalah 68.08% dalam kalangan individu yang mempunyai jangkitan batuk kering. Sementara itu, peratus positif untuk jangkitan batuk kering peringkat terpendam yang dikesan dalam kalangan yang mempunyai risiko tinggi untuk dijangkiti penyakit batuk kering adalah 46.42%. penyelidikan ini telah menunjukkan keputusan yang boleh dibandingkan dengan Negara yang berdekatan

seperti Singapura dan India. Kajian kami menunjukkan bahawa QFT juga sesuai digunakan untuk mengesan jangkitan batuk kering peringkat aktif dan terpendam.

CHAPTER 1 & 2: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

1.1.1 *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is the most common causative agent for the air-borne disease; tuberculosis (TB). Other species of mycobacterium that also cause TB are *M. africanum* and *M. bovis*. *M. tuberculosis* was isolated by Robert Koch in 1882. It is rod shaped and holds acid fastness which is responsible for its resistance in dry areas and virulence as an airborne pathogen. Its cell wall contains a high degree of mycolic acid that retains carbol fuchsin when stained with Ziehl Neelson (ZN) stain (Koul et al., 2011).

1.1.2 Tuberculosis infection

TB infection is an airborne disease since the transmission of TB occurs via airborne particles which are called nuclei droplets which range from 1-5μ in diameter. This also explains why TB is such a contagious disease even at a global level. These droplets are generated from persons who have pulmonary or laryngeal TB disease through sneezing, shouting, and coughing. Once the droplet enters a new host, it traverses into the mouth or nasal passages to the upper respiratory tract, and bronchi to reach the alveoli of the lungs.

At the lungs, the tubercle bacillus multiplies and a small number of tubercle enters the bloodstream and travels systemically to other parts of the body. Common sites where TB disease develops include the brain, larynx, lymph nodes, lung, bones, kidneys and spine. This explains why sometimes, when a sputum test is carried out, the results for TB might be negative but it does not rule out a TB infection. Evidently, the bacillus induces the immune system to response by activating the macrophages to engulf them

and form a granuloma. The state of the immune response will further determine whether the person develops TB symptoms or form Latent TB Infection (LTBI).

Typical TB symptoms include chronic cough, weight loss, night sweats, and fever. Besides that, people infected with TB are capable of spreading the TB bacteria to others and require isolation. In addition, the body is not capable of fighting off the bacterium in the body because the immune response is already weak and cannot induce enough macrophages to contain the bacillus. The granuloma shell breaks down and spreads the bacillus to other parts of the body as mentioned before. On top of that, other opportunistic organisms can cause infections to the person easily thus, rendering the person to be vulnerable to all sorts of disease and death.

On the contrary, for LTBI, the bacterium remains inactive. People with LTBI are not capable of spreading TB to others and do not require isolation. Most diagnostics like Interferon Gamma Release Assays (IGRA) and Tuberculin Skin Test (TST) detects LTBI by sensing the IFN□ produced due to the formation of the granuloma (Tsiouris et al., 2006). In fact, most of the diagnostics are able to detect for LTBI since IFN□ that are the biomarkers of these tests are available should the immune status of the patients remain intact. There are several factors that determine the susceptibility of a person towards a TB infection.

According to CDC in 2011, the general factors that need to be considered are the immune status of the patient, the infectiousness of the tubercle bacilli, the environment and the exposure rate. Relating to infectiousness, it is largely dependent on the patient being infected by TB. For example, should the patient is careless for his/her own distance with the community, the bacilli can spread easier via air though activities like coughing or sneezing (Organization, 2006).

1.1.3 Prevalence for Tuberculosis globally

Meanwhile, relating to environment, the air quality is most concerned as it can either cause the tubercle bacilli to remain in an area or not. Generally and geographically, the burden of TB is highest in Asia and Africa. India and China together account for almost 40% of the world's TB cases. About 60% of cases are in the South-East Asia and Western Pacific regions. The African Region holds account for 24% of the world's cases, and the highest rates of cases and deaths per capita (Global Tuberculosis Programme, 2010).

According to WHO in 2012, the latest estimates included in their annual TB report are that there were almost 9 million new cases in 2011 and 1.4 million TB deaths (990 000 among HIV negative people and 430 000 HIV-associated TB deaths). In 2011, there were an estimated 8.7 million incident cases of TB (range, 8.3 million–9.0 million) globally, equivalent to 125 cases per 100 000 population. Mainly, the estimated number of cases in 2011 occurred in Asia (59%) and Africa (26%); a less number of cases occurred in the Eastern Mediterranean Region (7.7%), the European Region (4.3%) and the Region of the Americas (3%). Countries with the largest number of incident cases in 2011 were India (2.0 million–2.5 million), China (0.9 million–1.1 million), South Africa (0.4 million–0.6 million), Indonesia (0.4 million–0.5 million) and Pakistan (0.3 million–0.5 million). India and China alone accounted for 26% and 12% of global cases, respectively (World Health Organization, 2006).

1.1.4 Prevalence for Tuberculosis in Malaysia

In Malaysia, the estimated mortality rate is 1.7 thousand (range, 0.77 thousand-2.9 thousand) equivalent to 5.7 cases rate per 100,000 population. The prevalence rate for TB cases including HIV positive cases are 29 thousand (range, 13 thousand -52

thousand). A mortality and prevalence chart showed that over the years (1990-2011) both mortality and prevalence for TB is slowly declining. However, the treatment success rate is unstable and is likely to remain so for some period of time. Having said this, eventhough the rate of TB infection in Malaysia is slowly declining; the treatment for TB is a long and painful process that might not be successful for severe cases. Therefore, the best way to prevent TB is to diagnose it at an early stage (World Health Organization, 2012).

1.1.5 Tuberculosis diagnostics

Currently, the diagnostics that are used for TB detection include:

- Medical history,
- Physical examination,
- Test for TB infection (TB skin test or TB blood test),
- Chest radiograph (X-ray)
- Appropriate laboratory tests

More popular tests for the diagnosis of TB are TST and the Interferon Gamma Release IGRA; the QuantiFERON–TB Gold In-Tube test (QFT-GIT) and T-SPOT TB test (T-Spot) which measures the amount of IFN-□ stimulated by the bacillus in the body(Mazurek et al., 2010). The gold standard for detection of TB is culture and AFB microscopy. The sensitivity reaches a minimum of 100 bacilli per ml of sputum and range from 80-90%. The specificity (approximately 99%) of culture holds the advantage of allowing the differentiation between Mycobacterium Tuberculosis and other bacteria. Media for culture which are usually used are Lowenstein-Jensen (egg-based medium) and Ogawa (agar-based medium). However, the big downfall for culture is that it takes up to 8 weeks of incubation. In normal cases, medical examiners will not wait for the

results from the culture to initiate TB treatment. Usually, AFB smears are conducted alongside culture technique. Results from AFB determine whether the patient is treated and isolated or not and the results from culture acts as a confirmation test for TB diagnosis.

In the TST or Mantoux test, a protein extract from TB bacilli, is injected into the patient to stimulate IFN□ that is measured from the formation of an induration on the skin. The problem is that the test will be positive after any contact with TB or also after vaccination with BCG, in the absence of active disease. In addition, TST tests are prone to false-positive due to other mycobacterial species. Therefore, TST is not recommended except in the case of children.

Other diagnostics include PCR, chromatography, serology etc. However, the evaluations of these techniques are not done extensively and there is a weakness to every technique which prevents them from being used routinely for TB detection. Some of the reasons why these techniques are not used include the expensive cost of machinery, the need for high-skilled personnel to operate the tests, and low specificity and sensitivity (World Health Organization, 2010).

1.2 QuantiFERON TB Gold In-Tube

Of late, diagnostic kits which are most effective for the detection of *M.tuberculosis* are IGRA. Commercially available kits approved by FDA are QuantiFERON-TB Gold assay (Cellestis Limited, Carnegie, Victoria, Australia) and the T SPOT-TB assay (Oxford Immunotec, Oxford, UK). QuantiFERON-TB Gold assay detects peptides from three main antigens of *M. tuberculosis* which is ESAT-6, CFP-10 and TB7 utilizes samples from whole-blood and that IFNγ response to PPD (purified protein derivative) with ELISA. The alignments of the peptides are shown in Figure 1.1, Figure 1.2, and

Figure 1.3 and the list of the amino acid sequence are shown in Table 1.1. For ESAT-6 and CFP-10, the alignments shown are the cocktail of peptides within the whole ESAT-6 and CFP-10 sequence. QFT only incorporated a part of the whole peptide cocktail into their kit. For TB7.7, QFT used only Peptide 4 (P4) in their kit.(GOLD, 2010, Brock et al., 2004a)

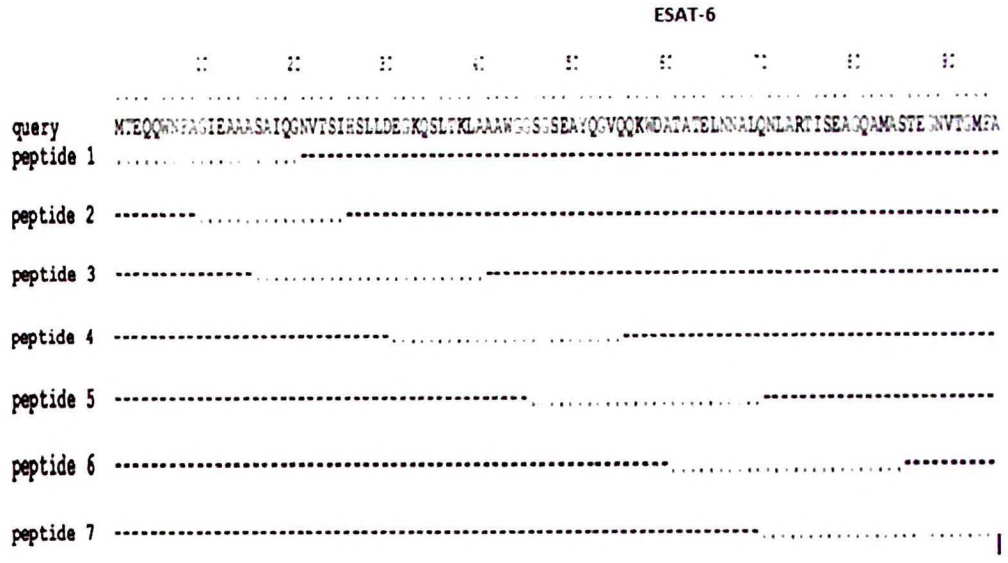

Figure 1.1: Alignment for ESAT-6 overlapping peptides

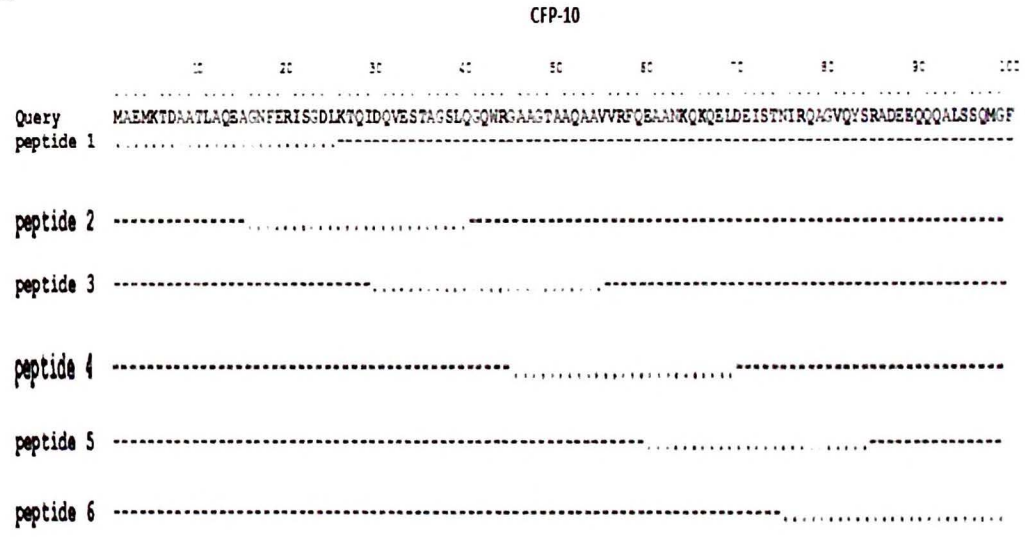

Figure 1.2: Alignment for CFP-10 overlapping peptides

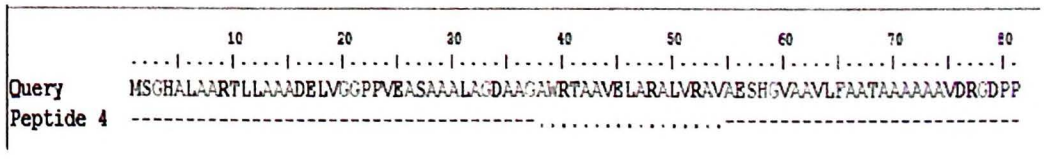

Figure 1.3: Alignment for TB7.7 (P4) peptide

Table 1.1: Amino acid sequences of overlapping peptides for ESAT-6, CFP-10 and TB7.7

Antigen	Amino acid sequence
CFP-10	
Peptide 1	MAEMKTDAATLAQEAGNFERISGDL
Peptide 2	GNFERISGDLKTQIDQVESTAGSLQ
Peptide 3	DQVESTAGSLQGQWRGAAGTAAQAAV
Peptide 4	AGTAAQAAVVRFQEAANKQKQELD
Peptide 5	AANKQKQELDEISTNIRQAGVQYSR
Peptide 6	IRQAGVQYSRADEEQQQALSSQMGF
ESAT-6	
Peptide 1	MTEQQWNFAGIEAAASAIQG
Peptide 2	GIEAAASAIQGNVTSI
Peptide 3	SAIQGNVTSIHSLLDEGKQSLTKLA
Peptide 4	EGKQSLTKLAAAWGGSGSEAYQGVQ
Peptide 5	SGSEAYQGVQQKWDATATELNNALQ
Peptide 6	TATELNNALQNLARTISEAGQAMAS
Peptide 7	NLARTISEAGQAMASTEGNVTGMFA
TB7.7	
Peptide 4	AWRTAAVELARALVRAV

Meanwhile, T SPOT-TB assay uses peripheral blood mononuclear cells (PBMCs), uses ESAT-6 and CFP-10, and detects (by use of ELISPOT) the number of T cells producing IFNγ. These tests have several advantages over the TST (Pai et al., 2004). Firstly, the tests are done in vitro and do not involve measurements of skin indurations thus, eliminating repeated visits to the hospitals from patients. Besides that, the results acquired are less subjective and even more sensitive than TST up to a certain degree.

1.2.1 Performance of QuantiFERON TB Gold In-Tube in different countries

It is now apparent that QFT exceeds the performance of TST in diagnosing for LTBI (Manuel and Kumar, 2008). The next issue of concern is that the performance of QFT varies across region and population. For example, a study involving patients from Melbourne, Australia compared the performance both QFT and TST tests with standardized protocols. Results show that the agreement between the tests are poor however, the concordance of QFT was better than TST as the positive results of QFT was associated with the country that has a high prevalence of TB and high-risk occupational contact. In addition, there were discordant results in situations where TST results were positive and QFT results were negative in recipients that had BCG vaccination (Vinton et al., 2009). These results show that QFT had a better degree of specificity and sensitivity than the TST method.

Another study done in South Africa compared gamma interferon release assay (IGRA) with sputum smears and TST. According to the study, the results revealed that the sensitivity of IGRA varied by clinical subgroup from 64% to 82% for the sputum smear and from 85% to 94% for the TST. Meanwhile, the sensitivity of the IGRA in human immunodeficiency virus (HIV)-infected TB cases was 81%. All in all, the combined

specificity for TST plus IGRA and TST plus a single sputum smear were 96% and 93%, respectively.

In addition, according to Mazurek, the sensitivity of the IGRA is similar to that of TST in infected persons with culture-positive TB. The IGRAs are thought to be more specific than the TST because they do not cross-react to BCG vaccine or to non-tuberculous mycobacteria. Updated 2010 guidelines suggest that IGRAs may be preferred for testing persons who have received BCG and persons unlikely to return for TST reading. Meanwhile, Ibrahim O. Al-Orainey claimed that, IGRAs and TST have similar degree of sensitivity and in some studies they correlated better with the degree of exposure. Finally, for immune-compromised patients, their sensitivity was better than that of the TST.

In Malaysia, the degree of specificity and sensitivity for QFT usage in diagnosing LTBI was 50.5% and 82.1% respectively (Rafiza et al., 2011). Meanwhile, a Korean study revealed a degree of specificity of 36% and 74% respectively. In Finland, a low-prevalence for TB, the sensitivity of IGRA compared to TST was 0.92 and specificity was 0.91 (Tavast et al., 2009). In Italy, 336 children at risk for TB was assessed and the results showed that the number of positive results for TST and QFT were 58 and 60 respectively. Half the children that produced a positive TST result had a negative QFT result. This suggests that TST might be more sensitive than QFT or alternatively, QFT is more specific than TST (Mazurek et al., 2010).

Based from past studies, the performance rate of QFT is exceptionally sensitive for low endemic countries especially when detecting active TB patients in low TB endemic countries. In contrast, the sensitivity of QFT in high endemic countries is relatively lower (Tsiouris et al., 2006). It was found that the lower sensitivity of QFT in high

burden countries are due to the HIV co-infection, additional diseases, malnutrition, host immune response and variation in the M. tuberculosis strain (Dheda et al., 2009). However, of particular interest, is the effect of different genetic or HLA makeup of the population that responds differently to the 3 peptides of QFT (CFP-10, ESAT-6 and TB7.7).

1.2.2 Future Implications for QuantiFERON TB Gold In-Tube

Another study states that the sensitivity of ELISA can be increased by using purified peptides that are specific to the TB complex (Zhang et al., 2009). These peptides must not only be specific towards the mycobacterium strain but must also be characterized and tailored to react to the population of concern. These characterizations are achieved by means of bioinformatics but are however, restricted to a limited number of genes or major histocompatibility complex (MHC) alleles. Nevertheless, the characterisation and build-up of a peptide that is potentially more sensitive in fishing out a response in Malaysian population is possible with the help of bioinformatics and gene libraries.

1.3 Objectives

The objectives of this study is to evaluate the performance of QuantiFERON-TB Gold In-Tube in Malaysian population, to assess the sensitivity and specificity of the QuantiFERON-TB Gold In-Tube, and to test whether QuantiFERON-TB Gold In-Tube can produce significant results between groups of different TB status.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

All the equipment used in this research project is as listed in Table 2.1 and chemical reagents used are listed in Table 2.2.

Samples

1. Whole blood sample from TB patients (Hospital Gombak TB patients, Orang Asli TB patients, Hospital Kota Bharu TB patients, Sg. Siput and Pahang individuals.)
2. Whole blood sample from high-risk TB patients (Hospital Gombak, Orang Asli, Sg. Siput and Pahang healthy contacts)
3. Whole blood sample from low-risk TB patients (USM students and Pahang individuals.)

Table 2.1: List of equipment and supplier

Equipments	Supplier
1. QuantiFERON TB Gold	Celestis, Qiagen, USA
2. ELISA plate washer	Multiwash III Tricontent, USA
3. ELISA reader	SoftMax Pro 5, USA
4. QuantiFERON analysing software	Celestis, Qiagen, USA
5. Centrifuge (bood samples)	Thermo Scientific (RVALL ST16 Centrifuge), USA
6. Incubator	Memmert, Germany
7. Vortex mixer	IKA MS 3 , Germany
8. Plate shaker	Heidolph Titromax x 101, Germany

Table 2.2: List of chemical reagents used

Chemical reagents	Supplier
Phosphate Buffered Saline (PBS) washing buffer	INFORMM
Distilled water	INFORMM

2.2 Methods

2.2.1 Blood collection and sample preparation

Blood from the respected individuals were collected via butterfly needle or venepuncture on a random basis. Then, the tubes were shook vigorously for 5 seconds to ensure the blood is well mixed and reacts with all the antibodies on the walls of the tubes. Next, the tubes were immediately incubated upright at 37°C for 16-24 hours as immediate incubation will reduce the probability of getting indeterminate results (Herrera et al., 2011). After that, the samples were centrifuged at 2000-3000 (RCF) for 15 minutes. Finally, approximately 200µl of plasma were harvested from each tube and stored in microtubes for storage prior ELISA procedure. The flowchart for blood collection is shown in Figure 2.1.

2.2.2 Human IFN-γ ELISA

The IFN-γ kit Standard must be first reconstituted with deionized water. The reconstitution volume for deionized water is indicated on the label of the vial. The reconstitution volume differs between batches. In our project, the vial was reconstituted with 2.61 mL of deionized water. After that, the mixture was shook gently to minimize frothing. The deionized water was added to produce a solution with an 8.0 IU/mL concentration.

After that, the reconstituted IFN-γ kit solution of 8 IU was used to produce a serial dilution to serve as the standard curve in ELISA. The standard curve consists of four standards of standard dilution. The concentrations of each standard are shown in Table 2.3 and the flowchart for the preparation of the standard curve as shown in Figure 2.2.

Table 2.3: Concentration for each standard

Standard	Concentration (IU/mL)
Kit Standard	8.0
Standard 1	4.0
Standard 2	1.0
Standard 3	0.25
Standard 4 (Green Diluent Only)	0.0

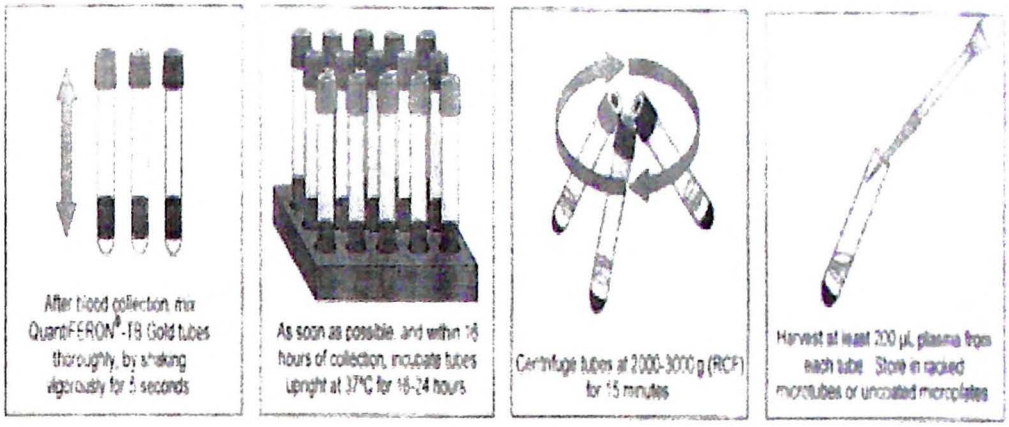

Figure 2.1: Blood collection procedures

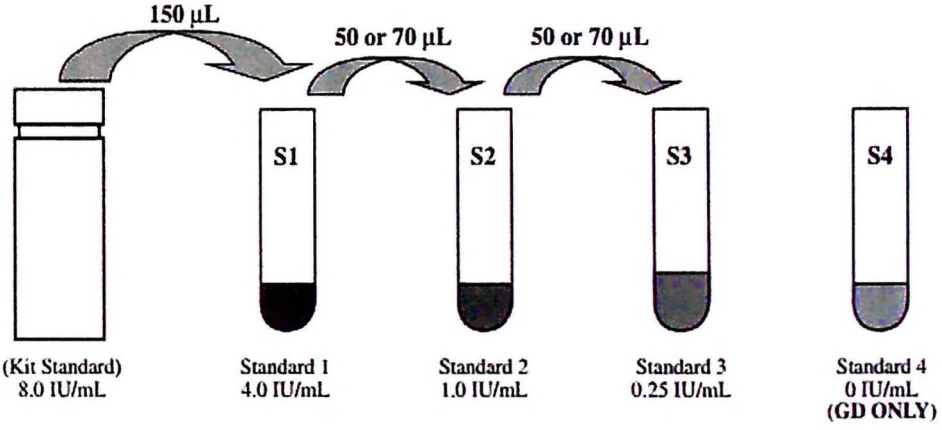

Figure 2.2: Preparation of standard curve

The Conjugate 100X Concentrate was reconstituted with 0.3 mL of deionized or distilled water. It was mixed gently to ensure a complete solubilisation of the conjugate. Then, the Working Conjugate was prepared by adding the Conjugate 100X Concentrate with an amount of Green Diluent which depends on the number of strips used. For our project, we used up 12 strips, thus the portion of Conjugate 100X Concentrate and green diluent is 60 μL and 6.0 mL respectively.

The wash buffer was prepared during the incubation period of the ELISA. One part of the Wash Buffer 20X Concentrate was diluted with 19 parts of deionized or distilled water and mixed thoroughly. However since the working strength for Wash Buffer has an expiry date of 2 weeks, we only prepared a small volume of working strength of Wash Buffer every time we performed ELISA.

The ELISA procedures are follows. Prior to assay, the plasmas were mixed thoroughly. Then, 50 μL of Working Strength conjugate was added into all ELISA wells. Next, 50 μL of test plasma samples were added into the appropriate wells. Finally, 50 μL of each standard (Standard 1 to 4) were added. Each standard were assayed in triplicates.

2.2.3 Validation of ELISA results with the Quantiferon TB Gold ELISA kit

According to the kit, the Mitogen control generally elicits the greatest IFN-γ response of the 3 samples from each subject. In some cases, the mitogen control OD value will be above the limit of the microplate reader; this has no impact on the test interpretation. The IFN- γ level of the Nil control is considered background and is subtracted from the TB Antigen and Mitogen results for that blood specimen.

The cut-off for the QFT test is 0.35 IU/mL above the Nil control (and TB Antigen minus Nil is more or less than 25% of the Nil control) for the TB Antigen stimulated

plasma sample. Individuals displaying a response to the TB Antigen above this cut-off are likely to be infected with *M.tuberculosis.*

The magnitude of the measured IFN- γ level cannot be correlated with stage or degree of infection, level of immune responsiveness, or likelihood for progression to active disease. A positive QFT result does not indicate the presence of active TB disease. Other diagnostic procedures, such as X-ray examination of the chest and microbiological examination of sputum, should be used when TB disease is suspected. The criteria for the interpretation of results are shown in Table 2.4.

2.3 Flowchart of overall study

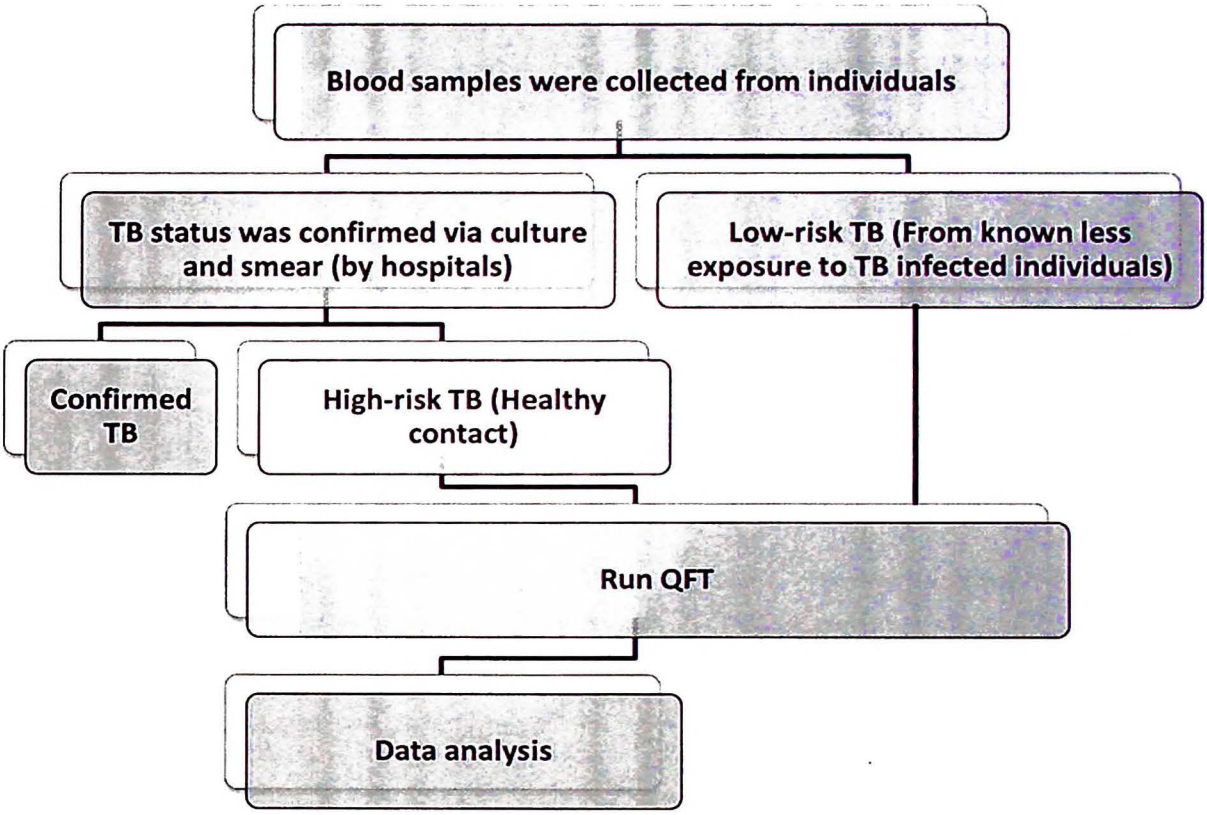

Table 2.4: Interpretation guidelines for QFT samples

Nil [IU/mL]	TB Antigen minus Nil [IU/mL]	Mitogen minus Nil [IU/mL][1]	QuantiFERON®-TB [IU/mL]	Report/Interpretation
≤ 8.0	< 0.35	≥ 0.5	**Negative**	*M. tuberculosis* infection NOT likely
	≥ 0.35 and < 25% of Nil value	≥ 0.5		
	≥ 0.35 and ≥ 25% of Nil value	Any	**Positive**[2]	*M. tuberculosis* infection likely
	< 0.35	< 0.5	**Indeterminate**[3]	Results are indeterminate for TB Antigen responsiveness
	≥ 0.35 and < 25% of Nil value	< 0.5		
> 8.0[4]	Any	Any		

CHAPTER 3: RESULTS

3.1 Samples

A total of 99 individuals which were randomly picked from Hospital Gombak, Hospital Kota Bharu, Universiti Sains Malaysia Health Campus, Sungai Siput, Pahang and some individuals from the Orang Asli were selected for this study. From the diagnostics done, 45 TB patients were identified and enrolled into the TB patient group, 30 were identified as having high-risk of TB and enrolled in the high-risk TB group and 24 were identified as having low-risk for TB and classified in the low-risk of TB group. Diagnostics used to classify TB patients from non TB patients were used on all individuals except on the low-risk TB group include chest radiograph, culture and sputum smear. All individuals have been vaccinated with bacillus Calmette-Guerin (BCG) as the Malaysian government commenced the BCG vaccine compulsory since the year 1961 (Merican and bin Yon, 2002) and were of random gender, age and health status. All individuals were tested with QFT.

All indeterminate results were repeated before finally reporting intermediate. Finally, the end result were calculated and analysed for sensitivity and specificity. Specificity and sensitivity calculations did not include indeterminate results to simplify the analysis and to prevent technical errors. Positive high-risk and low-risk individuals for TB were not given LTBI treatment.

3.2 Response towards QuantiFERON

Specificity was estimated by assuming all the subjects in the low-risk group are negative. Meanwhile, sensitivity was estimated by assuming all the TB patients are positive. The cut-off value used to distinguish between positive and negative results

from QFT were adapted from the kit itself, which is 8.0 IU/mL for Nil, 0.35 IU/mL for TB antigen and 0.5 IU/mL for Mitogen.

For the performance of QFT, 97.0% of the samples tested produced valid results while the remaining 3.0% produced invalid (indeterminate results). Therefore, the total samples that were used in the analysis of the specificity and sensitivity were 96 out 99 samples. All statistical analysis was done using IBM SPSS Statistics, version 20 (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp. Using the cut-off value from the kit, the TB patients showed 33 positives out of 45 (73.3 %), 12 (26.7 %) patients showed negative results. For high-risk TB individuals, 12 out of 29 (41.4%) produced positive results, 17 (58.6 %) showed negative results. Finally, for low-risk TB patients, 3 out of 22 (13.6 %) individuals produced positive results, 19 (86.4 %) individuals showed negative results. Finally, our sensitivity was calculated to be 73.3 % and our specificity was 86.4%. The results for all QFT samples are shown in Table 3.1 and Figure 3.1.

Table 3.1: Results for QFT for all individuals

		Count		Percentage		Total
		Positive	**Negative**	**Positive**	**Negative**	
Group	**Low-risk TB**	3	19	13.6 %	86.4 %	22 (100.0 %)
	High-risk TB	12	17	41.4%	58.6%	29 (100.0%)
	Confirmed TB	33	12	73.3 %	26.7%	45 (100.0%)
Total		48	48	50.0%	50.0%	96 (100.0%)

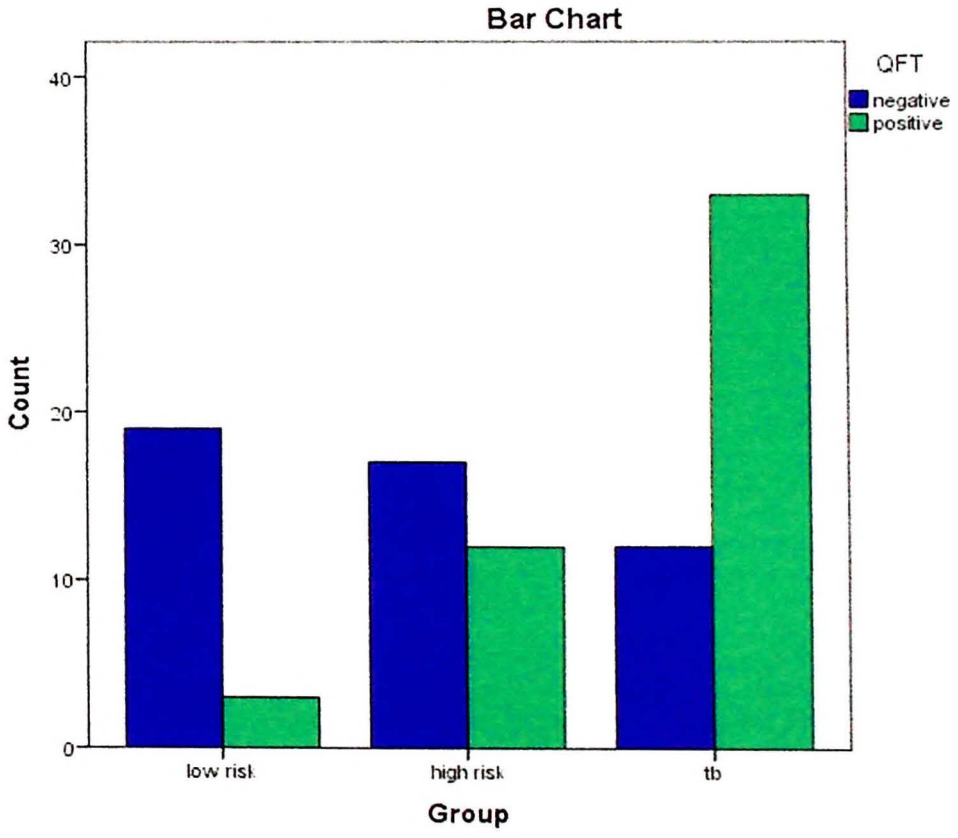

Figure 3.1: Results for QFT for all individuals

3.3 Statistical Analysis

Meanwhile, the association between groups of high-risk TB, low-risk TB and confirmed TB was tested using Chi-Square test. This was because Fisher's exact test could not be used to analyse all three relationships simultaneously. Thus, all relations between groups need to be assessed separately using Chi-Square test. All p values were presented in Table 3.2, Table 3.3, Table 3.4 and Table 3.5.

For Table 3.2, there was a significant association between the groups of non-Tb and confirmed TB with the results of QFT. ($p < 0.001$). The graph for the association between the groups of non-Tb and confirmed TB with the results of QFT is presented in Figure 3.2.

Table 3.2: Association between TB and non-TB groups in 96 samples

Group	Count (%)		p-value
	Positive	**Negative**	
Non-TB	15 (29.4)	36 (70.6)	<0.001
Confirmed TB	33 (73.3)	12 (26.7)	

*Chi-Square test, significant at p<0.001

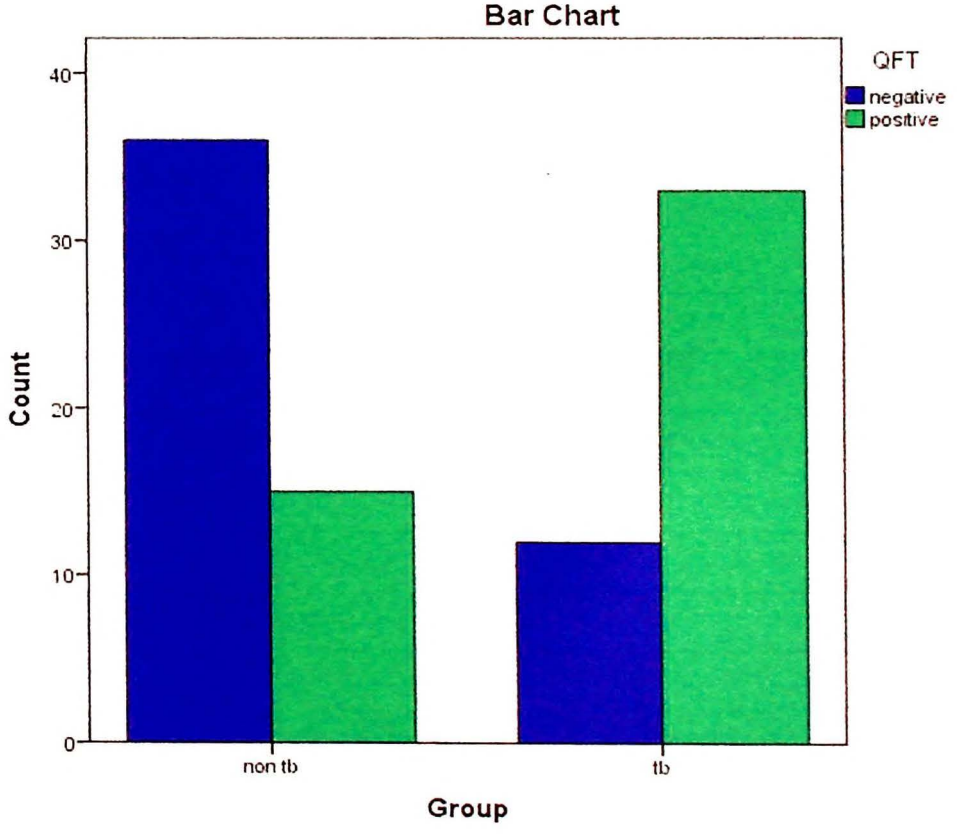

Figure 3.2: Association between TB and non-TB groups in 96 samples

There was a significant ($p<0.001$) association between groups of non-TB and confirmed TB with the results of QFT.

For Table 3.3, there was a significant association between the groups of high-risk TB and low-risk TB with the results of QFT. ($p= 0.031$). The graph for the association between the groups of non-TB and confirmed TB with the results of QFT is presented in Figure 3.3.

Table 3.3: Association between high-risk TB and low-risk TB groups in 51 samples

Group	Count (%)		p-value
	Positive	**Negative**	
High-risk TB	12 (41.4)	17 (58.6)	0.031
Low-risk TB	3 (13.6)	19 (86.4)	

*Chi-Square test, significant at $p<0.05$

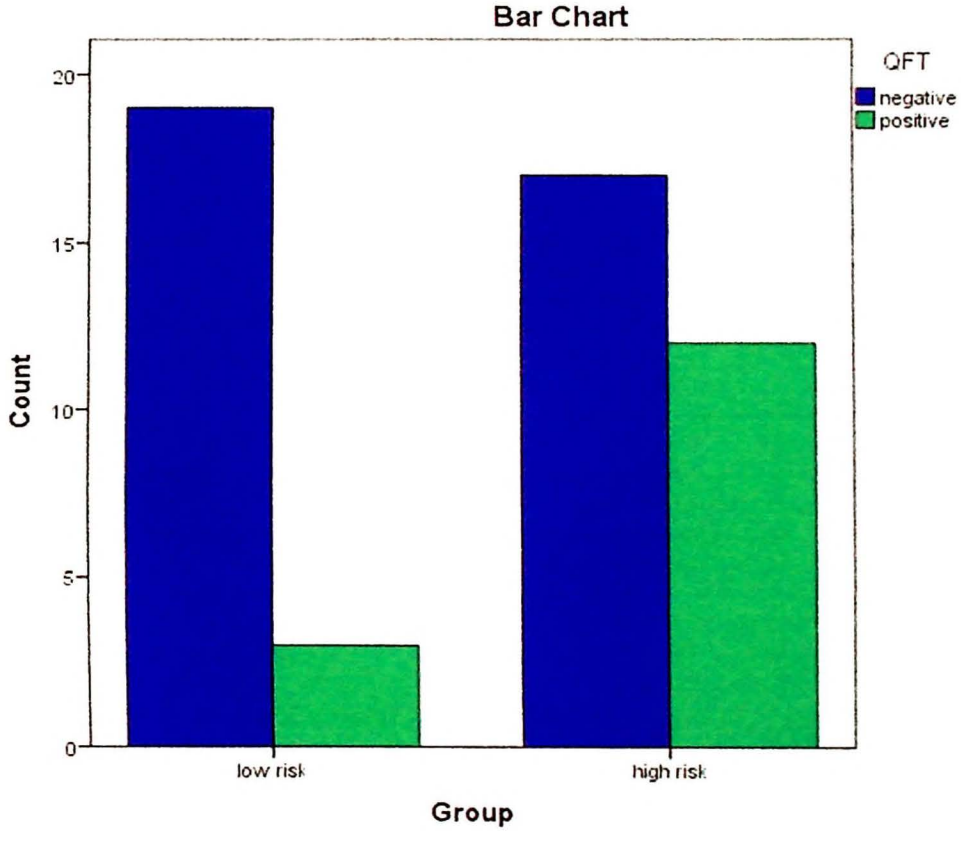

Figure 3.3: Association between TB and non-TB groups in 51 samples

There was a significant ($p= 0.031$) association between the groups of high-risk TB and low-risk TB with the results of QFT.

For Table 3.4, there was a significant association between the groups of low-risk TB and confirmed TB with the results of QFT. ($p< 0.001$). The graph for the association between the groups of non-Tb and confirmed TB with the results of QFT is presented in Figure 3.4.

Table 3.4: Association between low-risk and confirmed TB groups in 67 samples

Group	Count (%)		p-value
	Positive	**Negative**	
Low-risk TB	3 (13.6)	19 (86.4)	<0.001
Confirmed TB	33 (73.3)	12 (26.7)	

*Chi-Square test, significant at p<0.001

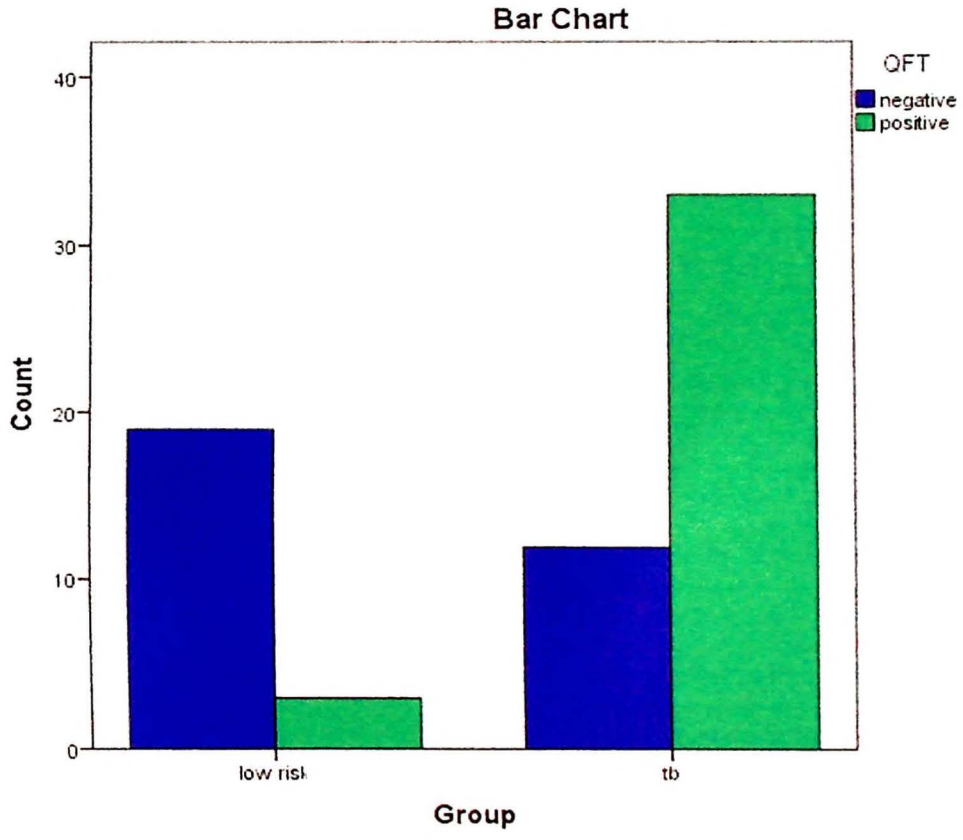

Figure 3.4: Association between low-risk and confirmed TB groups in 67 samples

There was a significant ($p<0.001$) association between the groups of low-risk TB and confirmed TB with the results of QFT.

For Table 3.5, there is a significant association between the groups of high-risk TB and confirmed TB with the results of QFT. ($p= 0.006$). The graph for the association between the groups of high-Tb and confirmed TB with the results of QFT is presented in Figure 3.5.

Table 3.5: Association between high-risk and confirmed TB groups in 74 samples

Group	Count (%)		p-value
	Positive	**Negative**	
High-risk TB	12 (41.4)	17 (58.6)	0.006
Confirmed TB	33 (73.3)	12 (26.7)	

*Chi-Square test, significant at $p<0.05$

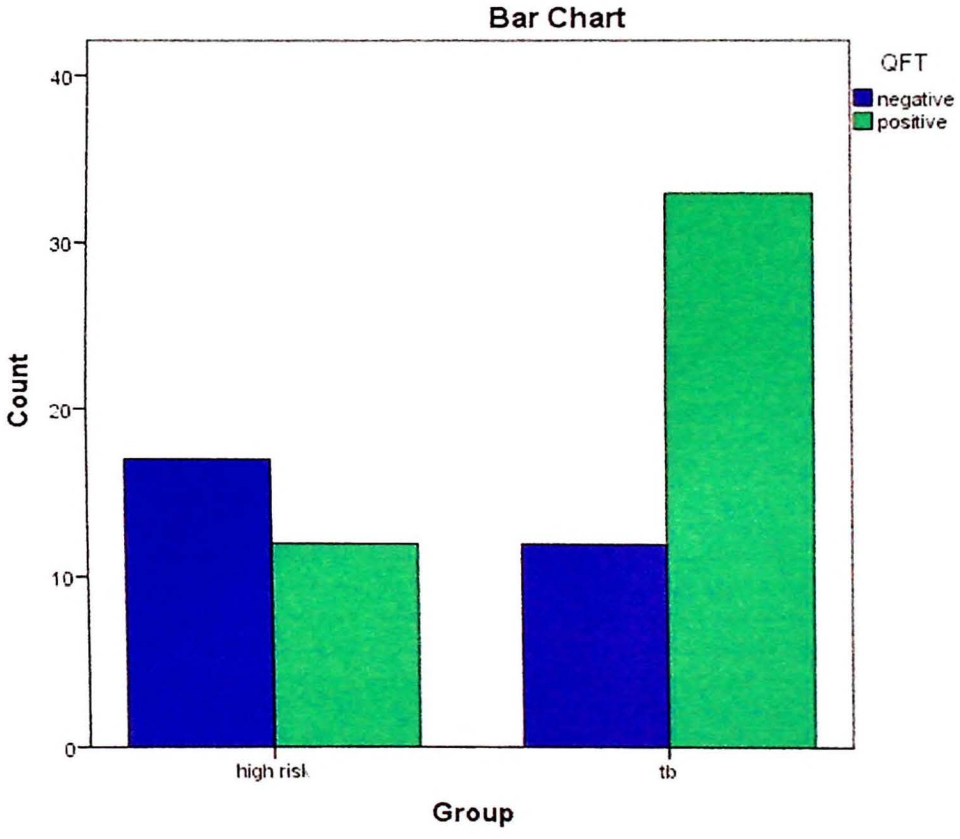

Figure 3.5: Association between high-risk and confirmed TB groups in 74 samples

There was a significant (p=0.006) association between the groups of high-risk TB and confirmed TB with the results of QFT.

Based on our results, the positive predictive value for our study is 91.6 % and our negative predictive value is 61.2 %. All in all, our results are quite similar to our neighbouring countries like Singapore and India whose sensitivity value was 83 % and 73 % respectively (Global Tuberculosis Programme, 2010)

CHAPTER 4: DISCUSSION

4.1 Discussion

This cross-sectional study provides data on the prevalence rate of TB among Malaysian population which is comparable among other countries using QFT. However, the lack of a gold-standard for LTBI (Herrera et al., 2011) diagnosis makes it difficult to compare the true performance value of QFT for LTBI diagnosis. Generally, the prevalence rate for TB infection in Malaysia is relatively low for an intermediate TB burden country (Rafiza et al., 2011). However, since there was no prevalence data for the general population of this country for LTBI, this study was done to provide that data along with the comparison of the performance of QFT among other countries.

Regarding our number of samples, using Singapore as a reference for prevalence studies, the total of samples for us needed to conduct a prevalence study was 185. This was calculated using the formula n= (z/delta) ^{2}*p (1-p). If we were to compensate for total of samples that were to produce invalid results or drop out from the study, a total of 10% of the sample must be added (n+10%) and thus, a total of 203 samples must be used in order to produce a valid prevalence study. However, due to the time constraint, we only managed to acquire a total of 99 samples of which 3 samples were inconclusive. In spite of this, we have managed to find significant association between all the groups and the results produced by QFT. In addition, our specificity and sensitivity did not differ much from our neighbouring country, Singapore (sensitivity; 83 %) (Mazurek et al., 2010).

The sensitivity of the QFT kit was estimated by using culture-confirmed TB infected patients as true positives (Mori et al., 2004). Meanwhile specificity of QFT was evaluated from true negative results from the low-risk TB group (Schablon et al., 2011).

Generally from our study, supported by previous papers which had done the same research for the quality of performance of IGRA, the specificity and sensitivity for QFT are apparently better than the Mantoux or Tuberculin-Skin Test (TST). This is due to the specific antigens used in QFT that eliminates the cross-reactivity from the BCG vaccination that occurs in the TST (Brock et al., 2004b).

Our results showed that the rate of performance for our kit was that it was able to produce up to 97% of valid results and 3% invalid results. Meanwhile for the sensitivity, 33 out of 45 (73.3 %) patients showed positive results against the true positives and for specificity, 19 out of 22 (86 .4 %) individuals in the low-risk TB group showed negative results against the assumed true negatives. In our calculation, we excluded the indeterminate results from our table to prevent erroneous calculation (Ferrara et al., 2006).

In our study, 3 individuals were identified producing indeterminate results. Therefore, they were not included in the analysis of the statistics of the samples. Among the factors thought to be associated with the indeterminate results based on previous studies were extremities of age (very old or very young) and the reduced function of the immune system (Ferrara et al., 2006, Cho et al., 2012), malnutrition such as hypoproteinemia, chronic and severely diseased individuals (Cho et al., 2012). In addition, indeterminate results could also resulted from the limitation of the kit itself, such as incomplete washing of ELISA plate, incorrect transportation or handling of the blood specimens, or longer than 16 hours of blood specimen incubation, or excessive levels of circulating IFN-γ or presence of heterophile antibodies (GOLD, 2010).

Our statistics showed significant association between all groups. This indicates that our QFT produced clear results that can be differentiated among different groups. There was

a significant association between non-TB and confirmed TB groups ($p<0.0001$), between high-risk TB and low-risk TB groups ($p=0.031$), between low-risk TB and confirmed TB groups ($p<0.0001$) and between high-risk and TB groups ($p=0.006$). This also proves that QFT can be used to diagnose LTBI and active tuberculosis. However, as indicated in the kit manual, QFT must not be used alone to diagnose for TB and must be accompanied with other forms of diagnostics for TB such as culture and sputum smear.(GOLD, 2010)

In addition, our positive predictive value (91.6 %) is higher than our negative predictive value (61.2%). This means that our kit is more likely to detect for positive results among samples by 91.6% compared to detecting negative results which is 61.2%. This quality is important for diagnosing TB as a false negative result could lead to the progress of LTBI to active tuberculosis.

Our sensitivity values are also relatively similar compared to our neighbouring countries like Singapore (sensitivity 83%), Indonesia (88.7%) and India (73%) (Rutherford et al., 2010, Mazurek et al., 2010). Meanwhile, data for specificity of QFT were especially high in low TB incidence countries for example Japan (99%) and Germany (100%).

Despite the fairly good value of specificity and sensitivity obtained, our discordance for sensitivity is due to the low positive results obtained. Previous studies claimed that it was correlated better with the degree of exposure (Al-Orainey, 2009). On the other hand, low sensitivity could result from the high degree of false negatives or due to the low reactivity of our samples towards the QFT peptides. Regarding false negatives, since we do not have the data on the characteristics of the samples, it is difficult to comment on why low sensitivity for this population is so. However, on a general scale, false negatives can result from low T-cell response, misclassification of groups, (Rose

et al., 2012), young age (Bergamini et al., 2009), and immunosuppression (Haustein et al., 2009). Having said all that, we should include all these factors that could be the characteristics to some of our samples which inevitably could explain the high amount of negative results from QFT run.

On the other hand, for the low reactivity, it is important to take note that although the samples came from different regions within the country, they still share the same set of HLA alleles; that may not respond well to the HLA epitopes of the peptide cocktail. This, inevitably may result in a low positive percentage (Betts et al., 2000). In addition, QFT utilizes the usage of human leucocyte antigens (HLA)-class II restricted epitopes of ESAT-6 and CFP-10 *M. tuberculosis* proteins whereby each peptide cocktail is a combination of more than one epitopes that would be able to bind to at least four different HLA-DR, two different HLA-DP and two different HLA-DQ specificities with a assumed binding ability of 80% maximum binding for any allele belonging to an HLA-class II serological specificity (Vincenti et al., 2007). Although previous studies claimed that the designed epitopes were able to cover more than 90% of the HLA-class II haplotypes present in different human populations, it is still a possibility that Malaysian populations might not respond well to the peptide cocktail used in QFT.

Meanwhile, for the specificity, it is relatively higher than the sensitivity due to the specific peptides transcribed from region of difference 1, which is a region on the mycobacterial genome specific for *M tuberculosis* and absent in BCG and most other mycobacteria incorporated in the QFT (Lighter et al., 2009). The peptides used eliminates cross reactivity from BCG vaccination and from other most non tuberculous mycobacteria except *M. kansasii, M. szulgai and M. marinum* (GOLD, 2010). However, our data for specificity is rather low compared to other countries for example, Japan (specificity 99%). This may be due to false positives. One of the sample within the low-

risk groups showed a positive result for the first run reverted to negative result on the second run. Reasons for this reversion of results were unknown (Gray et al., 2012). However, this data was not presented here. False positives are expected from low-risk individuals and it is recommended that the samples are repeated for better confirmation (GOLD, 2010). Besides that, a good confirmation of a non LTBI from low-risk individuals can eliminate the prescription of LTBI treatment and can save cost and the side effects of the treatment (Gray et al., 2012).

Limitations of this study include missing information regarding the samples for example gender, age, socioeconomic background and health status. More inferences can be made if more data was provided. In addition, QFT results must be accompanied and be in accordance to the epidemiological, historical, medical and diagnostic findings of the given sample (GOLD, 2010, Franken et al., 2007) therefore, the more data provided, the better analysis can be done. Besides that, the sample size for our study is too small. A bigger size sample with more confirmed-TB cases and more low-risk individuals would result in a better sensitivity and specificity analysis (Dogra et al., 2007).

Further implications of this study include population-tailored peptides for a better response towards the kit. A study by Richeldi suggested a tailoring application of the new blood IFN-gamma assays for LTBI in different high-risk groups (Richeldi et al., 2009). This can be beneficial especially for high prevalence countries like India and Gambia where high-risk population are at large. Besides that, the expensive cost of ELISA and QFT kit can be a huge limitation for countries which are poor financially. In addition, the procedure of ELISA can be quite time consuming and laborious. To top it off, highly skilled personnel are needed to run this test in a lab setting with complete apparatus (Mazurek et al., 2010). ELISA or QFT cannot be simply done anywhere and by anyone; which explains why technical errors are not impossible and needs to be

taken into account. The status for active TB or LTBI diagnosis can be improved with the invention of a point-of-care diagnostic kit. A lateral flow format of QFT would eliminate the high cost for ELISA and QFT kit, the need for highly skilled personnel and the expensive facilities needed to run the test (Oxlade et al., 2007). Besides that, a lateral flow format would produce results in a shorter time span and can be done virtually anywhere (Corstjens et al., 2011).

CHAPTER 5: CONCLUSION

5.1 Conclusion

As a whole, from previous studies QFT is a form of IGRA which is deemed more sensitive and specific for the diagnosis of LTBI. However, the usage of QFT in high TB prevalence countries are not recommended due to the immunosuppression, malnourishments, severely diseased and the high-risk population that could lead to misinterpretation of the results and false negatives.

QFT results should never be used alone and must be accompanied by other diagnostics tests before confirming the disease status of a person. It also must be in accordance with the epidemiological, historical, medical and diagnostic findings of the patient. However, a positive QFT result that is repeated and carefully analysed should be followed with proper LTBI treatment.

5.2 Future Implications

In the future, the design of more specific peptide cocktails that are tailored to the HLA of specific population should be explored as this could increase the level of specificity and sensitivity of the kit. In addition, a lateral flow format of this kit would also be beneficial in many ways as it can eliminate the physical and technical limitations of ELISA.

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APPENDIX

Presentation (Poster)

Liyana Bt Azmi, Irna Zulikha, Norazmi Mohd Nor, Aziah Ismail. QuantiFERON-TB Gold In-Tube performance for diagnosing active and latent Tuberculosis in Malaysian population. International Conference on Medical and Health Sciences (ICMHS) 2013 in conjunction with the 18th National Conference on Medical and Health Sciences (18th NCMHS), Malaysian Society of Human Genetics Inaugural Conference (MSHG) and the 12th Annual Scientific Meeting College of Pathologists, Academy of Medicine, Malaysia

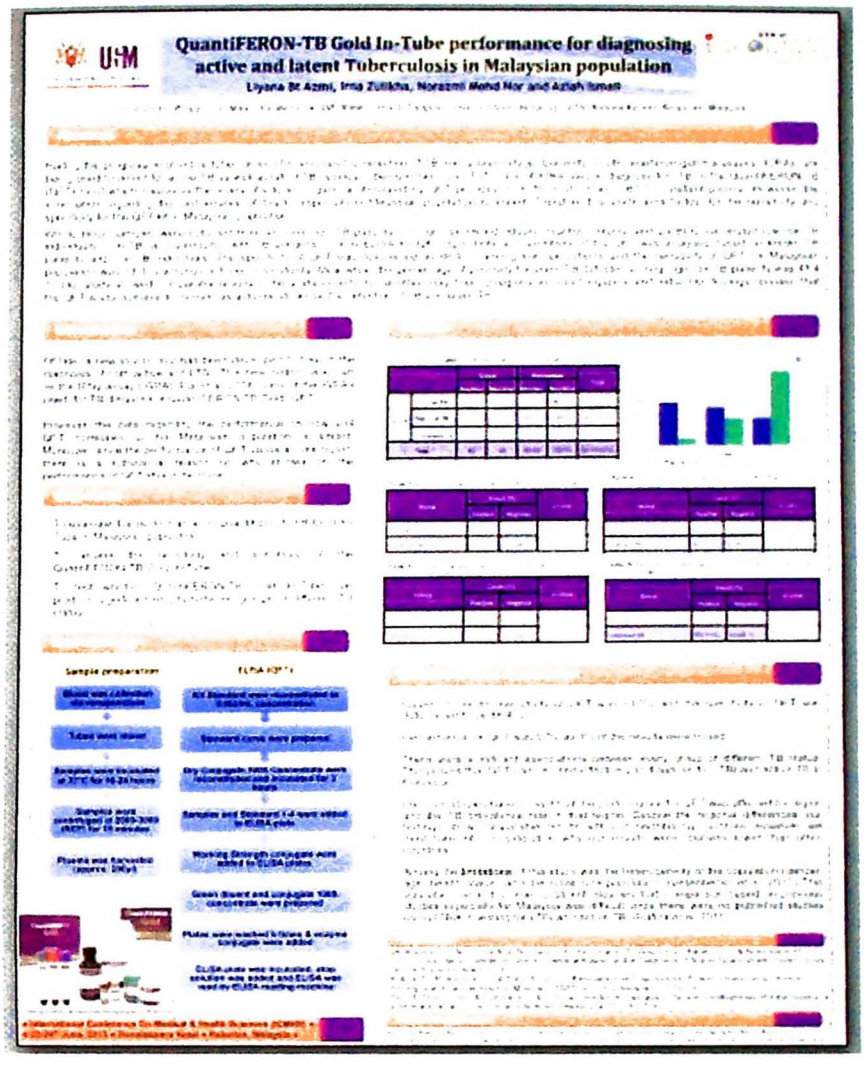

Figure 4: Poster Presentation: QuantiFERON-TB Gold In-Tube performance for diagnosing active and latent Tuberculosis in Malaysian population