

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.



**Utility of Newer and Novel Technologies for the Diagnosis and
Treatment-Monitoring of Tuberculosis Using Different
Biological Fluids**

By

LAURA MARY LENDERS

Student Number: LNDLAU002

SUBMITTED TO THE UNIVERSITY OF CAPE TOWN

In fulfilment of the requirements for the degree of

MASTER OF SCIENCE IN MEDICINE (by dissertation)

**Faculty of Health Sciences
UNIVERSITY OF CAPE TOWN**

Date of submission: 16 August 2010

Supervisor: Prof Keertan Dheda

Co-Supervisors: Dr Anke Binder and Dr Richard van Zyl-Smit

**Lung Infection and Immunity Unit
Division of Pulmonology
Department of Medicine
H.46 Old Main Building, Groote Schuur Hospital
Observatory, Cape Town, South Africa**

DECLARATION

I, Laura Lenders, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

DATE: 23 November 2010

STUDENT NAME: Laura Lenders

STUDENT NUMBER: LNDLAU002

SIGNATURE

A handwritten signature in black ink, appearing to read 'Lenders', is written over the printed word 'SIGNATURE'. The signature is written in a cursive style with a horizontal line underneath.

RESEARCH OUTPUT FROM THE PROJECT

Lenders LM, Meldau R, van Zyl-Smit RN, et al. (2010) Comparison Of Same Day Versus Delayed Enumeration of TB-Specific T Cell Responses, Journal of Infection, May 2010; 60(5): 313-408.

Dheda K, Davids V, Lenders L, Roberts T, Meldau R, et al. (2010) Clinical Utility of a Commercial LAM-ELISA Assay for TB Diagnosis in HIV-Infected Patients Using Urine and Sputum Samples. PLoS ONE, March 2010; 5(3): e9848.

University of Cape Town

ACKNOWLEDGEMENTS

I must especially thank my supervisor and mentor, Professor Keertan Dheda (Lung Infection and Immunity Unit), for his outstanding supervision, expertise, assistance and guidance.

With much appreciation, I thank my co-supervisors, Dr Anke Binder and Dr Richard van Zyl-Smit, for their expert guidance and support.

I sincerely thank the nursing staff, as well as other members of the Lung Infection and Immunity Unit who offered support and advice.

Thank you to the National Health Laboratory Service (NHLS) at Groote Schuur Hospital for conducting smear and culture testing on the specimens.

Thank you to the staff at the E16 respiratory clinic (Groote Schuur Hospital), especially Dr Greg Symons and Dr Hoosain Khalfey, for supplying bronchoalveolar lavage fluid from routine tuberculosis suspects who agreed to take part in the study.

I am grateful to all patients who took part in the study.

Thank you to the South African tuberculosis Vaccine Initiative (SATVI) laboratory (Institute of Infectious Diseases and Molecular Medicine, University of Cape Town) for the use of their ELISPOT reader and the Mycobacterial Immunology Group (Institute of Infectious Diseases and Molecular Medicine, University of Cape Town) for the use of their Gene Xpert® System.

Last but definitely not least, a very special thank you to my family and friends for their unwavering love, encouragement and support throughout my studies.

ABBREVIATIONS

AFB	acid-fast bacilli
AIDS	Acquired Immunodeficiency Syndrome
BAL	bronchoalveolar lavage
BCG	Bacille Calmette Guérin
CFP-10	Culture Filtrate Protein-10
CFU	Colony Forming Unit
CI	Confidence Interval
COPD	chronic obstructive pulmonary disease
CR3	Complement Receptor 3
CSF	cerebro-spinal fluid
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked Immuno-Spot assay
ESAT-6	Early Secretory Antigenic Target-6
FDA	Food and Drug Administration
GD	Green Diluent
HBHA	Heparin-Binding Haemagglutinin
HCW	Health Care Worker
HIV	Human Immunodeficiency Virus
HRP	Horse Radish Peroxidase
IFN- γ	Interferon Gamma
IGRA	Interferon Gamma Release Assay
IL	Interleuken
INH	isoniazid
IL-1RA	IL-1 receptor antagonist
IP-10	IFN- γ -inducible protein
IS	Insertion Sequence
LAM	Lipoarabinomannan
LAMP	Loop-mediated Isothermal Amplification
LIP	lymphoid interstitial pneumonia
LJ	Löwenstein-Jensen
LOD	limit of detection

LTBI	Latent tuberculosis infection
MAPc	Mycolyl-AG-peptidoglycan complex
MCP-1	monocyte chemotactic protein 1
MCP-2	monocyte chemo-attractant protein 2
MDR TB	Multi-Drug Resistant tuberculosis
MHC	Major Histocompatibility Complex
MIP	monokine-inducible protein
MODS	Microscopic-Observation Drug-Susceptibility
MR	Mannose Receptor
<i>M.tb</i>	<i>Mycobacterium tuberculosis</i>
NAATs	Nucleic Acid Amplification Tests
NPV	negative predictive value
NSIP	non-specific interstitial pneumonia
NTM	nontuberculous mycobacteria
OD	optical density
PBMCs	Peripheral blood mononuclear cells
PCC	probe check control
PCP	<i>Pneumocystis carinii</i> pneumonia
PCR	Polymerase Chain Reaction
PHA	Phytohaemagglutinin
POC	Point-of-care
PPD	Purified Protein Derivative
PPV	positive predictive value
QFT TB-G	QuantiFERON TB Gold
QFT-TB GIT	QuantiFERON TB Gold-In Tube
RBILD	respiratory bronchiolitis-associated interstitial lung disease
RD-1	Region of difference-1
RIF	rifampicin
ROC	Receiver Operating Characteristic
ROIs	Reactive Oxygen Intermediates
rpoB	RNA polymerase β
SAIGRA	South African IGRA study
SFC	Spot Forming Cell

SP-A	Surfactant protein A
SP-D	Surfactant protein D
SPC	sample processing control
SR	Sample Reagent
TACO	Tryptophane Aspartate-containing Coat Protein)
TB	tuberculosis
TBM	tuberculosis meningitis
TMB	tetramethylybenzidene
TGF- β	Transforming growth factor beta
Th1	helper T cell 1
TLR-2	Toll-like Receptor 2
TNF- α	Tumour Necrosis Factor alpha
Treg	Regulatory T-cell
TST	Tuberculin Skin Test
TCX	T-Cell Xtend
UCT	University of Cape Town
WHO	World Health Organisation
XDR TB	Extremely Drug Resistant tuberculosis

BACKGROUND. Smear microscopy has a poor sensitivity, cannot identify drug resistance, and when followed up with culture tests it takes several weeks to obtain a result. Furthermore, sputum-scarce and smear negative tuberculosis is difficult to diagnose. The use of biological samples other than sputum such as blood, urine and bronchoalveolar lavage fluid, are thus increasingly being used for diagnostic purposes. However, there is a low yield of rapid diagnostic tools, and the use of recently released commercially available technologies for tuberculosis diagnosis requires clarification.

AIMS/OBJECTIVES AND METHODOLOGY. Five-hundred tuberculosis suspects were sequentially recruited as part of a larger prospective parent study. Thus, where possible, blood, sputum, urine and bronchoalveolar lavage fluid samples were available for use in the current study. Blood samples were used to evaluate the following: i) treatment-related serial changes in the magnitude of antigen-specific IFN- γ release assay (IGRA: T-SPOT TB and QFT GIT) responses to a range of antigens (RD-1, HBHA and PPD) in active TB patients, ii) the comparison of same-day versus delayed enumeration of TB-specific T cell responses and iii) whether delayed sample processing *with* and *without* the use of T-Cell Xtend, a proprietary reagent for use with the T-SPOT.TB assay, impacts upon test accuracy in a clinical setting. Urine samples were used to evaluate the performance outcomes of the Clearview[®] urine LAM ELISA, and the diagnostic accuracy of urine LAM in HIV-infected patients stratified by CD4 T cell categories. The diagnostic utility of the Xpert[®] MTB/RIF Assay using bronchoalveolar lavage fluid was also evaluated.

RESULTS.IGRAs: There was poor agreement between changes (conversions/reversions) in peripheral quantitative T cell responses compared to culture status in active TB patients followed-up over a standard 6-month treatment period. T-Cell Xtend-independent median ELISPOT counts (spot forming cells per million peripheral blood mononuclear cells) were significantly higher at day 1 versus day 0 (114 vs.100; n=66;p=0.03); inter-time-point agreement between the results was 95.45% and the

conversion/reversion rate was 4.55%. By contrast, counts on day 0 *without* T-Cell Xtend versus day 1 *with* T-Cell Xtend were similar (56 vs. 56;n=215), inter-time-point agreement between the results was 97.17%, and the conversion/reversion rate was 2.83%. These findings were independent of HIV status. Clearview Urine LAM ELISA: Urine-LAM positivity was associated with HIV positivity (p=0.007) and test sensitivity, although very low, was significantly higher in HIV-infected compared to uninfected patients (21% versus 6%; p<0.001), and also in HIV-infected participants with a CD4 <200 versus >200 cells/mm³ (37% versus 0%; p = 0.003). Urine-LAM remained highly specific in all subgroups (95%-100%). 25% of smear-negative but culture-positive HIV-infected patients with a CD4 <200 cells/mm³ were positive for urine-LAM. MTB/RIF Gene Xpert: When using BAL fluid from patients with suspected TB, the Xpert MTB/RIF assay had a specificity of 97%. However, although the sensitivity (almost 60%) was higher than that of smear microscopy, this difference was not significant.

CONCLUSIONS. IGRAs: IGRAs do not appear to be a useful monitoring tool of tuberculosis in a high burden setting. Regarding delayed processing of blood for use in the T-SPOT TB assay, there was high agreement between results when samples were processed immediately and after a 24-hour delay. However, although the use of T-Cell Xtend appeared to reduce the number of conversions/reversions this reduction was not statistically significant. Larger studies are required to clarify these findings. Clearview Urine LAM ELISA: The overall usefulness of urine-LAM in a high burden primary care setting is limited. However, smear-negative HIV-infected TB patients with a CD4 count<200 cells/mm³, who would otherwise have required further investigation, may benefit from the use of urine LAM as a rule-in test. Although sensitivity in this group was modest, these preliminary findings suggest that future and adequately powered studies in a primary care setting should specifically target TB suspects who have advanced HIV infection. MTB/RIF Gene Xpert: The user-friendliness and the rapidity of acquiring results makes the MTB/RIF Gene Xpert assay a promising test for the diagnosis of TB using bronchoalveolar lavage fluid. However, larger studies in different geographical settings are required to clarify these findings.

TABLE OF CONTENTS

DECLARATION	i
RESEARCH OUTPUT FROM THE PROJECT	ii
ACKNOWLEDGEMENTS	iii
ABBREVIATIONS	iv
COMPREHENSIVE ABSTRACT	vii
LIST OF FIGURES	x
LIST OF TABLES	xv
INTRODUCTION, HYPOTHESIS AND SPECIFIC AIMS	1
<u>CHAPTER 1: LITERATURE REVIEW</u>	2
1.1. TUBERCULOSIS: CAUSE, DISEASE PRESENTATION AND EPIDEMIOLOGY	2
1.2. STRUCTURE AND MICROBIOLOGY	4
1.3. TRANSMISSION, PATHOGENESIS AND IMMUNE EVASION STRATEGIES	7
1.4. DIAGNOSIS	13
1.4.1. The diagnosis of TB mostly relies on a combination of factors	13
1.4.2. The Tuberculin Skin Test (TST) and the emergence of new technologies	14
1.4.3. Alternative sampling	15
1.5. INTERFERON-GAMMA RELEASE ASSAYS (IGRAs)	16
1.5.1. What are IGRAs?	16
1.5.2. Antigen-specific IGRAs versus the tuberculin skin test, and the utility of IGRAs for the diagnosis of latent tuberculosis infection	17
1.5.3. Utility of IGRAs for the diagnosis of Active TB disease	19
1.5.4. Utility of IGRAs for the diagnosis of HIV-TB co-infected patients	21

1.5.5. Utility of IGRAs for the monitoring of disease activity and efficacy of anti-tuberculosis treatment in high versus low burden settings	22
1.5.6. Can we do better?	30
1.6. LIPOARABINOMANNAN (LAM) ASSAYS	32
1.6.1. Urine lipoarabinomannan for diagnosis of tuberculosis	32
1.6.2. Performance outcomes of urine LAM in clinical studies	33
1.6.3. Clinical utility of lipoarabinomannan ELISA using biological samples other than urine	35
1.6.4. Future directions for urine-based diagnostics	36
1.7. NUCLEIC ACID AMPLIFICATION TESTS (NAATs)	37
1.7.1. NAATs for the diagnosis of TB	37
1.7.2. In-house versus commercial NAATs	39
1.7.3. Extra-pulmonary TB poses a diagnostic challenge	40
1.7.4. NAATs for the diagnosis of active pulmonary TB using bronchoalveolar lavage fluid	41
1.7.5. Future NAAT research directions	41
CHAPTER 2: The Use of a Range of Antigens in the Serial Evaluation of Antigen-specific Quantitative T Cell Responses of Active TB Patients on a Course of Anti-tuberculous Therapy	43
2.1. INTRODUCTION	43
2.2. AIM AND OBJECTIVE(S)	45
2.3. MATERIALS AND METHODS	46
2.3.1. Clinical recruitment	46

2.3.2. Laboratory methods	47
2.3.2.1. Isolation of peripheral blood mononuclear cells (PBMCs) for use in the T SPOT. TB assay	48
2.3.2.2. The ELISPOT (T-SPOT.TB) assay	50
2.3.2.3. QuantiFERON-TB Gold In-Tube (QFT-TB GIT)	53
2.3.2.4. Definition of IGRA conversions and reversions	56
2.3.3. Statistical Analyses	57
2.4. RESULTS	58
2.4.1. The frequency and magnitude of IGRA (T SPOT TB and QFT TB GIT) RD1 responses at serial time points during anti-TB treatment	61
2.4.1.1. T SPOT TB	61
2.4.1.2. QFT-GIT	63
2.4.2. Evaluation of the risk factors for change in the RD1 ELISPOT (T SPOT TB) and QFT-GIT results vs. culture and smear results	64
2.4.2.1. Changes at 2 months	66
2.4.2.2. Changes from 0 to 6 months	67
2.4.3. The frequency and magnitude of PPD and HBHA ELISPOT responses at serial time points during anti-TB treatment	68
2.4.3.1. Purified Protein Derivative (PPD)	68
2.4.3.2. Heparin-binding haemagglutinin (HBHA)	69
2.5. DISCUSSION	70
<u>CHAPTER 3: Delayed Enumeration of TB-specific T Cell Responses</u>	74
and the Evaluation of T Cell Xtend	
3.1. INTRODUCTION	74

3.2. AIM	76
3.3. MATERIALS AND METHODS	77
3.3.1. Clinical recruitment	77
3.3.2. Laboratory methods	78
3.3.2.1. Isolation of peripheral blood mononuclear cells	78
3.3.2.2. The use of T Cell Xtend	79
3.3.2.3. The ELISPOT (T-SPOT.TB) assay	80
3.3.3. Preliminary/optimisation phase	80
3.3.4. For the validation phase the cohort was divided into three study protocol groups	81
3.3.5. Definition of conversions and reversions	82
3.3.6. Statistical Analyses	82
3.4. RESULTS	83
3.4.1. Clinical and demographic information	83
3.4.2. OPTIMISATION PHASE	83
3.4.2.1. T-Cell <i>Xtend</i> independent of delay did not impact on test results at baseline (day 0)	83
3.4.2.2. Three-way serial analysis	85
3.4.3. VALIDATION PHASE	88
3.4.3.1. Delayed processing (~24 hours) <i>without</i> T-Cell <i>Xtend</i> resulted in significant changes in ELISPOT results	88
3.4.3.2. Delayed processing (~24 hours) with T-Cell <i>Xtend</i> maintained a high level of inter-time-point agreement, but the number of conversions/reversions was not significantly reduced	89

3.4.3.3. Delayed processing for up to 32 hours <i>with T-Cell Xtend</i>	91
resulted in high inter-time-point agreement, but the number of conversions/ reversions was not significantly reduced	
3.4.4. Individually scored RD1 antigen-specific spot counts	92
3.5. DISCUSSION	94
<u>CHAPTER 4: Clinical Utility of the Clearview-TB®-ELISA</u>	97
Lipoarabinomannan Assay Using Urine Samples from HIV-infected tuberculosis Patients	
4.1. INTRODUCTION	97
4.2. HYPOTHESIS AND AIMS	99
4.3. MATERIALS AND METHODS	100
4.3.1. Participants and study design	100
4.3.2. Laboratory methods	101
4.3.2.1. Detection of LAM in urine samples using the Clearview-TB®-ELISA	101
4.3.2.2. Interpretation of the results	102
4.3.3. Statistical methods	103
4.4. RESULTS	104
4.4.1. Clinical and demographic information	104
4.4.2. Sensitivity, specificity of urine LAM versus smear microscopy in different categories of TB suspects, and the association of urine LAM with HIV status and urine protein	105
4.4. DISCUSSION	109

CHAPTER 5: Diagnostic Utility of the Xpert® MTB/RIF Assay using Bronchoalveolar Lavage Fluid	112
5.1. INTRODUCTION	112
5.2. HYPOTHESIS AND AIM	113
5.3. MATERIALS AND METHODS	114
5.3.1. Participants and study design	114
5.3.2. Laboratory methods	116
5.3.2.1. The Gene Xpert Dx System: Principle of the procedure	116
5.3.3. Statistical methods and interpretation of the Gene Xpert results	118
5.4. RESULTS	119
5.5. DISCUSSION	123
GENERAL CONCLUSIONS AND RECOMMENDATIONS	125
REFERENCES	127

LIST OF FIGURES

Figure 1.1. (A) Scanning electron micrograph image of *M.tb* rods, and (B) Light microscope image of *M.tb* bacterial colonies growing on Middelbrook 7H11 agar medium.

Figure 1.2. *Mycobacterium tuberculosis* cell wall structure

Figure 2.1. Study flow for the investigation of the use of a range of antigens in the serial evaluation of antigen-specific quantitative T cell responses of active TB patients on a course of anti-tuberculous therapy.

Figure 2.2. Simplified diagram illustrating the main steps of the T-SPOT.TB assay.

Figure 2.3. An example of a T SPOT-TB test result in a subject classified as 'not infected with *M.tb*' (A), and a subject classified as 'infected with *M.tb*' (B).

Figure 2.4. Preparation of the kit standards for use in the QuantiFERON Gold-In Tube assay.

Figure 2.5. Plate layout template used for the QuantiFERON Gold-In Tube ELISA (28 tests per plate).

Figure 2.6. Quantitative antigen-specific IFN-gamma responses, in active TB patients on a six-month course of anti-TB therapy, to RD-1 antigens: (A) T SPOT.TB: summated results for ESAT-6 and CFP-10 at 0 months (baseline) and at 2 months post-treatment initiation [n=42], and (B) 0 months (baseline), 2 months post-treatment initiation and at treatment completion (6 months) [n=17]. The medians for the T SPOT.TB assay are indicated as horizontal

solid lines and the cut-points (24 SFCs/million PBMCs) are indicated as horizontal dotted lines.

Figure 2.7. Quantitative antigen-specific IFN-gamma responses, in active TB patients on a six-month course of anti-TB therapy, to RD-1 antigens: (A) QFT-GIT (ESAT-6, CFP-10, TB7.7) results at 0 months (baseline) and at 2 months post-treatment initiation [n=28], and (B) 0 months (baseline), 2 months post-treatment initiation and at treatment completion (6 months) [n=12]. The medians for the QFT-GIT assay are indicated as horizontal solid lines and the cut-points (0.35 IU/ml) are indicated as horizontal dotted lines.

Figure 2.8. Quantitative antigen-specific IFN-gamma responses, in active TB patients on a six-month course of anti-TB therapy, to PPD antigen: (A) PPD ELISPOT results at 0 months (baseline) and at 2 months post-treatment initiation [n=42], and (B) 0 months (baseline), 2 months post-treatment initiation and at treatment completion (6 months) [n=17]. The medians for the PPD ELISPOT assay are indicated as horizontal solid lines and the cut-points (24 SFCs/million PBMCs) are indicated as horizontal dotted lines.

Figure 2.9. Quantitative antigen-specific IFN-gamma responses, in active TB patients on a six-month course of anti-TB therapy, to HBHA antigen: (A) HBHA ELISPOT results at 0 months (baseline) and at 2 months post-treatment initiation [n=21], and (B) 0 months (baseline), 2 months post-treatment initiation and at treatment completion (6 months) [n=13]. The medians for the PPD ELISPOT assay are indicated as horizontal solid lines and the cut-points (24 SFCs/million PBMCs) are indicated as horizontal dotted lines.

Figure 3.1. Study flow diagram for the investigation of the delayed enumeration of TB-specific T Cell responses and the evaluation of T Cell Xtend. The study was conducted using two phases, namely, a preliminary/optimisation phase and a validation phase.

Figure 3.2. Simplified flow diagram showing how T-Cell Xtend should be incorporated into the T SPOT TB assay protocol for use with whole blood.

Figure 3.3. Summated response to ESAT-6 and CFP-10: day 0 *without* T-Cell Xtend vs. day 0 *with* T-Cell Xtend. The medians are indicated as horizontal solid lines and the cut-point (24 SFCs/million PBMCs) is indicated as a horizontal dotted line.

Figure 3.4. One sample was split into 6 (1.1-1.6), processed in parallel by the study operator, and seeded at a final concentration of 250 000 PBMCs per well. Intra-test variability was found to be minimal [average summated spot count =4.8 (values ranging from 4.5 to 5)].

Figure 3.5. Summated response to ESAT-6 and CFP-10 on day 0 *without* T-Cell Xtend vs. day 1 *without* T-Cell Xtend vs. day 1 *with* T-Cell Xtend (n=20). The medians are indicated as horizontal solid lines and the cut-point (24 SFCs/million PBMCs) is indicated as a horizontal dotted line.

Figure 3.6. Summated response to ESAT-6 and CFP-10 on day 0 vs. day 1 (both *without* T-Cell Xtend). A line graph of each individual's summated response is shown in figure A (n=66). For the whole group, 2 conversions and 1 reversion occurred. None of the conversions/reversions fall within the 'borderline zone' (van Zyl-Smit et al 2009) and were thus all considered to be true conversions/reversions (B). The medians are indicated as horizontal solid lines and the cut-point (24 SFCs/million PBMCs) is indicated as a horizontal dotted line.

Figures 3.7. Summated response to ESAT-6 and CFP-10 on day 0 vs. day 1 (both *without* T-Cell Xtend): response of the HIV negative subjects only (n=51; A) and the HIV positive group (n=15; B). The medians are indicated as

horizontal solid lines and the cut-point (24 SFCs/million PBMCs) is indicated as a horizontal dotted line.

Figure 3.8. Summated response to ESAT-6 and CFP-10 on day 0 *without* T-Cell Xtend vs. day 1 *with* T-Cell Xtend. A line graph of each individual's summated response is shown in figure A (n=215). For the whole group, a total of 9 conversions/reversions occurred. However, 3 of the 9 conversions/reversions fall within the 'borderline zone' (van Zyl-Smit et al 2009). Hence, only 6 (4 conversions and 2 reversions) were considered to be true conversions/reversions (B). The medians are indicated as horizontal solid lines and the cut-point (24 SFCs/million PBMCs) is indicated as a horizontal dotted line.

Figure 3.9. Summated response to ESAT-6 and CFP-10 on day 0 *without* T-Cell Xtend vs. day 1 *with* T-Cell Xtend: response of the subjects who had a negative HIV test result (n=115/159; A) and the HIV positive group (n=37/159; B). HIV results for 7/159 subjects were unknown. The medians are indicated as horizontal solid lines and the cut-point (24 SFCs/million PBMCs) is indicated as a horizontal dotted line.

Figure 3.10. Summated response to ESAT-6 and CFP-10 on day 0 *without* T-Cell Xtend vs. day 2 *with* T-Cell Xtend (n=12). The medians are indicated as horizontal solid lines and the cut-point (24 SFCs/million PBMCs) is indicated as a horizontal dotted line.

Figure 3.11. Group 1 (delayed processing at ~ 24 hours, independent of T-Cell Xtend): The median SFCs/million PBMCs for the summated RD1 antigen responses were 100 vs. 114, respectively, n=66, p=0.03 (as shown previously). A similar trend was seen when the results were displayed using (A) the individually scored SFCs/million PBMCs for ESAT-6, and (B) the individually scored SFCs/million PBMCs for CFP-10

Figure 3.12. Group 2 (delayed processing ~ 24 hours, with the addition of T-Cell *Xtend*): There were no significant differences between the median SFCs/million PBMCs for the summated RD1 responses (56 vs. 56, respectively, n=215, p=0.42 (as shown previously), and a similar pattern was seen when the when the results were displayed using (A) the individually scored SFCs/million PBMCs for ESAT-6, and (B) the individually scored SFCs/million PBMCs for CFP-10.

Figure 4.1. Simplified flow diagram illustrating the steps of the Clearview-TB®-LAM ELISA procedure.

Figure 4.2. Flow chart of patients recruited to the study stratified by patient subgroup, smear microscopy, HIV status, and CD4 T cell count.

Figure 4.3. The sensitivity, specificity (including 95% confidence intervals (%); top panel of table) and positive and negative predictive value (middle panel of table) of smear-microscopy alone, urine LAM alone, and a combination of urine LAM and smear-microscopy (using culture-positivity as a reference standard). The bottom panel of the table shows the test sensitivity in smear negative culture positive TB patients. For sensitivity calculations the definite TB group (n=141) was used. Specificity calculations were performed using the non-TB group (n=172) as a reference.

Figure 5.1. Study flow diagram for the investigation of the diagnostic utility of the Xpert® MTB/RIF assay using bronchoalveolar lavage fluid

Figure 5.2. Simplified flow diagram showing the main components of the Gene Xpert system, and the function of each component.

LIST OF TABLES

Table 1.1. Summary of longitudinal studies that have that have used IGRAs to monitor serial treatment-related responses in active TB adults on anti-TB treatment. The study location, sample group details, as well as information on the longitudinal follow-up time points, and type of IGRA assays used, are displayed in the table.

Table 1.2. Summary of commercially available nucleic acid amplification tests (NAATs) for tuberculosis.

Table 2.1. Guidelines for the preparation of the QFT-GIT kit Conjugate 100X Concentrate.

Table 2.2. Summary table of the evaluable subsets for the investigation of the use of a range of antigens in the serial evaluation of antigen-specific quantitative T cell responses of active TB patients on a course of anti-tuberculous therapy.

Table 2.3. Demographic and clinical characteristics of the patients who returned for the 2-month follow-up (n=42)

Table 2.4. Comparison of clinical and demographic characteristics of the T SPOT converters versus reverters.

Table 2.5. Risk factors for change in the RD1 ELISPOT (T SPOT TB) results vs. culture and smear results.

Table 2.6. Risk factors for change in the QFT GIT results vs. culture and smear results.

Table 2.7. Summary table of the McNemar test results used to assess whether there was agreement between the changes in IGRA and culture and smear changes (conversions/reversions) in TB patients after 2 months of treatment. Significant McNemar test p-values (≤ 0.05) denote that there is poor

agreement between the changes in the RD1 antigen-specific quantitative T cell responses (T SPOT TB and QFT-GIT) and the changes in culture and smear status at 2 months of treatment.

Table 2.8. Summary table of the McNemar test results used to assess whether there was agreement between the changes (conversion/reversion) in QFT-GIT results and culture and smear results in TB patients at treatment completion (6 months).

Table 3.1. A within-operator variability experiment showed that variability was minimal.

Table 3.2: Agreement between serial T-SPOT[®].TB results *with* and *without* T-Cell Xtend [agreement= the sum of those who remained T-SPOT positive (≥ 24 SFCs/million PBMCs) and those who remained T-SPOT negative (< 24 SFCs/million PBMCs) divided by the total sample size].

Table 4.1. Clinical and demographic information of the 440 evaluable TB suspects stratified by HIV status.

Table 5.1. Demographic and clinical characteristics of TB suspects and patients who had an unprocessed (raw) BAL fluid sample stored at -80°C for further analysis (n=59)

Table 5.2. Gene Xpert results stratified by confirmed diagnoses. There were no indeterminate Xpert MTB/RIF results. Hence, all of the results were evaluable (n=59).

Table 5.3. 2x2 tables of Gene Xpert results stratified by confirmed diagnoses.

Table 5.4. Sensitivity, Specificity, Positive and Negative Predictive Values for the evaluation of the diagnostic utility of the Xpert MTB/RIF assay using BAL fluid.

INTRODUCTION, HYPOTHESIS AND SPECIFIC

AIMS OF THE PROJECT

There is an ongoing search for rapid, inexpensive, easy-to-use, highly sensitive and specific point-of-care (POC) tests for tuberculosis diagnosis. Technologies such as Interferon Gamma Release Assays (T-SPOT.TB and the QuantiFERON TB Gold-In Tube test), the Clearview[®] LAM ELISA, and the Xpert[®] MTB/RIF Assay require clarification, especially in high burden settings. An additional concern is the difficulty in diagnosing smear negative or sputum-scarce tuberculosis, as well as patients who are co-infected with the human immunodeficiency virus (HIV).

The study hypothesis is that serial antigen-specific treatment-related response tracking and novel rapid diagnostic tests for use with alternative sampling (blood, urine and bronchoalveolar lavage) may be useful tools for the diagnosis and treatment-monitoring of tuberculosis in a high burden setting.

The first aim of the study is to evaluate treatment-related serial changes in the magnitude of antigen-specific IFN- γ responses to a range of antigens [RD-1 (ESAT-6, CFP-10 and TB 7.7), HBHA and PPD] in active TB patients on a 6-month course of anti-TB treatment. Secondly, we aim to investigate whether delayed blood sample processing *with* and *without* the use of T-Cell Xtend, a proprietary reagent for use with the T-SPOT.TB assay, impacts upon test accuracy of the assay. The third aim is to evaluate performance outcomes of the Clearview[®] LAM ELISA when using urine samples in HIV-infected and uninfected patients with suspected TB, and to determine the diagnostic accuracy of urine LAM in HIV-infected patients stratified by CD4 T cell categories. The final aim is to evaluate the diagnostic utility of the Xpert[®] MTB/RIF Assay in a group of pulmonary TB suspects, who are unable to expectorate sputum (sputum-scarce TB) or who are smear negative, using bronchoalveolar lavage fluid.

CHAPTER 1

LITERATURE REVIEW

1.8. TUBERCULOSIS: CAUSE, DISEASE PRESENTATION AND EPIDEMIOLOGY

Tuberculosis (TB) is a common, and often deadly, infectious disease that has been present in humans since ancient times (Zink et al 2001). TB, or 'tubercles bacillus', most commonly affects the lung-producing tubercles and, was discovered in the late 19th century by Dr Robert Koch. *Mycobacterium tuberculosis* (*M.tb*), is a pathogenic, rod-shaped bacillus responsible for the majority of cases of TB in humans (Malik & Godfrey-Faussett 2005).

Humans are the only reservoir for *M.tb*, and about two billion people, or one third of the world's population, are currently infected with this infectious pathogen, and two to three million people die from the disease annually (Cardona et al 2006; Corbett et al 2003; Malik & Godfrey-Faussett 2005). *M.tb* is spread through the air (mode of transmission and pathogenesis are described in detail later), when people who have the disease sneeze, spit or cough. TB most commonly affects the lungs, where it is referred to as 'pulmonary tuberculosis', while the term 'extra pulmonary tuberculosis' refers to TB in extrapulmonary sites such as the central nervous system (in meningitis), bones and joints (Pott's disease of the spine), the lymphatic system (scrofula of the neck), kidneys (urogenital TB) and skin (Golden & Vikram 2005). Miliary or disseminated TB, which is most common in young children and immunocompromised subjects, is a serious form of the disease. (Smith 2003)

The classic signs and symptoms of pulmonary TB are a chronic cough of more than 2 to 3 weeks, chest pain and expectoration of blood or blood-stained sputum. Systemic symptoms include weight loss, fatigue, appetite

loss, chills, fever, night sweats, and the production of sputum that changes from mucoid to purulent (Kumar 2007). However, about 90% of individuals infected with *M.tb* have asymptomatic latent TB infection (LTBI). World Health Organisation (WHO) TB reports reveal that new infections occur at a rate of about one per second (WHO Fact sheet N° 104, 2007). Approximately one in ten latent infections eventually progress to active disease, which kills more than 50% of its victims if left untreated (Onyebujoh & Rook 2004).

Other strains of Mycobacteria that commonly affect humans are *Mycobacterium leprae* (*M.leprae*) and *Mycobacterium avium* (*M.avium*). *M.leprae* is the causative agent of leprosy and *M.avium* causes a TB-like disease that is mostly prevalent in acquired immunodeficiency syndrome (AIDS) sufferers. Mycobacteria can also affect non-human hosts. The etiologic agent of TB in cows is *Mycobacterium bovis* (*M.bovis*). If humans ingest unpasteurised milk, they can be infected with *M.bovis*, which is most closely related to *M.tb*. (Thoen et al 2006) The Bacille Calmette Guérin (BCG) vaccine is a live vaccine against TB in humans and is derived from an attenuated (or weakened) strain of *M.bovis* (Hope & Villarreal-Ramos 2007). It was in 1921 that two French doctors -Camille Guérin and Albert Calmette- developed the BCG vaccine (Hawgood 2007), which was found to be the first genuine success in immunising against TB and is currently still the only partially successful vaccine that is used worldwide (Hope & Villarreal-Ramos 2007). However, the incidence of TB has been increasing since the 1980s, with sub-Saharan Africa and South-East Asia being the worst affected regions.

In 2005 the rate of TB infection in the Western Cape region of South Africa was the highest in the world, with an annual incidence of 1037 cases per 100 000 population (Cowley et al 2008). In 2007 there were an estimated 13.7 million people with chronic active disease, 9.3 million newly infected cases, and 1.8 million deaths, mostly in developing countries (World Health Organization 2009). Although TB is less common in developed countries, where it is mostly distributed in urban areas, people are more susceptible to

infection if their immune systems are compromised due to the use of immunosuppressive drugs, other forms of substance abuse (for example, smoking and alcoholism), and AIDS (Griffith & Kerr 1996). Co-infection with human immunodeficiency virus (HIV) is one of the many known host factors that make people more susceptible to TB infection and the rise of TB infection levels has been directly linked to the HIV/AIDS pandemic (WHO Fact sheet N° 104, 2007). The above-mentioned factors, coupled with the neglect of TB control programs, have contributed to the resurgence of the epidemic (Iademaro & Castro 2003). Furthermore, the emergence of multi- and extremely drug resistant (MDR and XDR, respectively) TB has exacerbated the problem (Dheda et al 2010c).

1.9. STRUCTURE AND MICROBIOLOGY

M.tb is a non-motile, acid-fast, obligate aerobe that is rod-shaped. The rods are usually 1-4 μm in length and 0.3-0.6 μm in width (Figure 1.1. A) (Kubica & Wayne 1984).

1 A



1 B



Figure 1.1. (A) Scanning electron micrograph image of *M.tb* rods, and (B) Light microscope image of *M.tb* bacterial colonies growing on Middelbrook 7H11 agar medium.

M.tb is an intracellular parasite, and its primary host cell is the macrophage. This bacterium has a very slow generation time (15-20 hours), which contributes to the chronic nature of TB. *M.tb* cannot be classified as Gram-positive or -negative, because this pathogen does not possess the characteristics of either of these classes of bacteria. However, certain structural characteristics of Gram-positive bacteria are present (Brennan & Nikaido 1995). The common layers of the bacterial Gram-positive cell wall are the inner plasma membrane, consisting of a lipid bilayer, and a thick layer of peptidoglycan which surrounds it. Both of these layers are present in *M.tb*, but there are also additional layers (Figure 1.2.), making the cell wall of *M.tb* thick and also unique in its complex composition.

A layer composed of arabinogalactan surrounds the peptidoglycan layer and the outermost layer is composed of mycolic acid. Thus, the core unit of the envelope is referred to as the mycolyl-AG-peptidoglycan complex (MAPc) (Brennan & Nikaido 1995). More than 60% of the cell wall is composed of complex lipids, which consist of three major components, namely, mycolic acids, Cord factor and wax-D. The mycolic acid layer is thick, hydrophobic and prevents disinfectants from entering the cell. Thus, when colonies are grown *in vitro*, there are distinctive chains of cells present in the smears. Cord factor is responsible for the distinctive s-shaped cords that are formed by these chains of cells and is most abundant in virulent strains of *M.tb*. Wax-D is present in the cell envelope and is associated with immunogenicity (Brennan & Nikaido 1995).

The outer surface of *M.tb* also contains complex surface glycolipids, which are most likely involved in mediating interactions between the pathogen and specialised cell surface receptors on the surface of macrophages (Schlesinger 1993). The mannose receptor (MR) is one of the many receptors present on the cell surface of macrophages.

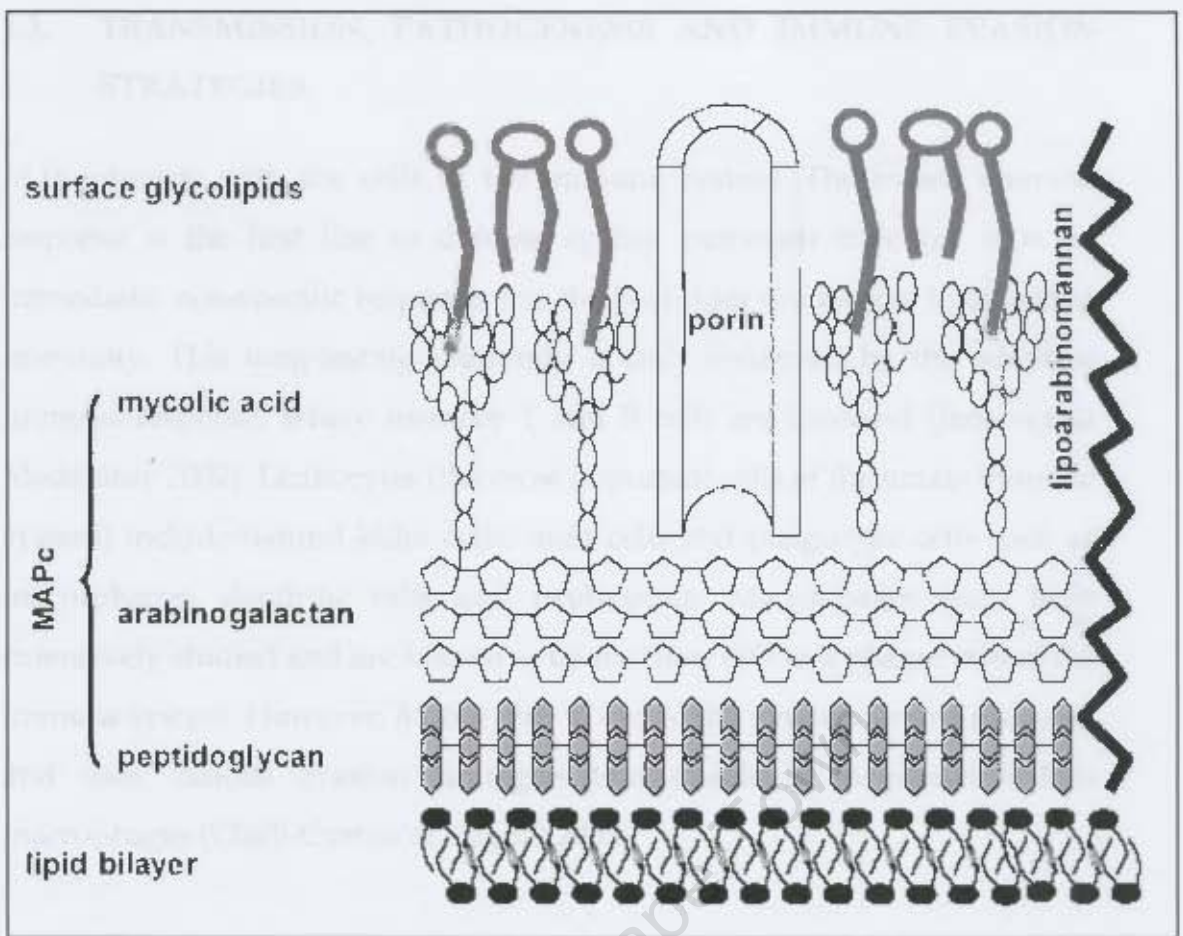


Figure 1.2. *Mycobacterium tuberculosis* cell wall structure

Lipoarabinomannan (LAM), a major surface lipoglycan and virulence factor associated with *M.tb*, serves as a ligand for the mannose receptor. LAM's primary function is to inactivate macrophages, which allows for the dissemination of mycobacteria to other parts of the body, and to scavenge oxidative radicals, which enables the bacteria to survive (Chan et al 1991; Knutson et al 1998). Assays using antibodies specific to LAM antigen have been developed to detect the presence of LAM in TB infected individuals. The detailed literature regarding the use of LAM assays for TB diagnosis will be reviewed later (Section 1.7).

1.3. TRANSMISSION, PATHOGENESIS AND IMMUNE EVASION STRATEGIES

M.tb interacts with the cells of the immune system. The innate immune response is the first line of defense against pathogen infection. It is an immediate, non-specific response, but the host does not receive long-lasting immunity. This long-lasting immunity is only conferred by the adaptive immune response, where memory T and B cells are involved (Janeway & Medzhitov 2002). Leukocytes (the most important cells of the innate immune system) include natural killer cells, mast cells and phagocytic cells such as macrophages, dendritic cells and neutrophils. Macrophages have been extensively studied and are known to be the most efficient phagocytes of the immune system. However, *M.tb* is able to evade the innate immune response and uses various evasion strategies that enable it to persist within macrophages (Clark-Curtiss & Haydel 2003).

M.tb pathogenesis can generally be divided into five stages. **Stage one** occurs when droplet nuclei are inhaled. These droplet nuclei are expelled by active pulmonary TB sufferers during coughing, sneezing and also via any other form of contact with their sputum. Droplet nuclei containing the infectious organisms can remain suspended in the air for several hours and people who are in constant contact with active TB sufferers are at an increased risk of being infected with the bacteria (Clark-Curtiss & Haydel 2003).

When *M.tb* enters the alveolar passages, the first cells that the pathogen encounters are the resident alveolar macrophages. However, the macrophages are not activated and are thus unable to destroy the intracellular organisms (Prescott et al 2005). Pattern recognition receptors (these include toll-like receptors) are present on the cell surface of macrophages. There are distinct receptors that recognise and bind to specific pathogens. The specific binding of a pathogen by cell surface receptors initiates phagocytosis, which is a mechanism whereby cells take up

extracellular particles and microorganisms. The macrophage mannose receptor, TLR-2 and complement receptor 3 (CR3) are a few of the receptors that are involved in the uptake of *M.tb* into macrophages (Ernst 1998; Schlesinger 1993).

Generally, phagocytosis is initiated when bacteria bind to the host pattern recognition receptors. A signal cascade is initiated inside the cell which triggers the rearrangement of the cortical actin skeleton of the host cell, which is regulated by small Rho-dependent GTPases. The rearrangement of the actin cytoskeleton leads to the formation of pseudopods (invaginations of the plasma membrane) and the engulfment of the bacteria. These pseudopods fuse to form a phagosome. When most bacteria are phagocytosed, the phagosome fuses with an early endosome and acquires early endosomal proteins. Maturation into a late phagosome occurs after the exchange of material with late endosomes. The final step of phagosome maturation involves fusion with a lysosome to produce a phagolysosome. Lysosomal enzymes are released into the mature phagosome and toxic molecules (free radicals or reactive oxygen species) are produced (Aderem & Underhill 1999; Nathan & Shiloh 2000). However, research to date suggests that once *M.tb* has been phagocytosed, it evades the host's immune response by releasing virulence proteins that somehow inhibit the fusion of phagosomes into early endosomes within the macrophages, thereby preventing early endosomal maturation (Russell 2001). Hence, the formation of the phagolysosome cannot occur and there is no acidification of the phagosomal environment, which enables *M.tb* to survive within the macrophage.

The exact mechanism whereby inhibition of phagosomal maturation occurs remains largely unknown, but it has been speculated that it is due to the modification of the phagosome membrane by a particular secreted protein, termed TACO (tryptophane aspartate-containing coat protein), which has been shown to be associated with the prevention of lysosomal delivery and

thus the prolonged survival of *M.tb* in the intracellular environment (Ferrari et al 1999; Gatfield & Pieters 2000). However, it is actually not clear whether the blocking of phagosomal maturation is absolutely vital for *M.tb* survival. The main point, though, is that *M.tb* is able to remain in the early phagosome, where it multiplies and grows (Russell 2001).

The capsule of *M.tb* contains various polysaccharides, such as glucan, mannan and arabinomannan. Thus, *M.tb* has many other special mechanisms for cell entry. Besides binding indirectly via certain complement or Fc receptors, it can also bind directly to macrophage mannose receptors via the mannosylated glycolipid (LAM), as previously mentioned (Schlesinger 1993). The evasion tactic in the case of cell entry involves receptor selection. Binding to CR3 does not effectively stimulate the macrophage to the same extent as binding to other phagocytic receptors, because the production of reactive oxygen intermediates (ROIs) by the host cell is reduced. *M.tb* interferes with the production of these ROIs by bypassing the activation of respiratory burst and thus decreases cytotoxicity and cell cytotoxicity is also downregulated by compounds such as glycolipids and LAM. *M.tb* secretes a group of proteins known as the Antigen 85 complex. This complex is known to bind fibronectin, which may aid in the sheltering of bacteria from the cells of the immune system and prevent granuloma formation, but there is not enough evidence to prove this.

The human toll-like receptor 2 (TLR-2) also plays a role in the uptake of *M.tb* (Noss et al 2001). In previous studies it has been mentioned that Surfactant protein A (SP-A), a glycoprotein found on the surface of the alveolar passages, is able to upregulate the expression of the mannose receptor and therefore enhance the binding and uptake of *M.tb* by macrophages (Gaynor et al 1995). Ferguson and colleagues investigated another alveolar surface glycoprotein, surfactant protein D (SP-D). SP-D binds to lipoarabinomannan (manLAM) on the surface of *M.tb*. SP-D was shown to reduce uptake and

inhibit intracellular growth of *M.tb* within human macrophages by enhancing phagolysosome fusion (Ferguson et al 1999).

The high lipid concentration in cell wall of *M.tb* results in resistance to antimicrobial agents, whereas the cord factor is known to be toxic to mammalian cells. However, the exact role of cord factor in *M.tb* virulence is unclear. Mycothiol is another compound that is secreted by *M.tb* and research to date suggests that it probably acts as an antioxidant for protection against toxic ROIs. KatG (catalase:peroxidase) is also an *M.tb* protein capable of detoxifying ROIs (Ng et al 2004).

Stage 2 of *M.tb* pathogenesis usually begins 7-21 days post-infection. The *M.tb* bacilli start to multiply within the naïve macrophages until the macrophages apoptose. Macrophages from the peripheral blood migrate to the site of infection and begin to phagocytose the bacteria. These macrophages are still not activated and cannot destroy the pathogen.

During **stage 3**, T lymphocytes, which are constantly circulating in the blood, start to converge at the site of infection. This late stage of infection is referred to as acquired or adaptive immunity and is a more specific response, because it is only activated if the innate immune system fails to control infection (Orme 2004). Infected macrophages are able to migrate via tissue fluid to secondary lymphoid regions. The lymphocytes come into contact with the infected macrophages in secondary tissues, where bacterial antigen is then presented to either cytotoxic T cells or to helper T cells. Dendritic cells, which are also important phagocytes of the immune system, are also the most efficient antigen presenting cells and play a role in T cell activation in response to *M.tb* antigens. Dendritic cells are able to migrate and thus may also play a role in dissemination or spreading of infection in the body (Lipscomb & Masten 2002). Antigen presentation occurs via MHC class II receptors, on the surface of antigen presenting cells, to CD4⁺ T lymphocyte receptors, helper T cell pathways are activated to secrete cytokines, such as interferon gamma (IFN- γ) which activates macrophages in conjunction with

Tumour Necrosis Factor alpha (TNF- α) to destroy the intracellular pathogen through reactive oxygen and nitrogen intermediates (Romani et al 1997). This response is referred to as the Th1 (helper T cell 1) response. IFN- γ is an important mediator of immunity and inflammation that and its key functions are mediated by cross-regulation of cellular responses to other cytokines (interleukin-2 and interleukin-12) and inflammatory factors. When antigens are presented via MHC class I receptors to CD 8⁺ T lymphocyte receptors, killer- or cytotoxic T cells directly kill the infected cells. In addition, long-lasting immunity is conferred, because memory cells are generated, which, as the name implies, are long-lived cells that have undergone clonal expansion after encountering antigen (Prescott et al 2005). Therefore, a rapid immune response can be elicited if the pathogen is encountered in future. Interferon-gamma release assays (IGRAs), which will be discussed in detail later, rely on the principle that sensitised T cells (effector memory cells) will rapidly produce IFN- γ when they encounter the *M.tb*-specific antigens. By contrast, central memory T-cells are more likely to require several days in the presence of antigen before IFN- γ is produced.

As cellular mediated immunity builds up, the activated macrophages are thus capable of destroying *M.tb* and during this process granuloma formation takes place. Infected macrophages aggregate together in order to prevent further spread of the pathogen. T lymphocytes and other cells of the immune system surround the entire aggregation structure and release cytokines in order to activate the macrophages to destroy the bacteria. This entire fibrous structure is called a granuloma. The destruction of the *M.tb* bacilli results in the formation of the caseous, necrotic centre of the granuloma or tubercle (Houben et al 2006; Nguyen & Pieters 2006). The *M.tb* bacilli are maintained in a steady, non-replicating viable state within the caseous centre of the granuloma. It has been postulated that this is due to the low oxygen and low pH environment that exists within the granulomatous centre. Therefore, some organisms enter into this state of dormancy and remain so for long periods of time. This state of infection is referred to as latent TB infection, which is non-transmissible and asymptomatic. The role

of the host immune system in the outcome of mycobacterial infection has been studied extensively for this state of infection. In a study performed by Dormans et al. 2004, the authors investigated the control of mycobacterial infections in humans and mice (Dormans et al 2004). It was found that the infections depend mainly on the macrophage activation, through the effect of Th1 type cytokines secreted by the infected macrophages. IL-12 regulates the gamma-interferon response and forms a feedback-loop to control infection (Romani et al 1997). Monocytes secrete IL-12 as part of a cellular-mediated response in order to stimulate immature helper T cells to differentiate into Th₁ cells. The action of Th1 type cytokines thus causes inflammation, which may lead to the development of the tissue pathology associated with granulomas (Dormans et al 2004). Therefore, during this stage (**stage 4**), many activated macrophages can be found surrounding the tubercles (granulomas).

It is important to emphasise that there is a difference between infection and disease. TB infection means that transmission has occurred (or in other words, *M.tb* is in the body), but the immune system is able to control the bacterial load. The problem arises when latent infections progress to the contagious active TB disease. Progression to active TB typically occurs as a result of immune suppression, for example in people who become immunocompromised due to HIV infection, malnutrition, drug usage or any other factors that may cause extreme weakening of the immune system (Griffith & Kerr 1996). This is referred to as **stage 5** of pathogenesis. For unknown reasons, the caseous centers of the granulomas liquify. *M.tb* is then able to multiply rapidly extracellularly, because the liquid creates a favourable environment in which the bacilli can grow. The infection spreads out of the granulomatous tissue within the lungs (active pulmonary tuberculosis) or in secondary tissues (extrapulmonary tuberculosis). This person then has progressive active TB disease, which is symptomatic, highly transmissible and lethal if left untreated.

1.4. DIAGNOSIS

1.4.1. The diagnosis of TB mostly relies on a combination of factors

A complete medical evaluation of the individual suspected of having TB needs to be conducted, which includes recording the details of their medical history, and conducting a physical examination, a chest X-ray, microbiological examination (culturing of sputum or some other appropriate biological sample) and blood tests to exclude other diagnoses. The evaluation may include the tuberculin skin test (TST, also known as a Mantoux test), and in some cases even a surgical biopsy. Other details of the medical history include demographic risk factors for TB, prior exposure to TB, whether the patient's received TB treatment in the past, and whether there are any other medical conditions that may increase the risk for TB disease, for example HIV infection. In the case of the physical examination, the physician's main objective is to assess the patient's general condition and observe other factors that may affect the treatment regimen. However, a physical examination alone cannot be used to confirm or rule out TB, because the only definitive way of diagnosing TB is by identifying *M.tb* in a biological sample (for example, sputum, cerebrospinal fluid, biopsied tissue, etc). However, the main problem is the difficulty in culturing this slow-growing bacterium in the laboratory (it may take 4 to 10 weeks for a positive blood or sputum culture). *M.tb* has historically been grown using Löwenstein-Jensen (LJ), Kirchner or Middlebrook (7H9 and 7H10) media, but it takes up to 4-6 weeks to obtain visual colonies on these media (Drobniewski et al 2003). Even when using a more specialised form of Middlebrook media (7H11), *M.tb* still takes at least 2 weeks to grow (Brennan & Nikaido 1995). If the patient is producing sputum, sputum smears and cultures are undertaken for acid-fast bacilli (AFB). The preferred method for microscopy is fluorescence microscopy (auramine-rhodamine staining), which has been shown to be more sensitive than the formerly used conventional method of Ziehl-Neelsen staining (Steingart et al 2006). Culture results may take 4-8 weeks before a

conclusive answer is obtained. However, new automated systems that are faster include the MB/BacT (2-3 weeks) (Brunello et al 1999), the BACTEC Mycobacterial Growth Indicator Tube (~13 days) (Diaz-Infantes et al 2000) and BACTEC 9000 (~10 days) (Pfyffer et al 1997). On average, the Microscopic-Observation Drug-Susceptibility (MODS) assay (Moore et al 2006) takes only about 7 days to diagnose TB and is able to identify the presence of MDR TB much faster than conventional culture (Caviedes & Moore 2007). Thus, it has potential for assisting clinicians to help control the spread of infectious TB by diagnosing and treating the disease at an earlier stage. However, the specific limitations and benefits of MODs in clinical settings is that it is labour intensive and newer test developments are underway. Thus, the lengthy time to diagnosis, especially in high burden countries, using any of the above-mentioned culture testing options remains a limitation and there is an ongoing search for newer and novel rapid diagnostic tools that will help to significantly decrease the time-to-diagnosis gap. Without culture, any diagnosis made would be classified as 'presumed' or 'probable' TB by using X-rays or scans and/or TST results.

1.4.2. The Tuberculin Skin Test (TST) and the emergence of new technologies

The interpretation of the TST depends on the individual's risk factors for infection such as exposure to other cases of TB (Pai & Menzies 2007b). Currently, LTBI is diagnosed in non-immunized subjects by a TST, which yields a delayed hypersensitivity type response to the *M.tb*-prepared extract of purified protein derivative (PPD). A major disadvantage of Tuberculin tests is false positives in the following instances: 1) if the patient has previously been immunised for TB, which is particularly the case in countries where routine BCG vaccination at birth is common (Rothel & Andersen 2005), 2) if the patient has past-cleared infection, or 3) if the patient is infected with environmental bacteria (Pai et al 2008b). Tuberculin tests also have the disadvantage of producing false negatives, especially when the patient

has malnutrition, Hodgkins lymphoma, sarcoidosis, or active TB disease (Pai & Menzies 2007b).

Other laboratory methods are being developed and evaluated that offer the hope of cost-effective, rapid and more accurate TB testing. The Interferon release assays (IGRAs) are more specific markers of TB infection (Dheda et al 2004b; Diel et al 2008; Pai et al 2008b). The detailed literature regarding the use of IGRAs for TB diagnosis will be reviewed later (**Section 1.5**). Furthermore, nucleic acid amplification tests (NAATs) have also emerged with the intended goal of enabling the rapid and accurate diagnosis of TB (Drobniewski et al 2003; Ling et al 2008). The detailed literature regarding the use of NAATs for TB diagnosis will be reviewed later (**Section 1.6**).

1.4.3. Alternative sampling

Another problem that clinicians are faced with, which has hugely contributed to the under-diagnosis of TB, is the difficulty of diagnosing sputum-scarce and smear-negative pulmonary TB (Gordin et al 1989; Moore & Roper 2007). In patients who are unable to produce a sputum sample, common alternative sources for diagnosing pulmonary TB include performing sputum induction, bronchoscopies (with bronchoalveolar lavage, bronchial washings, and/or transbronchial biopsies), gastric washings, and fine-needle aspiration (trans-tracheal or -bronchial). In some cases, a more invasive technique is necessary, such as tissue biopsy. However, the low yield of rapid diagnostic tests for use with alternative sampling remains a problem.

TB treatment is difficult to monitor as it requires long courses of multiple antibiotics. Furthermore, as mentioned previously, antibiotic resistance (MDR and XDR TB) is a growing problem. Prevention mainly relies on vaccination strategies and efficient screening programs. Thus, we cannot over-emphasise the urgent need for the development of new rapid and

inexpensive diagnostic tests and the evaluation of existing tests, especially in the developing world. For the current study, we were particularly interested in using samples other than sputum (blood, urine and bronchoalveolar lavage fluid) to evaluate the use of IGRAs, the Clearview LAM assay, and MTB/RIF Gene Xpert assay, respectively, in a high burden clinical setting.

1.5. INTERFERON-GAMMA RELEASE ASSAYS (IGRAs)

1.5.1. What are IGRAs?

Currently, there is no gold standard for diagnosing latent TB infection (LTBI). However, over the past decade, the development, validation and global recognition of antigen-specific interferon-gamma release assays (IGRAs) represents a 100-year change in the use of the traditional tuberculin skin test for the diagnosis of latent tuberculosis infection (LTBI) (Barnes 2001; Lalvani & Millington 2008; Pai et al 2008b), and have improved our understanding about TB in humans (Dheda et al 2009a; Pai et al 2006c; Pai & Menzies 2007b). IGRAs are based on the ability of the *M.tb* region of difference 1 (RD1) antigens, early secretory antigen target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10), to stimulate host production of interferon-gamma. The T-SPOT.TB (Oxford Immunotec Ltd., London) and QuantiFERON-TB Gold (QFT-G) [Cellestis Ltd., Australia] assays are commercially available blood tests which use the *M.tb* RD1 antigens to identify the presence of TB by detecting the frequency of IFN- γ -secreting CD4+ antigen-specific effector T cells in peripheral blood (Dheda et al 2009a; Dheda et al 2005) of infected individuals. Although both measure overnight (<24 hours) IFN- γ responses to the overlapping antigen-specific RD1peptides, these two IGRA tests are not equivalent (Ferrara et al 2006). The QFT-G is based on an ELISA format and detects the whole blood production of IFN- γ (Mol & Koethe 2006; Todd 2006). QFT-G quantifies the total amount of IFN- γ when whole blood is exposed to RD1 antigens,

assumption that LTBI test sensitivity should be at least as good as, or better than, sensitivity in active TB, investigators have used quantified TB exposure as a surrogate gold standard for LTBI. Using this strategy, several studies have shown test sensitivity between 78 and 100% when using both RD1 antigens in either an ELISPOT or ELISA assay (Brock et al 2001; Chapman et al 2002; Lalvani et al 2001a). However, the optimal strategy that should be utilised to detect LTBI using IGRAs is controversial, especially due to the lack of sufficient prospective data.

In 2006, a study by Wilkinson et al. (London) 16 LTBI patients receiving treatment were followed-up 2-6 weeks into treatment and at the end of treatment (12 weeks). Furthermore, an additional 17 subjects were recruited to determine the phenotype of the expanding T cell population in these LTBI patients. Briefly, PPD stimulated PBMCs were subjected to flow cytometry staining for CD4, CD45RO, IFN- γ , CD38 and CD62L. The ELISPOT results showed that treatment of LTBI with isoniazid (INH) and rifampicin for 3 months decreased the number of IFN- γ -secreting T cells at the end of treatment, but not significantly (Wilkinson et al 2006). However, the fact that there were changes in the T cell responses early during the treatment of LTBI could be an indication of a possible surrogate marker of efficacy that cannot be dismissed and might be useful early during the early stages of treatment-related clinical studies. Diagnosing and treating LTBI is a less important strategy in developing countries. In order to limit ongoing transmission, the main priority in the developing world is the diagnosis and treatment of large numbers of active TB cases. Currently, international guidelines mostly recommend the use of IGRAs for the diagnosis of LTBI, either alone (Mack et al 2009), or in conjunction with the TST (Dheda et al 2009a). One approach, as suggested by UK and Canadian guidelines, is to first perform a screening TST, and if the test is positive an IGRA can then be performed. However, this two-step approach relies on the assumption that the TST does not evoke boosting of IFN- γ responses resulting in 'false-positive' IGRA responses.

Recently published data from our laboratory indicate that definite boosting of IFN- γ responses occurs by day 7 after TST administration, and with other antigens by day 3 (van Zyl-Smit et al 2009). Thus, our findings suggest if a two-step strategy is used for IGRA testing, the IGRA should be performed by day 3 (i.e. at the time of reading of the TST). This approach is consistent with recent Canadian IGRA guidelines (Pai et al 2008a).

1.5.3. Utility of IGRAs for the diagnosis of Active TB disease

Timely diagnosis of active TB cases is essential for TB control. Isolation of *M.tb* can take up to 6 weeks and there is the risk of false negative culture results. Thus, an ideal test of active disease to enable clinicians to rule out a possible diagnosis is one that is rapid, highly specific and also has very few false-negatives, or in other words, is highly sensitive. The major limitations of current tests mostly arise from intra-patient immunogenetic differences that result in heterogeneous immune responses, and variability of antigen recognition according to the stage of the disease (Dheda et al 2007; Lyashchenko et al 1998).

A study conducted in Korea, which is an area with a very high LTBI prevalence, showed that QFT-TB Gold and T-SPOT.TB have high sensitivity but reduced specificity for diagnosing patients with active TB (Kang et al 2007). In a recently published meta-analysis of data from developed and developing countries, Diel and colleagues (Diel et al 2010) reported that QFT-TB GIT had a pooled specificity of almost 100% and sensitivity of 81%, whereas T-SPOT.TB had a pooled specificity and sensitivity of 86% and 88%, respectively. In direct comparisons, the sensitivity of IGRAs surpassed that of tuberculin skin testing, and the authors concluded that IGRAs are superior to the TST for detecting confirmed active TB disease. However, it should be noted that although not precise, the IGRA and TST are the only available proxy markers of TB infection. Both tests are measures of T-cell priming and IGRAs are particularly useful because they correlate well with the

magnitude of exposure to *M.tb* (Dheda et al 2009a; Pai et al 2006c). However, exposure does not necessarily indicate the presence of potentially viable *M.tb* organisms in the tissue of individuals with LTBI, and there is a substantial amount of evidence for the TST, but not the IGRA, that treating individuals who test positive reduces the risk of possible subsequent development to active TB (Comstock 1978; Grzybowski et al 1975).

A major drawback is that IGRAs cannot differentiate between active TB disease and LTBI (Dheda et al 2005; Lalvani 2007). Thus, IGRAs are not viewed as good rule-in tests. However, in individuals who are highly suspected of having active disease, but whose other diagnostic tests are negative, a positive IGRA result can help to support a presumed active TB diagnosis. Thus, in cases such as these, a positive result can be of value in initiating anti-TB treatment (Dheda et al 2005). A classic example of this scenario was reported by Richeldi and colleagues in the *Annals of Internal Medicine* (Richeldi et al 2004). The authors described a case of MDR TB in an asymptomatic patient with a cavitating lesion that was detected on a CT scan (ie. highly suggestive a TB disease). The patients TST was found to be negative. However, an ELISPOT IGRA yielded a positive result.

Thus, the question remains whether IGRAs can somehow be improved to accurately diagnose active disease. It is feasible to postulate that patients with a non TB diagnosis, for example cancer, should not have a high frequency of antigen-specific T cells at the site of disease. Therefore, it should be possible to diagnose active TB if there is a high frequency of *M.tb*-antigen-specific T cells at the specific disease site. Indeed, quantitative antigen-specific T-cell responses (using T-SPOT TB) have been shown to be accurate and useful for the diagnosis of pulmonary TB when using bronchoalveolar lavage (BAL) cells in both high and low-burden clinical settings (Dheda et al 2009c; Jafari et al 2009). Recent work conducted by our laboratory (Lung Infection and Immunity Unit, Department of Medicine, University of Cape Town) suggests that although IGRAs may be useful for the immunodiagnosis of active TB using cells from induced sputum, with the

current format of the assay, this diagnostic approach is not feasible in clinical practice (Cashmore et al 2010). However, is it perhaps possible to make use of the same approach using biological fluid obtained from other compartments? Although there is promising preliminary data from studies conducted by our group (Dheda et al 2009d) and other groups (Baba et al 2008; Chegou et al 2008), it has been shown that IGRAs are not clinically useful for the diagnosis of pleural TB in high burden settings. The poor specificity reported in the above-mentioned studies, is most likely due to the migration of the *M.tb*-specific T lymphocytes of non-TB patients, for example those with bacterial infection or cancer and other diseases, into the pleural compartment (Dheda et al 2009d). However, these findings may be different in a low burden setting. Preliminary data regarding the investigation of the clinical utility of IGRAs for the diagnosis of TB meningitis (TBM) are also encouraging (Thomas et al 2008). Moreover, we have recently found that T-SPOT.TB is an accurate and useful clinical diagnostic tool for TBM (Patel et al 2010). Thus, although IGRAs cannot be used to distinguish LTBI from active TB disease, they appear to be useful tools for the diagnosis of active TB when using cells from the site of disease (for example, cerebrospinal fluid and lung).

1.5.4. Utility of IGRAs for the diagnosis of HIV-TB co-infected patients

TB is the most common opportunistic infection in HIV-infected individuals and it is challenging, especially in high burden countries, to accurately diagnose TB in this group (Dheda et al 2004a). An easy-to-use, rapid blood test that is highly accurate and specific would thus be a particularly useful diagnostic option in this group. There are limited data in low and high burden settings on the diagnostic utility of IGRAs in HIV-TB co-infected individuals. Studies in Italy and the UK, using less than 40 HIV-TB co-infected subjects, found the overall range of the sensitivity and specificity of T-SPOT TB to be between 70-95% and 64-100%, respectively (Clark et al 2007; Goletti et al 2007; Vincenti et al 2007).

Three studies conducted in a high burden setting (Africa) used either QFT-GIT or an in-house ELISPOT assay to evaluate the sensitivity and specificity of the assay in HIV-TB co-infected subjects (Chapman et al 2002; Raby et al 2008; Rangaka et al 2007). The sample size ranged from between 40-75 individuals in each of the three studies, and IGRA sensitivity varied between 74-100%. The ELISPOT performed better than the TST in all studies. However, an overall observation was that IGRA sensitivity decreased with advancing immune-suppression. Interestingly, two studies, namely the UK study conducted by Clark et al and the study conducted by Rangaka et al. in Africa, found that the IGRA:CD4 T cell count ratio in the study participants was useful in distinguishing LTBI from and active TB disease (Clark et al 2007; Rangaka et al 2007). However, these were preliminary findings and a major limitation of IGRAs is the high rate of indeterminate results at very low CD4 T cell counts. Thus, further studies are required to determine the CD4 T cell count cut-point at which IGRA responses are attenuated. Furthermore, studies are required to evaluate whether IGRAs have incremental value over smear microscopy in HIV-TB co-infected patients.

1.5.5. Utility of IGRAs for the monitoring of disease activity and efficacy of anti-tuberculosis treatment in high versus low burden settings

Data evaluating active TB patients from high burden settings are limited, mostly using in-house/standard ELISPOT (Aiken et al 2006; Carrara et al 2004; Ferrand et al 2005; Lalvani et al 2001a; Pathan et al 2001; Wilkinson et al 2006) and ELISA (Al-Attayah et al 2003; Turner et al 2000) assays using ESAT-6 or CFP-10 (or both) and/or PPD as stimulants. Tuberculin purified protein derivative (PPD) is an immunological reagent that can be purchased for research purposes. The product is produced from virulent *M.tb* culture filtrates, and has been used as a stimulatory antigen in the ex vivo ELISPOT assays (Aiken et al 2006; Dheda et al 2009d; Hill et al 2005; van Zyl-Smit et al 2009).

Existing treatment-related data are discordant. Some studies report a failure of correlation with exposure status and a failure to return to negative after

treatment (Pai et al 2006d; Pai & Menzies 2007b). As previously described, the IGRAs rely on the principle that sensitised T cells (effector memory cells) will rapidly produce IFN- γ when they encounter the *M.tb*-specific antigens. By contrast, central memory T-cells are more likely to require several days in the presence of antigen before IFN- γ is produced. Therefore, incubation of whole blood or peripheral blood mononuclear cells (PBMCs) is for 24 hours (16-20 hours for T-SPOT.TB and 16-24 hours for QFT-GIT). If longer incubation times are used it is possible for infection that has been treated, or resolved, to yield positive tests. This may explain the discordant results of studies that evaluated the change in IFN- γ responses with anti-TB treatment (Dhedra et al 2005). Studies that show substantially decreased IFN- γ responses with treatment, used short incubation times (ELISPOT with < 24 hours in all except one study) whilst those that showed equivalent or increasing responses all had considerably longer incubation times (3 or more days and usually 4 to 5 days) (Pai et al 2006a).

The following table (table 1.1) outlines the longitudinal studies that have used IGRAs to monitor serial treatment-related responses in active TB adults on anti-TB treatment. The majority of the studies have been conducted in low burden settings using in-house ELISPOT and/or ELISA assays.

Table 1.1. Summary of longitudinal studies that have that have used IGRAs to monitor serial treatment-related responses in active TB adults on anti-TB treatment. The study location, sample group details, as well as information on the longitudinal follow-up time points, and type of IGRA assays used, are displayed in the table.

Study (First author, journal, year)	Study location	Study population (N) & treatment status/details	Follow-up time points	Type of assay and antigens
Al-attiyah, Fems Imm and Med Micro, 2003	Kuwait	N=15 confirmed cases of pulmonary TB	Baseline N=15, 2 months n=12, and 6 months n=7	ELISA (6 days incubation); recombinant ESAT-6 expressed in <i>E.coli</i>
Aiken, BMC Infectious Diseases, 2006.	The Gambia*	151 adults with active TB (sputum smear and culture positive TB) were recruited N=89 evaluable, paired ELISPOT results for baseline recruitment vs. 12 months	follow-up at 12 months	In-house ELISPOT assay overnight incubation with ESAT-6, CFP-10, PPD
Bossard, Respiratory Medicine, 2008.	Geneva	N=89 HIV negative subjects either treated for culture-proven TB or having completed their treatment within the past 6 months, N=41 evaluable results at 0 months, 6 months & 12 months	N=62 at T0 (2 weeks before treatment initiation), N=55 at 6 months, and N=41 at 6 months post-treatment completion	T SPOT TB: ESAT-6 and CFP-10
Carrara, CID, 2004	Italy	N=18 patients (enrolled from a previous study) with microbiologically confirmed TB	Time of diagnosis and 3 months after they started therapy	In vitro ELISPOT assay: ESAT-6, PPD
Dheda, J Inf, 2007	UK	N=33 patients with culture proven TB recruited: Early phase (=4 months treatment; n=12) Continuation/late phase (>4 months of treatment or after treatment completion, N=21) N=3 patients followed longitudinally	Mean duration of treatment=8.7 months Early phase (=4 months of anti-TB treatment) & continuation/late phase (>4 months of treatment or after treatment completion)	T-SPOT TB: ESAT6 and CFP10
Domínguez, Diagnostic Microbiology and Infectious Disease, 2008	Spain	N=10 HIV negative, pulmonary TB patients followed-up	Baseline (0 months), between 1 to 3 months of treatment, and after more than 3 months	T SPOT TB (ESAT 6 & CFP 10) and QFT GIT (ESAT 6, CFP 10 & TB 7.7)
Golletti et al, BMC Infect Dis, 2008	Uganda*	N=12 active TB patients	Followed-up at 3 months and 6 months post-treatment initiation	In-house overnight RD ELISPOT assay (ESAT-6, CFP-10)
Kobashi, Inter Med, 2008	Japan	N=22 patients with active TB	6 months treatment, 3 years of follow-up	QFT-TB GIT: ESAT6, CFP10, TB7.7
Lalvani, J Infect Dis, 2001	India* and UK	N=50 patients with Culture positive TB recruited in India; N=5 culture-confirmed TB patients were followed up longitudinally in the UK	N=50: 35 patients <1 month treatment or no treatment at the time of blood-draw; 15 patients at later time points in treatment (<1 year) N=5: longitudinal follow-up over 20 weeks of treatment	In-house ELISPOT assay: ESAT6 and CFP10 & PPD
Pathan, J Immunol, 2001	London	N=12: longitudinally tracked 12 patients on anti-TB treatment	Follow-up over 34 weeks of treatment	In-house ELISPOT assay: ESAT-6
Ribeiro, S et al., BMC Infect Dis, 2009	Brazil	N=58 active TB patients with T SPOT TB results at baseline, 4 months and at treatment completion (6 months)	Baseline, 16 and 24 weeks of treatment	T-SPOT TB assay (using frozen PBMCs): ESAT-6 & CFP-10, note: they displayed summated RD-1 SFCs in the results section
Turner et al., Tuber Lung Dis, 2000.	The Gambia*	N=7 active TB patients	6 months treatment: follow-up at 9 months	In-house ELISA: PPD

The developing countries/high burden TB settings are marked with an asterisk (*)

In 2006, Aiken and colleagues evaluated antigen-specific IFN- γ responses to ESAT-6, CFP-10 and PPD using an in-house ELISPOT assay (overnight incubation). The study group comprised 151 active TB patients (sputum smear and culture positivity was used as a reference standard for diagnosis) from The Gambia, who were on a standard 6-month course of anti-TB treatment. The patients were followed-up for a period of 12-months, and there were evaluable, paired ELISPOT results for 89/151 (59%) of the patients (Aiken et al 2006). The outcomes reported in their study were as follows: 96% of the patients who had evaluable, paired results completed treatment and were cured, 3% defaulted from treatment, and treatment only failed (remained sputum positive) in 1%. At 12 months, 55% were ELISPOT negative. Another study conducted in The Gambia used an in-house ELISA assay to longitudinally track the levels of IFN- γ production in response to stimulation with PPD in 7 definite TB patients on a standard 6-month course of anti-TB treatment (Turner et al 2000). The patients were followed-up at 9 months. However, the levels of IFN- γ remained unchanged throughout the study. A treatment-related follow-up study conducted in another high burden setting (Uganda) used an in-house RD1 ELISPOT assay to evaluate serial changes in antigen-specific T cell responses in 12 active TB patients at 3 months and 6 months post-treatment initiation (Goletti et al 2008). Although responses decreased by end of treatment, the responses remained above the cut-point for positivity and here were no statistically significant changes observed over time. The median SFCs at diagnosis and after 3 and 6 months of therapy were 167, 103 and 100, respectively.

In Kuwait, a study by Al-Attiyah et al. evaluated the use of an in-house ELISA (6-day stimulation assay using recombinant ESAT-6 expressed in *Escherichia coli*) to assess the responses of 15 confirmed cases of pulmonary TB patients on a standard course of therapy. However, there were only evaluable results for 12 of the subjects 2 months into treatment and 7 at 6 months (treatment completion). Prior to treatment initiation, there were 13-60% responders in the IFN- γ assays. After 2 months of treatment, the percentage of responders increased (46-81%), and at the end of treatment all

patients had responded. In 2004, Carrara et al. conducted a study (in Italy) using an in-house ELISPOT assay (ESAT-6 and PPD) to evaluate treatment-related responses in a group of 18 patients with microbiologically confirmed TB (Carrara et al 2004). The patients were on a standard 6-month course of treatment and their responses to ESAT-6 and PPD were assessed at the time of diagnosis (baseline, T₀) and 3 months after treatment was initiated (T₁). All patients had positive ELISPOT results at diagnosis. At 3 months, the responses to ESAT-6 peptides were still detectable only in 5 patients, who had an absence of clinical improvement after treatment completion. Another study using an in-house ELISPOT assay (ESAT-6 CFP-10 and PPD) was conducted by Lalvani and colleagues in 2001, which evaluated treatment-related data from India. The study, based in the United Kingdom (UK), assessed a group of 50 culture positive patients recruited in India. Only 5/50 (10%) of these culture-confirmed TB patients were longitudinally followed-up over 20 weeks of treatment. Eighty percent of these patients responded to ≥1 antigen, and many donors had high frequencies of T-cells that were specific for certain immune-dominant peptides. In contrast, of 40 mostly BCG-vaccinated, healthy UK adult residents, none responded to either antigen (Lalvani et al 2001a). Another study conducted in London (Pathan et al 2001) longitudinally tracked 12 patients (on anti-TB therapy) for a period of 34 weeks. Using an in-house ELISPOT assay, the investigators observed a decline in the overall frequency of ESAT-6 peptide-specific T cells ($p=0.005$).

Certain studies, such as the one published by Ferrand and colleagues in 2005 (London) have focused on the comparison of ELISPOT assays, or more specifically, an in-house ELISPOT assay that uses overnight ESAT-6 stimulation versus a 6-day stimulation assay. The aim of the study was to determine which type of assay provided the best indicator of a memory T-cell response, and also to assess the relationship between ESAT-6 specific T-cells and protective immunity (Ferrand et al 2005). In the overnight ELISPOT assay, the median IFN- γ responses in all of the TB patients, irrespective of treatment status, were significantly higher than in the healthy BCG-vaccinated individuals who served as controls. On the other hand, the

median IFN- γ responses in the group who had completed treatment were higher than in the patients who were in the early phase of anti-TB treatment. Furthermore, the investigators found a considerable amount of variability in the degree of expansion of the ESAT-6 specific T-cells over the 6-day period in both groups. However, it was concluded that further studies were required to assess the phenomenon (Ferrand et al 2005).

Although several studies have investigated the use of T-SPOT TB and QFT-TB GIT using samples obtained from active TB patients on anti-TB therapy (Bosshard et al 2009; Dheda et al 2007; Dominguez et al 2009; Kobashi et al 2008; Ribeiro et al 2009), to our knowledge, there are currently no data from a high burden setting comparing T SPOT TB and QFT GIT results alongside PPD and HBHA responses to monitor treatment-related responses in active TB patients.

The majority of the existing studies have only used the T-SPOT TB assay to monitor treatment-related responses in active TB patients from low burden settings. In 2007, a study entitled 'Interpretation of Mycobacterium tuberculosis antigen-specific IFN- γ release assays (T-SPOT.TB) and factors that may modulate test results' was published by Dheda and colleagues in the UK (Dheda et al 2007). A total of 33 patients with culture-proven TB were recruited. The mean duration of treatment was 8.7 months. A total of 12 (36%) of the patients were in the early phase of treatment (≤ 4 months), and 21 were in the continuation/late phase of treatment completion (> 4 months). Only 3 patients were followed longitudinally for the duration of the study. Significantly more patients in the early rather than the late phase had positive IGRA responses (10/12 (83%) vs. 4/21 (19%); $p \leq 0.01$). 17/21 (81%) in the late phase or who had completed treatment were IGRA-negative, despite having robust antigen-specific recall proliferative responses. Furthermore, experiments using prolonged incubation (5 days vs. overnight), the addition of endotoxin, and different protein and peptide antigen preparations failed to elicit positive responses in these 17 IGRA-negative patients. The authors concluded that although discordant IGRA data remain unexplained by

variation in laboratory protocols, the variability displayed in the results was more likely due to environmental or host factors. The T-SPOT TB responses were found to be persistently positive in the 3 patients (initially tested at 4 months, 4 and 6 months after commencing treatment, and then again at 8, 18, and 18 months, respectively) who were followed longitudinally.

In 2008, Bosshard et al. published a study detailing the treatment-related responses (T-SPOT TB: ESAT-6 and CFP-10) of active TB patients who were followed-up at the time of treatment completion (6 months) and 6 months after treatment completion. There were evaluable results for baseline (T_0 : 2 weeks before treatment initiation) versus 6-month follow-up (T_E : treatment completion) versus 12-month follow-up (T_{E+6} : 6 months after treatment completion) for 41 culture-proven TB patients. The mean spot forming cells (SFCs) were 75 at T_0 , 46 at T_E , and 33 at T_{E+6} , and the positive rates were 98%, 93% and 98%, respectively. The SFCs decreased significantly between T_0 and T_E , and 2 (6%) reversions occurred over the course of treatment. However, no reversions occurred between T_E and the follow-up at 6 months post-treatment completion (T_{E+6}). Of 6 patients (17%) with an increase in SFCs between T_0 and T_E , 5 had a favourable outcome at T_E and $T_E + 6$. (Bosshard et al 2009) Ribeiro and colleagues (Brazil) used T-SPOT TB to evaluate 58 active TB patients at baseline, 4 months and at treatment completion (6 months). The investigator's used summated RD-1 SFCs for their analyses. Their findings showed that the mean baseline ESAT-6, CFP-10 and summated RD1 specific SFCs decreased from 43, 41 and 84, respectively, to 23 ($p=0.01$), 23 ($p=0.18$) and 47 ($p=0.02$) at completion of 6 months of treatment. Furthermore, only 10% of subjects with a reactive test at baseline reverted to negative at treatment completion. However, for the entire study, frozen PBMCs were used in the T-SPOT TB assays. The overall sensitivity of the T-SPOT TB assay, as measured by baseline reactivity of the frozen samples, was 72.4% (95%; CI 59–83%). (Ribeiro et al 2009).

A study conducted in Japan (Kobashi et al 2007) evaluating the utility of QFT TB GIT (ESAT6, CFP10, TB7.7 antigens), used samples obtained from 22 patients with active TB followed up at 6 months and 3 years after treatment completion. The investigators found that the positive rate of QFT test results decreased by 50% at treatment completion, and thereafter, although the positive rate of results decreased 45% six months later (even after treatment was completed). Two years post treatment completion, the responses decreased slightly to 41%, and one later (ie. 3 years post-treatment completion) the responses had decreased further, but not significantly, to 36%.

To our knowledge, there is only one study from a low burden setting (Spain) that has used both the T SPOT TB and QFT TB GIT tests to monitor treatment-related responses in a group of active TB patients (Dominguez et al 2009). Only ten HIV-uninfected, pulmonary TB patients were followed up over 6 months of anti-TB treatment. Samples were collected from the patients at the beginning of treatment, between 1-3 months of treatment, and after more than 3 months of treatment. Furthermore, the study was mostly focused on the sensitivity and specificity of the assays. The sensitivities of T-SPOT TB and QFT-GIT for the patients at the beginning of treatment were 83.3% and 69.4%, respectively. During treatment, the sensitivity decreased to 69.8% for T-SPOT.TB and 48.8% for QFT-GIT. When compared with the values during treatment, the responses to the specific antigens increased significantly after treatment completion. However, the main finding was that the T-SPOT.TB test was more sensitive in diagnosing active TB than the QFT TB GIT. (Dominguez et al 2009)

In summary, studies from low burden countries generally show rapidly declining IGRA responses, whereas those from high burden countries show highly inconsistent changes that range from minimal to modest. These observations are more likely to be due to biological factors and perhaps other factors such as residual post-treatment, persistent infection, re-infection or persistent exposure to environmental mycobacteria, rather than technical

factors. Another possibility is that there is possible maintenance of a circulating pool of effector memory T-cells. The diagnostic utility of IGRAs may be improved if they can be shown to be good proxy markers of disease activity.

1.5.6. Can we do better?

The key question throughout the IGRA literature is “Can we do better?” (Arend et al 2000; Lalvani & Millington 2008; Menzies 2008; Pai & Menzies 2007a). One approach that has been utilised to increase the diagnostic accuracy of T Cell-based ELISPOT and ELISA assays is to incorporate additional antigens of established high specificity (Liu et al 2004), because it has been hypothesised that assays that use cocktails of antigens may overcome the existing problem of heterogeneous immune responses (Houghton et al 2002). In this study by Houghton and colleagues, genomic expression libraries from *M.tb* were screened with sera from TB patients or rabbit anti-serum to *M.tb*. By doing so the authors identified novel antigens, namely Mtb11 (also known as CFP-10), Mtb8, and Mtb48, which were used in an ELISA to detect specific antibodies to *M.tb*. These 3 proteins, together with a previously tested 38-kDa protein associated with active TB disease, were also produced as a genetically fused poly-protein, and tested with two additional antigens (DPEP, or MPT32) and Mtb81, using sera from pulmonary and extra-pulmonary TB patients, TB patients co-infected with HIV, and patients with PPD positive and negative statuses with no evidence of disease. The authors discovered that in samples from HIV-negative individuals, the ELISA detected antibodies in more than 80% of smear positive subjects and ~60% smear negative subjects, with a high specificity (98%), and the antigen combination detected a significant number of HIV/TB co-infections and extra-pulmonary infections. Thus, their data suggests that the use of multiple antigens can be used to develop a highly sensitive test for *M.tb* antibody detection. In another recent study, Dosanjh and colleagues used RV3879c, a novel RD-1 encoded antigen, alongside ESAT-6 and CFP-10

to create the 'ELISpotPLUS assay', which was shown to have significantly improved sensitivity compared to that of the standard ELISPOT test (Dosanjh et al 2008). In another study, the use of novel antigen Rv2645 in QFT-GIT significantly improved the sensitivity of the assay in diagnosing active TB cases without compromising the assay's specificity (Harada et al 2008). Heparin Binding Haemagglutinin (HBHA) is another TB-specific antigen that appears to be promising for immuno-diagnosis. HBHA, which is a 28-kDa methylated surface-exposed mycobacterial protein antigen that is involved in mediating the interaction of *M.tb* with the host (Delogu et al 2006; Menozzi et al 1996) and may play a role in the protection against extrapulmonary dissemination of *M.tb* into alveolar epithelial cells (Pethe et al 2001; Shin et al 2006), has also been used in IGRA studies (Dheda et al 2009c). Furthermore, HBHA is referred to as a latency antigen (Hougardy et al 2007). Evidence has emerged showing that cells from individuals with LTBI release IFN- γ in response to HBHA, whereas cells from patients do not (Temmerman et al 2004).

It is evident that further studies are urgently required to evaluate alternative testing platforms and to investigate the incorporation of additional novel antigens (for example, HBHA) of established high specificity. Furthermore, a useful approach would be to evaluate different biomarker readouts, for example, IFN- γ -inducible protein (IP-10), Monocyte Chemotactic Protein 1 (MCP-1) and IL-1 receptor antagonist (IL-1RA), for the rapid diagnosis of TB (Dheda et al 2010b).

1.6. LIPOARABINOMANNAN (LAM) ASSAYS

1.6.1. Urine lipoarabinomannan for diagnosis of tuberculosis

The use of urine for TB diagnostic purposes is increasingly becoming an attractive test option, because it is a convenient, easily obtainable biological fluid that can be transported, processed and stored without much hassle. Almost half a century ago (late 1950s/early 1960s), scientists postulated that *M.tb* bacilli would be present in the urine of active pulmonary TB patients. However, the use of urine specimens in routine diagnosis of active pulmonary TB is currently not recommended, mainly due to the poor clinical utility of conventional TB diagnostics when applied to urine samples. Thus, the development of novel tools to detect mycobacteria in the urine of TB-infected individuals, such as tests that utilise mycobacterial lipoarabinomannan (LAM) and urine mycobacterial deoxyribonucleic acid (DNA) have provided a promising way forward from using conventional TB diagnostics. (Peter et al 2010)

The recognition of the urgent need for the development of novel and improved technologies for detecting TB in urine was mainly spurred on by the release of two large retrospective urine diagnostic reviews (Mortier et al 1996; Nour et al 2007), which both reported that the yield of urine smear and culture was very low (<2%), but the yield of urine culture improved significantly, by approximately 39-fold (>77%), in HIV-infected patients in the stages of advanced immune-suppression. Moreover, recent reports reveal that the sensitivity of urine smear and the low incremental yield of urine *M.tb* culture (<5%), compared to sputum smear microscopy or culture, remains a problem in HIV-infected and -uninfected TB patients (Monkongdee et al 2009).

1.6.2. Performance outcomes of urine LAM in clinical studies

Studies by Daffe and Draper, and Lee et al. suggest that LAM is specific for mycobacteria (Daffe & Draper 1998; Lee et al 1996). Our recently published urine LAM findings (please refer to chapter 4: urine LAM specificity of 95-100%) are consistent with the high specificities reported in two other recent studies (Lawn et al 2009; Shah et al 2009). However, these findings contradict earlier data from other studies showing poor urine LAM specificity (83-89%) (Daley et al 2009; Mutetwa et al 2009; Reither et al 2009). The low specificity displayed in these early studies could have been due to undiagnosed occult TB disease, contamination of the sample by environmental mycobacteria or other bacteria, or possibly even nonbacterial contamination. Indeed, recent studies have revealed that several species of bacteria, and even some fungi, possess LAM-like glycolipids in their cell walls. Interestingly, we have found that anti-LAM polyclonal antibodies cross-react with various micro-organisms found in the normal mouth flora of non-TB patients (Dheda et al 2010a).

The very first tests for the detection of LAM antigen in urine, namely, a basic LAM ELISA and a dipstick test, were designed and evaluated almost a decade ago (Hamasur et al 2001). The Clearview-TB® LAM ELISA (Inverness Medical Innovations, Waltham, Massachusetts, USA), is the most recently developed urine LAM test, and is based on the earlier developmental studies (Hamasur et al 2001; Tessema et al 2001). Moreover, the Clearview-TB® LAM ELISA supersedes a pre-commercial prototype (MTB LAM ELISA Test®, Chemogen, Portland, USA) that was first tested 5 years ago in Tanzania (Boehme et al 2005). In the evaluation of this pre-commercial prototype (MTB LAM), Boehme and colleagues reported a sensitivity of 80.3% and a specificity of 99% (Boehme et al 2005). Although the high sensitivity reported by Boehme et al. is promising, several recently published studies have reported very low sensitivities (ranging between 13-51%) in unselected TB suspects (Daley et al 2009; Mutetwa et al 2009; Reither et al 2009), thus

suggesting that the urine LAM assay has little clinical utility in this particular sub-group of patients. Furthermore, the accurate diagnosis of TB in HIV-infected patients, particularly those with advanced immune-suppression, is difficult. Studies in HIV/TB co-infected patients are of particular importance, because these patients suffer from increased rates of extra-pulmonary TB, often with atypical presentations. However, the use of standard tests is not feasible, and there is an urgent need to evaluate rapid tools such as the Clearview-TB® LAM ELISA.

Recent studies have shown that urine LAM may have diagnostic value in HIV-infected (Lawn et al 2009; Mutetwa et al 2009; Reither et al 2009; Shah et al 2009) suspects but not in those who are HIV uninfected (Daley et al 2009). Moreover, the Clearview TB ELISA has shown strong performance in detecting TB in individuals co-infected with HIV. The aim of the study conducted by Shah et al. was to evaluate the diagnostic accuracy of the Clearview LAM ELISA for TB in hospitalised patients in a high HIV prevalence setting. Thus, the authors conducted a nested cohort study of 499 South African TB suspects who were inpatients, and the majority of these were co-infected subjects (84.6% HIV-infected). In the confirmed TB cases, the sensitivity of the urine LAM ELISA was suboptimal (59%, 95% CI). Interestingly, the urine LAM positivity in the smear-negative TB patients, in particular, was also poor (56%). However, the sensitivity improved to 71% in patients with a CD4 cell count of 50-100 cells/ml³. The sensitivity was even further improved to 85% in patients who were in the most advanced stages of immune-suppression (CD4 cell counts < 50 cells/ml³). It was suggested that the improved sensitivity was related to a high antigen burden and disseminated disease, because the investigators observed that HIV positivity, and positive sputum smear and mycobacterial blood culture were all factors associated with LAM positivity. The overall conclusion was that urine LAM in combination with sputum smear microscopy is an attractive test option in high HIV prevalence settings. (Shah et al 2009)

We have also postulated that the Clearview[®] LAM ELISA may be a useful rapid tool for TB diagnosis in HIV-infected TB suspects from primary care facilities in a high burden setting. The detailed evaluation of the performance outcomes of our study using the Clearview[®] urine LAM ELISA are contained in chapter 4. This work, along with sputum- LAM data generated during the course of the parent study, has recently been published (Dhedha et al 2010a). Other studies have also investigated the utility of LAM ELISA using samples other than urine.

1.6.3. Clinical utility of lipoarabinomannan ELISA using biological samples other than urine

Although most studies have evaluated the diagnostic utility of LAM-ELISA using urine, several studies have investigated urine LAM using various specimens, including cerebrospinal fluid (CSF) (Patel et al 2010), pleural fluid (Dhedha et al 2009b), and serum (Sada et al 1990). Furthermore, besides our study (Dhedha et al 2010a), sputum has also been used in another LAM detection study (Pereira Arias-Bouda et al 2000). In the study conducted in a resource-poor high HIV prevalence setting by Patel and colleagues, it was shown that the detection of LAM may be a promising tool for the diagnosis of TB meningitis (TBM) (Patel et al 2010). In a collaborative effort between our laboratory and Patel and colleagues, a larger prospective study evaluating LAM antigen detection in CSF, yielded promising results and has recently been completed (V. Patel and K. Dheda; personal communication). In pleural fluid, LAM has been shown to be of no diagnostic use (Dhedha et al 2009b). Furthermore, detection of LAM in sputum (and induced sputum) has been found to be complicated due to cross-reactivity with oral flora (Dhedha et al 2010a).

1.6.4. Future directions for urine-based diagnostics

Given the advantages of using urine as a diagnostic sample, coupled with the fact that several host and organism-related metabolites and proteins are likely to be found in urine, there is much promise for the identification of signature molecules that may be helpful for TB diagnosis. The use of proteomic and metabolomic technologies for screening urine may be a promising tool to detect TB-specific protein signatures. Other approaches using urine include gas chromatography spectrometry for the characterisation of TB-specific volatile organic compounds. Urine nucleic acid amplification tests (NAATs) such as such as loop-mediated isothermal amplification (LAMP) are also available (Boehme et al 2007).

University of Cape Town

1.7. NUCLEIC ACID AMPLIFICATION TESTS (NAATs)

1.7.1. NAATs for the diagnosis of TB

NAATs such as Polymerase Chain Reaction (PCR) are radically changing the detection of infectious pathogens such as *M.tb*. As previously mentioned, currently used conventional tools such as smear microscopy have a very low sensitivity and culture results are available only after several weeks. Hence, better efforts to control TB require more rapid and accurate diagnostic tests. NAATs have been developed to address these issues, because amplification technology offers the potential for the rapid diagnosis of TB (within a few hours of sample receipt) with a high degree of sensitivity and specificity (Ling et al 2008; Pai & Ling 2008).

A summary of the commercially available nucleic acid amplification tests (NAATs) for tuberculosis is shown in table 1.2. Two of the commercial tests, namely Roche Amplicor® MTB (Roche Molecular Systems Branchburg, New Jersey) and the Amplified *M.tuberculosis* Direct® Test (AMDT; Gen-Probe Inc. San Diego, California), have been approved by the US Food and Drug Administration (FDA) for testing of smear-positive sputum samples in diagnostic laboratories. Moreover, the Gen-Probe has also received FDA approval for use on smear-negative sputum samples. Other manufacturers have developed molecular assays for rapid TB diagnosis that utilise other amplification methods such as strand-displacement amplification (SDA) technology (Hellyer et al 1996) and ligase chain reactions (O'Connor et al 2000; Rantakokko-Jalava et al 2001). While the Amplicor® MTB (Roche Molecular Systems Branchburg, New Jersey) and Cobas® Amplicor (Roche Molecular Systems Mannheim, Germany) have been in existence for quite some time, the BD-ProbeTec ET (a newly improved version of the BD-ProbeTec Direct SDA technology) and Loop-mediated Isothermal Amplification (LAMP) tests (Boehme et al 2007) are relatively new. In 2009, Cepheid announced the European release of the Xpert® MTB/RIF Assay,

which is now also available in other parts of the world. The Xpert® MTB/RIF Assay is on-demand molecular test for simultaneous detection of *M.tb* and RIF resistance. This is the only system that delivers answers directly (TB or no TB) from unprocessed samples by combining on-board preparation of the sample with real-time PCR in less than 2 hours (Cepheid 2009).

Table 1.2. Summary of commercially available nucleic acid amplification tests (NAATs) for tuberculosis

NAAT	Method/Principle	Manufacturer
Amplified <i>M.tuberculosis</i> Direct® Test (AMDT)	Transcription-mediated amplification of rRNA transcripts, with product detection performed via chemiluminescence	Gen-Probe Inc. San Diego, California
Roche Amplicor® MTB	PCR amplification of 16s rRNA, with product detection performed via colorimetric detection	Roche Molecular Systems Branchburg, New Jersey
Cobas® Amplicor	PCR amplification of 16s rRNA	Roche Molecular Systems Mannheim, Germany
Abbott LCx® assay*	Ligase chain reaction amplification of 38kDa protein	Abbott Laboratoroies, Illinois
BD-ProbeTec Direct And the BD-ProbeTec ET	Strand Displacement Amplification (SDA) technology for the direct, qualitative detection of 16s rRNA and the <i>M.tb</i> insertion sequence IS6110	Becton-Dickinson Diagnostic Systems Sparks, Maryland
Xpert® MTB/RIF	Semi-quantitative nested real-time PCR <i>in-vitro</i> diagnostic test for the detection of <i>M.tb</i> complex DNA, and rifampicin-resistance associated mutations of the <i>rpoB</i> gene	Cepheid, Sunnyvale, California
Loop-mediated Isothermal Amplification (LAMP)	Isothermal amplification and visual read-out with ultra violet fluorescence	Eiken Chemical Co. Ltd.,Japan

*Discontinued in 2003

1.7.2. In-house versus commercial NAATs

In a meta-analysis by Flores et al., the authors reviewed data from 65 published studies on in-house NAATs for pulmonary TB. The main objective was to determine factors associated with the heterogeneity in test accuracy, and to evaluate the higher accuracy estimates of accuracy in studies that evaluated in-house PCR for the diagnosis of pulmonary TB. The confounding factors that contribute to heterogeneity in test accuracy are not well characterised, and it is difficult to address the issue in individual studies. The variability displayed among in-house NAATs may be due to the several factors, namely, differences in study design, or differences in laboratory techniques and assay characteristics, for example, the method of sample preparation (DNA extraction), the amplification step, and the method of detection used. Thus, increasing the power of analyses by combining data from multiple studies is a useful method which may enable researchers to identify the sources of heterogeneity in study findings. In their meta-analysis, Flores and colleagues reported that the use of *IS6110* as an amplification target, and the use of nested PCR methods appeared to enhance test accuracy, and suggested the inclusion of these elements in PCR protocols. Their analyses also suggested that the methods used for DNA extraction and detection were not critical. However, a potential limitation of targeting *IS6110* is that certain strains from different parts of the world lack this insertion sequence (Das et al 1995). Thus, a possible solution may be to use multiple targets. However, the meta-analysis revealed that that multiplex PCR failed to increase the diagnostic accuracy of in-house assays. At the time, the overall sensitivity and specificity of in-house assays could not be adequately summarised due to the significant amounts of heterogeneity in the results. However, several years later, studies determined that in-house NAAT sensitivities have been found to vary to some extent based on the bacillary load in the specimen. Compared to higher sensitivities (85-90%) in smear-positive samples, sensitivities range from 50-60% in smear-negative samples (Kaul 2001).

Commercial NAATs have been shown to give more consistent results compared to in-house assays, and several meta-analyses have evaluated the accuracy of commercial NAATs in pulmonary and extra-pulmonary TB (Daley et al 2007; Pai et al 2004; Pai et al 2003; Piersimoni & Scarparo 2003; Sarmiento et al 2003). Overall, the majority of studies evaluating commercial NAATs have reported high and consistent specificity (95-100%), but low and varied sensitivity (33-96%) (Ling et al 2008; Pai et al 2004; Pai et al 2003; Piersimoni & Scarparo 2003). Furthermore, when comparing smear negative and smear positive TB, studies have shown that sensitivity is lower in smear-negative TB (Greco et al 2006; Pai et al 2004; Pai et al 2003; Piersimoni & Scarparo 2003). Sarmiento et al. conducted a meta-analysis on the use of commercial NAATs for smear negative TB, and found that the sensitivity estimates were low and variable in this group (Sarmiento et al 2003). If the sensitivity of commercial NAATs, especially in smear negative TB, can be improved, this group of patients are most likely to benefit from the use of NAATs, because a faster diagnosis can lead to earlier treatment initiation if a result is positive.

1.7.3. Extra-pulmonary TB poses a diagnostic challenge

The lack of a diagnostic gold standard remains one of the biggest obstacles for evaluating new diagnostics, especially in HIV-infected patients and extrapulmonary TB. In cases of suspected extrapulmonary TB, rapid and accurate diagnosis is important, because, as previously emphasised, conventional methods of detecting AFB have many limitations. As mentioned earlier, the majority of studies evaluating commercial NAATs have reported high and consistent specificity, but low and varied sensitivity in both pulmonary and extra-pulmonary TB. However, given the paucibacillary nature of extra-pulmonary disease, it is particularly difficult to diagnose extra-pulmonary TB (Pai & Ling 2008). Although several meta-analyses have investigated the use of NAATs in studies that have used cerebrospinal fluid (Pai et al 2003), pleural fluid (Pai et al 2004) and lymph node aspirate or tissue (Daley et al 2007) from extra-pulmonary TB suspects,

none of the commercial NAATs are FDA-approved for use in extra-pulmonary samples. Overall due to the low sensitivity of the test, it is suggested that NAATs should be used in combination with the conventional methods and further research is required to improve the sensitivity of NAATs for extra-pulmonary (Ling et al 2008).

1.7.4. NAATs for the diagnosis of active pulmonary TB using BAL fluid

Using various in-house PCR assays, several studies have demonstrated that PCR is more sensitive than smear microscopy in detecting *M.tb* in the BAL fluid of sputum-scarce or smear negative patients with active pulmonary TB (Chen et al 2002; Liam et al 1998; Tueller et al 2005). A study conducted in Taiwan by Chen et al. found that when compared to smear, the sensitivity and specificity of an in-house NAAT in the BAL fluid of smear negative, pulmonary TB patients was 36% and 96%, respectively (PPV=94% and NPV=45%) (Chen et al 2002). Liam et al. (Malaysia) found that an in-house NAAT had a positivity rate of 80.9% (55/68) compared with lower smear and culture positivity rates of 8.8% and 7.4%, respectively, for detecting *M.tb* in BAL fluid of smear negative active pulmonary TB patients (Liam et al 1998). A study conducted by Tueller and colleagues (Switzerland) used an in-house NAAT in an investigation of 71 patients who had smear negative or sputum-scarce TB. BAL fluid (smear or *M.tb* complex-PCR) allowed an accurate rapid diagnosis in 10 (59%) out of 17 sputum smear negative patients, and 49 (91%) out of 54 patients who could not produce sputum (sputum-scarce TB). In all of these studies it was evident that the combined use of BAL fluid smear and *M.tb* NAAT had a good diagnostic yield in sputum-scarce or smear-negative TB.

1.7.5. Future NAAT research directions

Studies need to be conducted to evaluate the performance outcomes of commercial NAATs in different geographical settings, especially in high burden countries. A key concern is that commercial tests are expensive, for

example, the Xpert MTB/RIF assay is available in the private sector at R980 (US \$130) per test (Pathcare). However, the potential benefits may outweigh the cost when considering the rapidity of assay (result can be obtained in under 2 hours) and that RIF resistance can be detected simultaneously. A key study, conducted by Helb and colleagues (Helb et al 2009), who performed the first analysis of the Cepheid Gene Xpert System's MTB/RIF assay (using sputum samples obtained from 107 consecutively enrolled Vietnamese patients suspected of having TB), has been referred to in more detail in chapter 5 (pp.112 and 123). Furthermore, promising data on the analytic sensitivity, analytic specificity, and dynamic range for the new Xpert MTB/RIF assay has recently been published (Blakemore et al 2010). Seventy nine *M.tb* isolates, including 42 drug-susceptible and 37 RIF-resistant isolates, containing 13 different *rpoB* mutations or combinations of mutations were used to test the sensitivity of the new Xpert MTB/RIF assay. Assay specificity was tested using 89 non-tuberculous bacteria, fungi and viruses. The assay correctly identified all 79 *M.tb* isolates (ie. 100% sensitivity) and correctly excluded all 89 non TB isolates (ie. 100% specificity). Furthermore, RIF resistance was detected in all of the RIF-resistant isolates and in none of the drug-susceptible isolates. The dynamic range of the assay was also assessed by conducting spiking experiments (concentration range of 10^2 to 10^7 CFU of *M.tb* into non TB samples) which showed a log-linear relationship between T_c (cycle threshold) and input CFU over the entire range.(Blakemore et al 2010)

The above-mentioned data is very promising. However, due to the issue of smear negative and sputum-scarce TB, and the difficulty of diagnosing extra-pulmonary TB, we cannot over-emphasise that research is also needed to enhance the sensitivity of NAATs using biological fluids other than sputum. Furthermore, the lack of a diagnostic gold standard remains a serious problem when evaluating new commercial NAATs, especially in high burden settings.

CHAPTER 2

The Use of a Range of Antigens in the Serial Evaluation of Antigen-specific Quantitative T Cell Responses of Active tuberculosis Patients on a Course of Anti-tuberculous Therapy

2.1. INTRODUCTION

To date, the T-SPOT.TB test and similar in-house ELISPOT tests have reported sensitivities of 83–97% in active TB subjects (Goletti et al 2006; Lalvani et al 2001a; Meier et al 2005; Pathan et al 2001; Ribeiro et al 2009; Wilkinson et al 2005). Disease activity studies are required for providing clues about clinically important changes, which may be important in the management of disease monitoring. More specifically, good biomarkers for tuberculosis disease activity, cure and relapse are required because they may have an important role in providing validated surrogate endpoints, which can be used to enhance feasibility and thus accelerate the evaluation of new drugs, vaccines and other therapies. However, data on IGRAs as a promising marker of disease activity is conflicting (Dhedda et al 2005; Pai et al 2006a; Pai et al 2006d; Pai & Menzies 2007b). Moreover, the role of T-SPOT.TB in monitoring response to TB treatment is not well-established and responses to treatment studies are controversial.

Longitudinal data from high burden settings are limited, mostly using in-house/standard ELISPOT and ELISA assays (as discussed in the literature review, chapter 1 section 1.5.5) and to our knowledge, there are currently no data from a high burden setting comparing T SPOT TB and QFT GIT results alongside PPD and HBHA responses in a group of active TB patients on a course of anti-TB therapy. There is only one other study that has used both the T SPOT.TB and QFT-GIT tests to monitor treatment-related responses in active TB patients (Dominguez et al 2009), and the study was conducted in a low burden setting. Thus, in order to contribute towards already-existing

studies that have assessed the potential utility of IGRAs for monitoring treatment efficacy, we evaluated the frequency and magnitude of antigen-specific IFN- γ -secreting T cells responses, in patients with active TB receiving a 6-month course of anti-TB therapy, using a range of antigens, namely RD1 antigens ESAT-6 and CFP-10 (T SPOT.TB), ESAT-6, CFP-10 and TB7.7 (QFT-GIT), PPD (ELISPOT), and HBHA (ELISPOT). We also evaluated the relationship between the RD1 SFC counts/million PBMCs and markers of disease activity at baseline and 2 months post-treatment initiation, and at baseline, 2 months and at treatment completion (6 months).

University of Cape Town

2.2. AIM AND OBJECTIVE(S)

Aim:

The main purpose of this section of the study was to evaluate treatment-related serial changes in the magnitude of antigen-specific IFN- γ responses to a range of antigens [RD-1 (ESAT-6, CFP-10 and TB 7.7), HBHA and PPD] in active TB patients on a 6-month course of anti-TB treatment.

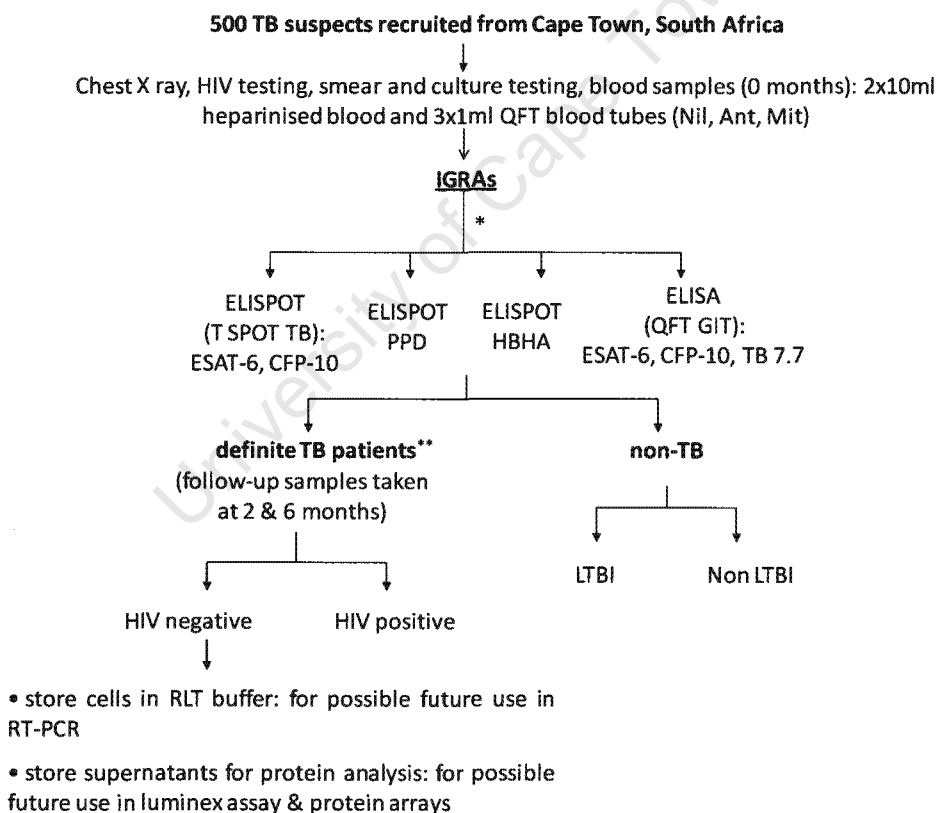
Objectives

1. What is the frequency and magnitude of IGRA (T-SPOT.TB and QFT TB GIT) RD1 responses at serial time points during anti-TB treatment (baseline versus 2 months post-treatment initiation, and versus treatment completion at 6 months)?
2. Is there agreement between the changes in RD1 antigen-specific quantitative T cell responses and changes in culture and smear status at 2 months?
3. Is there agreement between the changes in RD1 antigen-specific quantitative T cell responses and changes in culture and smear status at treatment completion (6 months)?
4. What is the frequency and magnitude of PPD and HBHA responses at serial time points during anti-TB treatment (baseline versus 2 months post-treatment initiation, and versus treatment completion at 6 months)?

2.3. MATERIALS AND METHODS

2.5.1. Clinical recruitment

The subjects for this study were recruited as part of a larger prospective parent study involving the evaluation of the utility and interpretation of antigen-specific T cell interferon gamma release (IGRA) assays in a high incidence tuberculosis and HIV setting in South Africa (SAIGRA). The sample recruitment sites, namely, Langa Clinic (Langa), Chapel Street Clinic (Woodstock), and Ikwezi Clinic (Lwandle) operate within the Cape Town Metro Region. Blood samples from five hundred sequentially recruited adult TB suspects from Cape Town (South Africa) were collected and processed at the Lung Infection and Immunity Unit Laboratory. The study flow diagram is shown in figure 2.1.



* Perform IGRAs for 500 TB suspects at 0 months (baseline). After overnight stimulation with RD1 antigens, PPD and HBHA, collect supernatants and store for later analysis.

** Only know whether subject has definite TB at 2 months (ie. culture and smear results confirmed). Invite all definite and probable TB subjects to return for follow-ups (2 & 6 months).

Figure 2.1. Study flow for the investigation of the use of a range of antigens in the serial evaluation of antigen-specific quantitative T cell responses of active TB patients on a course of anti-tuberculous therapy.

Ethical approval was obtained from the University of Cape Town Health Sciences Faculty Research Ethics Committee and all subjects signed informed consent prior to entering the study. A detailed questionnaire was completed by each participant and all underwent clinical, radiological, and laboratory evaluation.

2.5.2. Laboratory methods

Blood (~20ml) was drawn from all TB suspects at baseline (0 months) into lithium-heparin tubes (BD Europe) and transported to the laboratory within 3-4 hours where peripheral blood mononuclear cells (PBMCs) were isolated from ~10ml blood (within 8 hours of venipuncture) for use in the T-SPOT®.TB assays. The remaining 10ml was used in a different experimental set-up (chapter 2). An additional 3ml of blood was collected as follows: 1 mL of blood was drawn directly into each of 3 QuantiFERON®-TB Gold-IT blood collection tubes, namely, a Nil control tube (grey cap), TB antigen tube (red cape) and Mitogen control tube (purple cap).

Individuals were diagnosed based on detailed patient characterisation criteria, as shown below. The characterisation was performed by a clinician blinded to the IFN gamma release assay (IGRA) results.

1. **Definite TB:** A clinical presentation compatible with TB with at least 1 positive culture (from any specimen) for *M.tb* with response to anti-TB therapy (Wilson et al 2006).
2. **Probable TB:** A clinical-radiological picture highly suggestive of TB and/or anti-TB treatment initiated by the attending clinician based on clinical suspicion, but not meeting the above-mentioned criteria for definite TB.
3. **Non-TB:** no evidence of TB based on smear microscopy and culture, and no radiological evidence to support the diagnosis of active TB, with

or without an alternative diagnosis being established on patient follow-up.

4. **Indeterminate TB/uncertain diagnosis:** either the culture or chest x-ray results (or both) were unavailable, and the patient was lost to follow-up or transferred to another centre, thus making it impossible to confidently rule-out or rule-in TB. These patients were excluded from the analysis.

The definite and probable TB patients were invited to return for follow-up sessions at 2 months and 6 months. However, the patients were ambulatory. Thus, it was anticipated that a certain percentage of patients would be lost to follow-up if they transferred to other clinics, interrupted treatment during the course of their therapy, or opted out of the study for various other reasons.

For all subjects, blood was collected between 2 weeks and 1 day prior to the possible initiation of TB therapy (0 months, baseline) and once diagnosed as definite or probable TB, blood was collected during follow-up (2 months and/or 6 months) from those who returned. Thus, the current study comprised a group of active TB patients on a 6-month course of anti-TB therapy who were assessed at baseline and followed-up, if possible, at 2 months of treatment and at completion of treatment at 6 months.

2.5.2.1. Isolation of peripheral blood mononuclear cells (PBMCs) for use in the T SPOT. TB assay

Blood (~10ml) was centrifuged in the collection tube at 1800rpm for 15 minutes. After centrifugation, the layer of plasma was removed with a Corning® Disposable Pasteur Pipette (Sigma-Aldrich) prior to carefully transferring the white cloudy layer of cells (~2-4ml; mostly composed of

PBMCs) above the red blood cell pellet to a 15ml conical centrifuge tube. The volume was made up to 10ml with 1x phosphate buffered saline (PBS, pH 7.2 10X liquid, GIBCO®, Invitrogen), layered on top of 3-4 ml Ficoll-Paque™ (Sigma-Aldrich) in a new 15ml conical centrifuge tube, and centrifuged at 1800 rpm for 25 min at room temperature, with no brake. After ficoll-density centrifugation, the further condensed and separated PBMC layer was collected and transferred, using a pipette, to a clean 15ml conical tube. Two wash steps were performed as follows: 1) for the first wash step, the volume was made up to 10ml with PBS and the sample was centrifuged at 600×g for 10 min at room temperature and no brake, and 2) after decanting the supernatant, the cell pellet was re-suspended by making the volume up to 10ml with PBS prior to centrifuging at 350×g for 7 min at room temperature and no brake. After the second wash, the cell pellet was re-suspended in 1ml of pre-warmed AIM-V medium (37°C for use with T-SPOT.TB.) [Adcock Ingram Scientific Group]. A 1:10 staining dilution was made by mixing 10ul of the re-suspended cells with 90 µl Turk's blood counting fluid. 10 ul of the stained cell suspension was loaded onto an Improved Neubauer haemocytometer, and the lymphocytes were manually counted using a light microscope (20x magnification). The ruled area of the haemocytometer consists of several, large 1 mm² squares (4x4 grids) and the volume of each grid is 10⁻⁴ ml (0.1µl). Thus, the total number of cells per ml was calculated by using the following formula:

$$\text{Counts/ml} = \frac{\text{total counts}}{\text{number of 4X4 grids counted}} \times 10^4 \times \text{sample dilution}$$

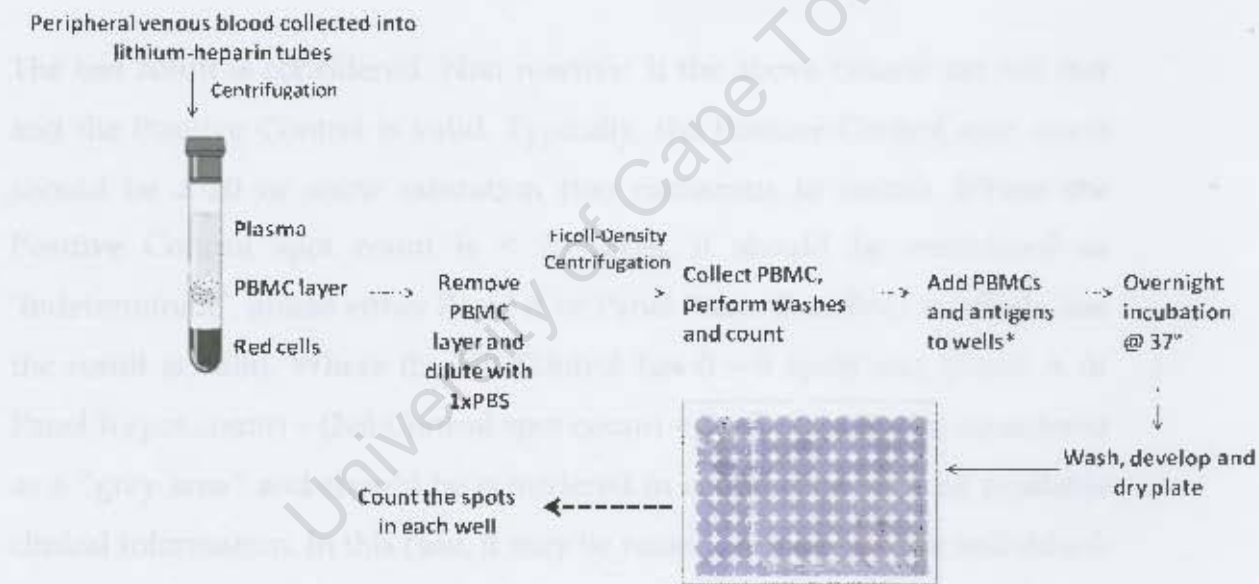
A 1ml aliquot of the final cell suspension was made at a concentration of 2.5×10⁶ cells/ml in pre-warmed AIM-V medium for use in the T-SPOT®.TB assay.

2.5.2.2. The ELISPOT (T-SPOT.TB) assay

The T SPOT.TB test was performed following the manufacturer's recommendations (T-SPOT®.TB [package insert] 2008), as follows: The assay requires four wells of the 8-well strip to be used for each patient sample. Two of the wells are for the TB-specific antigens (1 well for RD1 antigen Panel A/ESAT-6, and a second well for RD1 antigen Panel B/CFP-10). A Positive Control well containing phytohaemagglutinin (PHA, a known polyclonal activator) is required as a test for PBMC functionality, and a well for the Nil Control (AIM-V medium) is also required to identify non-specific cell activation. It is recommended that the samples are arranged vertically on the plate (ie. 2 patient samples can thus be run on an 8-well strip of the 96-well micro-titre plate. Each well on the strip is pre-coated with a mouse monoclonal antibody to the cytokine interferon gamma). For the purpose of the current evaluation, wells containing PPD and HBHA were also included. The PPD (Statens Serum Institut, Copenhagen, Denmark) was used at a working concentration of 20µg/mL, and the HBHA at a working concentration of 100µg/mL (derived from optimisation experiments). Thus, for each patient, PBMCs were stimulated in each well of the 8-well test strip by medium alone (as nil control), phytohemagglutinin (as positive control), 2 main peptide panels containing the antigens ESAT-6, CFP-10, and an additional 2 peptide panels containing PPD (in duplicate) and HBHA (in duplicate).

Thus, the assay was conducted by seeding the PBMCs at a final concentration of 250,000 PBMCs per T SPOT well (ie. 100ul of the 2.5×10^6 cells/ml cell suspension into each well), and adding 50 µl of the relevant antigens and controls to the wells (according to the above-mentioned layout). The plate was incubated at 37 °C in a CO₂ incubator for 16-24 hours. After the overnight incubation, the antigen-driven culture supernatants were collected in clean, labelled 1.5ml microfuge tubes and stored in cryogenic boxes at -20°C for possible later analysis. Each well was washed four times with 200 µl

1xPBS. The plate was gently, but thoroughly, tapped on a clean paper towel to remove excess PBS from the wells prior to adding 50ul of conjugate solution to each well (for each 8-well strip, a conjugate solution was made by adding 2.5 μ l T SPOT TB kit conjugate, equilibrated to room temperature prior to use, to 497.5 μ l 1xPBS). The plate was incubated at 4 °C for 1 hour. The conjugate was discarded and the plate was washed, as previously described, prior to adding 50ul T SPOT TB kit substrate (at room temperature). The plate was developed by incubating for 7 min at room temperature, and the reaction was stopped by rinsing each well four times with tap water. The developed plate was placed in a 37 °C incubator to dry for 24 hours. Please refer to figure 2.2, which is a simplified diagram illustrating the main steps of the T-SPOT.TB assay).



- * 96 well microtitre plate pre-coated with a mouse monoclonal antibody to the cytokine interferon gamma (IFN- γ): Four wells are required for each sample:
1. TB-specific antigen, Panel A (ESAT-6).
 2. TB-specific antigen, Panel B (CFP10).
 3. A Positive Control containing phytohaemagglutinin (PHA, a known polyclonal activator) as a test for PBMC functionality.
 4. A Nil Control to identify non-specific cell activation.

Figure 2.2. Simplified diagram illustrating the main steps of the T-SPOT.TB assay.

The antigen-specific T cell responses were scored using an automated AID ELISPOT plate reader (AID Autoimmun Diagnostika GmbH) at the Institute of Infectious Diseases and Molecular Medicine (Department of Medicine, University of Cape Town). The results were interpreted according to manufacturer's instructions as follows: A typical result would be expected to have few or no spots in the Nil Control and greater than 20 spots in the Positive Control. The test result is considered 'Reactive' if either or both of Panel A or Panel B respond, according to the following criteria:

- 1) Where the Nil Control has 0 - 5 spots and (Panel A or Panel B spot count) - (Nil Control spot count) ≥ 6 .
- 2) Where the Nil Control has 6 - 10 spots and (Panel A or Panel B spot count) $\geq 2 \times$ (Nil Control spot count).

The test result is considered 'Non reactive' if the above criteria are not met and the Positive Control is valid. Typically, the Positive Control spot count should be ≥ 20 or show saturation (too numerous to count). Where the Positive Control spot count is < 20 spots, it should be considered as 'Indeterminate', unless either Panel A or Panel B are 'Reactive', in which case the result is valid. Where the Nil Control has 0 - 5 spots and (Panel A or Panel B spot count) - (Nil Control spot count) = 5 - 7, this may be considered as a "grey area" and should be considered in conjunction with all available clinical information. In this case, it may be necessary to re-test the individual. Please refer to figure 2.3, which shows examples of a T SPOT TB result in a patient who is not infected with *M.tb* (figure 2.3 A) and a patient with *M.tb* infection (figure 2.3 B).

The SFCs for each RD-1 antigen were calculated using the above-mentioned criteria. However, for the purpose of graphical representation, the ESAT-6 and CFP-10 spot counts were summated using the spot count of the antigen that yielded the most spots (for example, if the ESAT-6 count was 20 and the CFP-10 count was 8, then the ESAT-6 count would be used to represent the RD1 response value, and vice versa). All counts were expressed as spot

forming cells (SFCs) per million PBMCs. The manufacturer (Oxford Immunotec Ltd., London) sets the cut-off for positivity at ≥ 6 spots per well containing 250,000 PBMCs, which thus translated into ≥ 24 SFCs/million PBMCs per well.

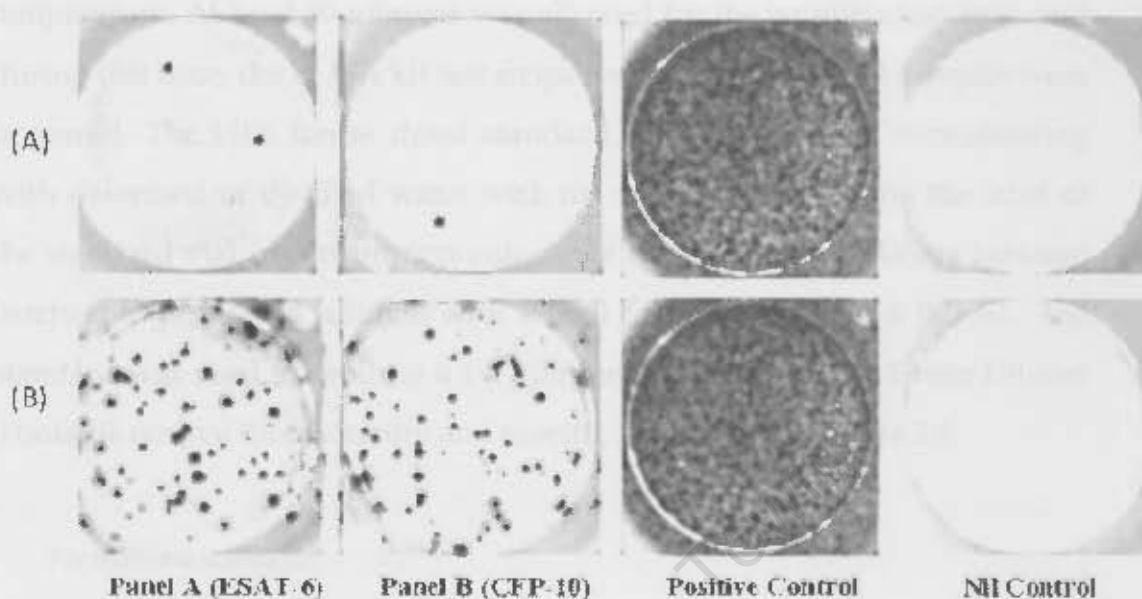


Figure 2.3. An example of a T SPOT-TB test result in a subject classified as 'not infected with *M.tb*' (A), and a subject classified as 'infected with *M.tb*' (B).

2.5.2.3. QuantiFERON-TB Gold In-Tube (QFT-TB GIT)

This QFT-TB GIT test was performed according to the manufacturer's instructions (QuantiFERON®-TB Gold In-Tube [Package Insert] 2007), as follows: 3ml of blood was collected from each participant into 3 tubes of 1 ml. each (Nil Control, Positive Control, and TB-specific antigens). Immediately after venepuncture each tube was vigorously shaken up and down 10 times to ensure the thorough mixing of the blood with the tube's contents. Upon arrival at the Lung Infection and Immunity Laboratory (within 3-4 hours of venepuncture, as previously mentioned), the QFT tubes were shaken again and immediately incubated at 37°C in an upright position. After 16-24 hours of incubation, the QFT tubes were centrifuged for 15 minutes at 3000 RCF (g), in order to separate the plasma from the cells, and the QFT tubes were then stored at 4°C for use in the enzyme linked immunosorbent assay (ELISA) assay.

Thus, the amount of IFN- γ released was measured by ELISA. Briefly, the plasma from the batched tubes (stored for a maximum of 2-3 weeks) was transferred to 1.5ml microfuge tubes. The samples and QFT-GIT ELISA kit reagents, except for the Conjugate 100X Concentrate, were brought to room temperature. At least 60 minutes was allowed for the equilibration step, and during this time, the ELISA kit test strips for the standards and samples were prepared. The kit's freeze dried standard was prepared by reconstituting with deionised or distilled water with the volume indicated on the label of the standard vial (reconstitution volume of the kit standard differs between batches) to produce a solution with a final concentration of 8.0 IU/mL. The standard was used to produce a 1:4 dilution series of IFN- γ in Green Diluent (contains normal mouse serum and casein), as depicted in figure 2.4.

For triplicate standards:

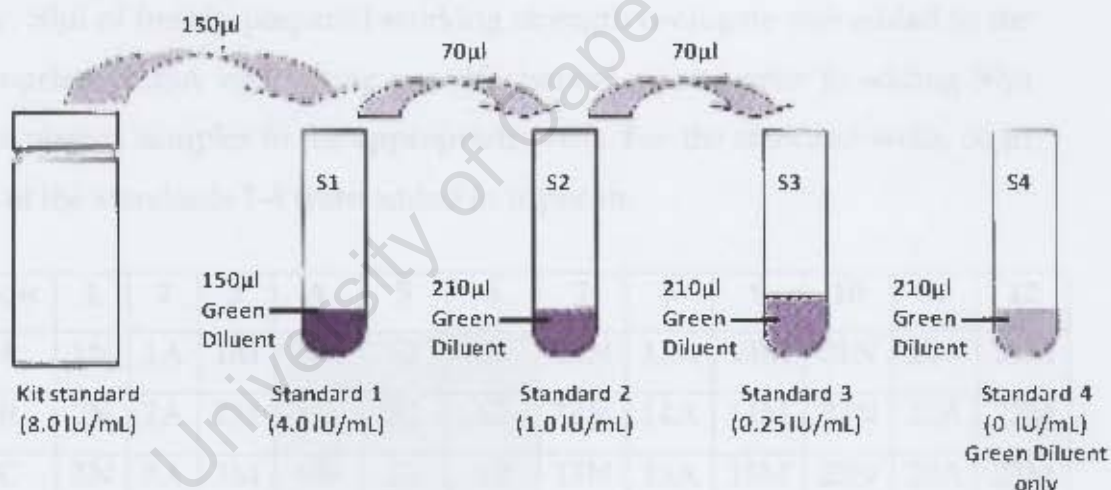


Figure 2.4. Preparation of the kit standards for use in the QuantIFERON Gold-In Tube assay

The freeze-dried kit Conjugate 100X Concentrate was prepared by reconstituting the concentrate with 0.3mL deionised or distilled water and mixing gently. Working strength conjugate was prepared from the solution by diluting the required amount of reconstituted conjugate in Green Diluent (as outlined in table 2.1).

Table 2.1. Guidelines for the preparation of the QFT-GIT kit Conjugate 100X Concentrate

Number of strips	Volume of Conjugate 100X Concentrate (μ l)	Volume of Green Diluent (ml)
2	10	1.0
3	15	1.5
4	20	2.0
5	25	2.5
6	30	3.0
7	35	3.5
8	40	4.0
9	45	4.5
10	50	5.0
11	55	5.5
12	60	6.0

The plate layout was prepared according to the template shown in figure 2.5. The plasma samples were thoroughly mixed prior to proceeding with the assay. 50 μ l of freshly prepared working strength conjugate was added to the appropriate ELISA wells using a multi-channel pipette prior to adding 50 μ l of the plasma samples to the appropriate wells. For the standard wells, 50 μ l each of the standards 1-4 were added in triplicate.

Row	1	2	3	4	5	6	7	8	9	10	11	12
A	1N	1A	1M	S1	S1	S1	13N	13A	13M	21N	21A	21M
B	2N	2A	2M	S2	S2	S2	14N	14A	14M	22N	22A	22M
C	3N	3A	3M	S3	S3	S3	15N	15A	15M	23N	23A	23M
D	4N	4A	4M	S4	S4	S4	16N	16A	16M	24N	24A	24M
E	5N	5A	5M	9N	9A	9M	17N	17A	17M	25N	25A	25M
F	6N	6A	6M	10N	10A	10M	18N	18A	18M	26N	26A	26M
G	7N	7A	7M	11N	11A	11M	19N	19A	19M	27N	27A	27M
H	8N	8A	8M	12N	12A	12M	20N	20A	20M	28N	28A	28M

S= Standard, N= Nil control plasma, A= TB Antigen plasma, M= Mitogen control plasma.

Figure 2.5. Plate layout template used for the QuantiFERON Gold-In Tube ELISA (28 tests per plate)

The conjugate and plasma samples/standards were thoroughly mixed for 1 minute using a micro-plate shaker. After mixing, the plate was covered with the lid and incubated at room temperature in a dark draw for 2 hours. During this incubation step, working strength wash buffer was prepared as follows: one part Wash Buffer 20X Concentrate was diluted with 19 parts deionised or distilled water and mixed. Thus, after incubation, the wells were washed 6-7 times, with 5 seconds in between each wash cycle, using 400µl of 1x working strength wash buffer. The plate was tapped face down on absorbent towel and 100 µl of Enzyme Substrate Solution (tertamethylbenzadine) was added to each well. The plate was covered with the lid and incubated at room temperature in a dark drawer for 30 minutes. The reaction was stopped by adding 50 µl Enzyme Stopping Solution (contains sulfuric acid) to the wells. The plate was read immediately after stopping the reaction (Anthos Labtec HT3 ELISA plate reader set on 450nm filter and 620nm-650nm reference filter).

The results were calculated using the manufacturer's analysis software. The software was used to conduct a quality control assessment of the assay, generate a standard curve and provide the Nil control, Antigen and Mitogen control results for each sample by using the OD values. For analysis purposes, the Nil control result was subtracted from the mitogen control and the antigen-stimulated sample result to obtain the final QFT-GIT read-out and a result for each subject. The cut-off value for a positive test was ≥ 0.35 IU/mL of IFN- γ in the sample after simultaneous stimulation with the specific antigens. A result was considered as indeterminate if the positive control was less than 0.5 IU/mL and the antigen-stimulated sample negative.

2.5.2.4. Definition of IGRA conversions and reversions

The conventional method, as used by the manufacturer, defines an IGRA conversion as a change from a negative to positive result relative to the cut-point, and a reversion as a change from a positive to negative result relative

to the cut-point) (Pai & O'Brien 2007). The conventional method was thus utilised during the analysis stage when the results were interpreted.

2.5.3. Statistical Analyses

Analysis was performed using the Wilcoxon matched pairs test when comparing two non-parametric data sets and the Friedman test was applied for comparison of results when more than two data sets were involved. The McNemar test was utilised to evaluate whether there was agreement between the antigen-specific quantitative T cell responses and culture and smear conversion/reversion at 2 months, and treatment completion at 6 months. The risk factors for change were evaluated using the relevant non-parametric test (Wilcoxon Signed Ranks/Mann-Whitney U test).

2.6. RESULTS

The definite and probable TB patients were invited to donate blood at 2 and 6 months. Only 42/269 (16%) were available for 2-month follow-up. The remainder, 227/269 (84%), were not available for follow-up due to the following reasons: 20 transferred to another clinic, 5 moved to another town, 2 were lost to an unrelated trial (REMOX) at the clinic, 1 was untraceable (occupation: seaman), 2 were convicted of crimes (unable to follow-up in jail), 3 died, 9 interrupted their treatment course, 17 probable TB patients returned but had no follow-up notes/no entry, 5 untraceable/lost folders, 3 refused to return (opted out of study), 160 did not return (unknown, no reason recorded on the database). We deal with a hugely migrant population and presume that the patients transferred to other clinics or defaulted treatment. There is no regional or national monitoring system in place to track such patients.

Only 21/42 (50%) patients who returned at 2 months donated blood at 6 months. The breakdown of the remainder (50%) who did not return to donate blood at 6 months was as follows: 2 died, 5 interrupted their treatment course, 1 was pregnant, 4 moved to another clinic or town, 6 refused to return (opted out of study), and 3 were not contactable. Furthermore, there were only 17/21 (81%) that were evaluable for 6-month follow-up, because 4 were excluded due to logistical reasons (4 ELISPOT results unavailable due to development errors). Moreover, due to other minor logistical reasons that arose during the course of the study (for example, HBHA availability), and the exclusion of indeterminate results, the analysis was divided into different subsets based on the evaluable results. Please refer to table 2.2 for a summary of the evaluable subsets. There was no systematic distribution of the groups analysed with respect to clinical background, HIV status or IGRA test results.

The evaluation of the subset with the largest number of patients (baseline vs. 2-month follow-up, n=42) was considered as the main analysis. However, detailed analyses were conducted for all of the subsets

Table 2.2. Summary table of the evaluable subsets for the investigation of the use of a range of antigens in the serial evaluation of antigen-specific quantitative T cell responses of active TB patients on a course of anti-tuberculous therapy.

0 months (baseline) vs. 2-month follow-up	Sample size (n)
T SPOT (RD1)	42
ELISPOT (PPD)	42
ELISPOT (HBHA)	21 [*]
QFT GIT (RD1 & TB77.7)	28 ^{**}
0 months (baseline) vs. 2-month follow-up vs. 6-month follow-up	Sample size (n)
T SPOT (RD1)	17 [#]
ELISPOT (PPD)	17
ELISPOT (HBHA)	13 ^{##}
QFT GIT (RD1 & TB7.7)	12 [¥]

* 21/42 no HBHA available at the time of conducting the HBHA ELISPOT

** 14/28 excluded due to an indeterminate QFT-GIT result either at baseline or 2 months.

Only 21/42 (50%) patients who returned at 2 months donated blood at 6 months.

Furthermore, there are only 17/21 (81%) evaluable results for the 6-month follow-up patients, because 4 were excluded due to logistical reasons (4 ELISPOT results unavailable due to development errors).

4/17 no HBHA available at the time of conducting the HBHA ELISPOT

¥ 5/17 excluded due to an indeterminate QFT-GIT result either at baseline, 2 months or 6 months.

The clinical and demographic characteristics of the patients who returned to donate blood at 2 months is shown in table 2.3 (n=42). Statistical analyses used to compare the clinical and demographic characteristics of the 21/42 (50%) who returned for the 6-month follow-up versus the 50% who did not

return yielded no significant differences (for example, when number of HIV-infected individuals were compared, $p=0.264$).

Table 2.3. Demographic and clinical characteristics of the patients who returned for the 2-month follow-up ($n=42$)

Characteristics	No. (% of total)
<i>Median Age, years (range)</i>	42 (20-71)
<i>Gender</i>	
Male	30 (71)
Female	12 (29)
<i>Ethnic origin</i>	
Black African	35 (83)
Coloured	7 (17)
<i>Tuberculin skin test</i>	
Positive	17 (40)
Negative	2 (5)
Not done/ no result	23 (55)
<i>M. tuberculosis culture positive</i>	36 (86)
<i>M. tuberculosis smear positive</i>	24 (57)
<i>Received previous TB treatment</i>	17 (40)
<i>Clinical/radiological findings highly suggestive of Tuberculosis</i>	33 (79)
<i>Pre-existing conditions and morbidity</i>	
Current smoker	24 (57)
<i>HIV status</i>	
HIV-infected	11 (27)
HIV uninfected	30 (71)
Refused test	1 (2)
<i>Other diseases</i>	
Diabetes	1 (2)
Epilepsy	2 (5)
Hypertension	1 (2)
<i>Chronic medication</i>	3 (7)
<i>Diagnosis</i>	
Definite TB	36 (86)
Probable TB	6 (14)

2.6.1. The frequency and magnitude of IGRA (T SPOT TB and QFT TB GIT) RD1 responses at serial time points during anti-TB treatment

2.6.1.1. T SPOT TB

The median T SPOT TB assay spot counts for baseline (0 months) versus 2-month follow-up (figure 2.6 A) remained above the cut-point for positivity, and were not found to be significantly different (86 vs. 98, respectively; $n=42$; $p=0.641$). After 2-months of treatment, 26% (11/42) subjects showed a change across the cut-point (6 converted and 5 reverted). Furthermore, there were no significant differences between those who converted and those who reverted (refer to table 2.4 for the comparison of the relevant clinical and demographic characteristics of the T SPOT converters vs. reverters).

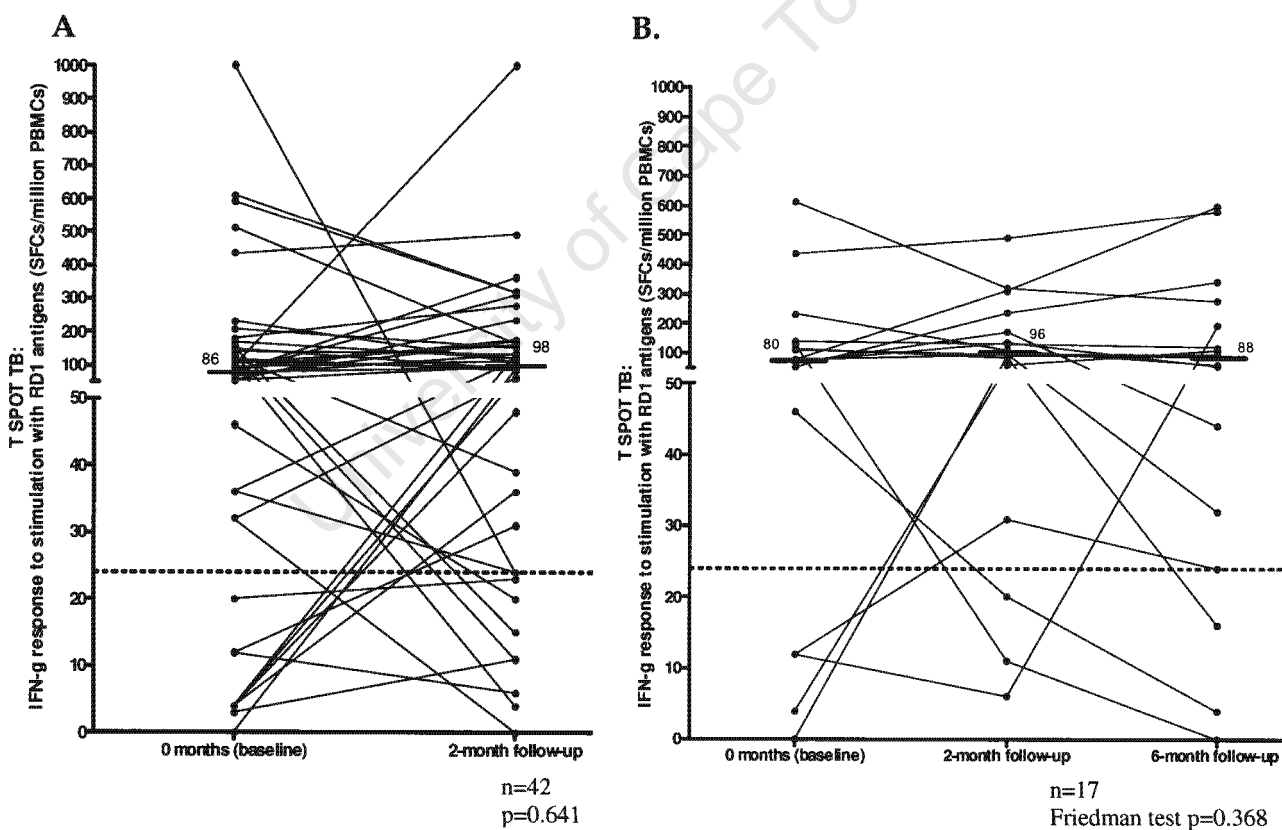


Figure 2.6. Quantitative antigen-specific IFN-gamma responses, in active TB patients on a six-month course of anti-TB therapy, to RD-1 antigens: (A) T SPOT.TB: summated results for ESAT-6 and CFP-10 at 0 months (baseline) and at 2 months post-treatment initiation [$n=42$], and (B) 0 months (baseline), 2 months post-treatment initiation and at treatment completion (6 months) [$n=17$]. The medians for the T SPOT.TB assay are indicated as horizontal solid lines and the cut-points (24 SFCs/million PBMCs) are indicated as horizontal dotted lines.

Table 2.4. Comparison of clinical and demographic characteristics of the T SPOT converters versus reverters.

Characteristic	T SPOT conversion N (%)	T SPOT reversion N (%)	p-value
Sex			0.265
Male	6 (100)	3 (60)	
Female	0 (0)	2 (40)	
Race			0.877
Black African	3 (50)	5 (100)	
Coloured	3 (50)	0 (0)	
HIV status			0.151
HIV infected	1 (17)	4 (80)	
HIV uninfected	5 (83)	1 (20)	
Previous TB			0.589
Yes	3 (50)	3 (60)	
No	3 (50)	2 (40)	
Other diseases			0.964
Yes	0 (0)	0 (0)	
No	6 (100)	5 (100)	
Current smoker			0.603
Yes	3 (50)	1 (20)	
No	3 (50)	4 (80)	
On chronic medication			0.964
Yes	0 (0)	0 (0)	
No	6 (100)	5 (100)	

Assessing the results of the subset of patients who returned for both the 2- and 6-month follow-up (figure 2.6 B) revealed similar findings when compared to the 2-month follow-up data. The medians were above the cut-point for positivity and that there was no significant change in the median spot counts (80 vs. 96 vs. 88, respectively; n=17; Friedman test p-value=0.368). By the end of treatment, 41% (7/17) subjects showed a change across the cut-point (4 converted and 3 reverted), and there were no significant differences between those who converted and those who reverted (data not shown).

2.6.1.2. QFT-GIT

The median IFN- γ levels (IU/ml) for the baseline (0 months) versus 2-month follow-up (figure 2.7 A) were above the QFT cut-point for positivity and were not significantly different (4 vs. 3, respectively; $n=28$; $p=0.737$). Only 11% (3/28) subjects showed a change across the cut-point (2 converted and 1 reverted).

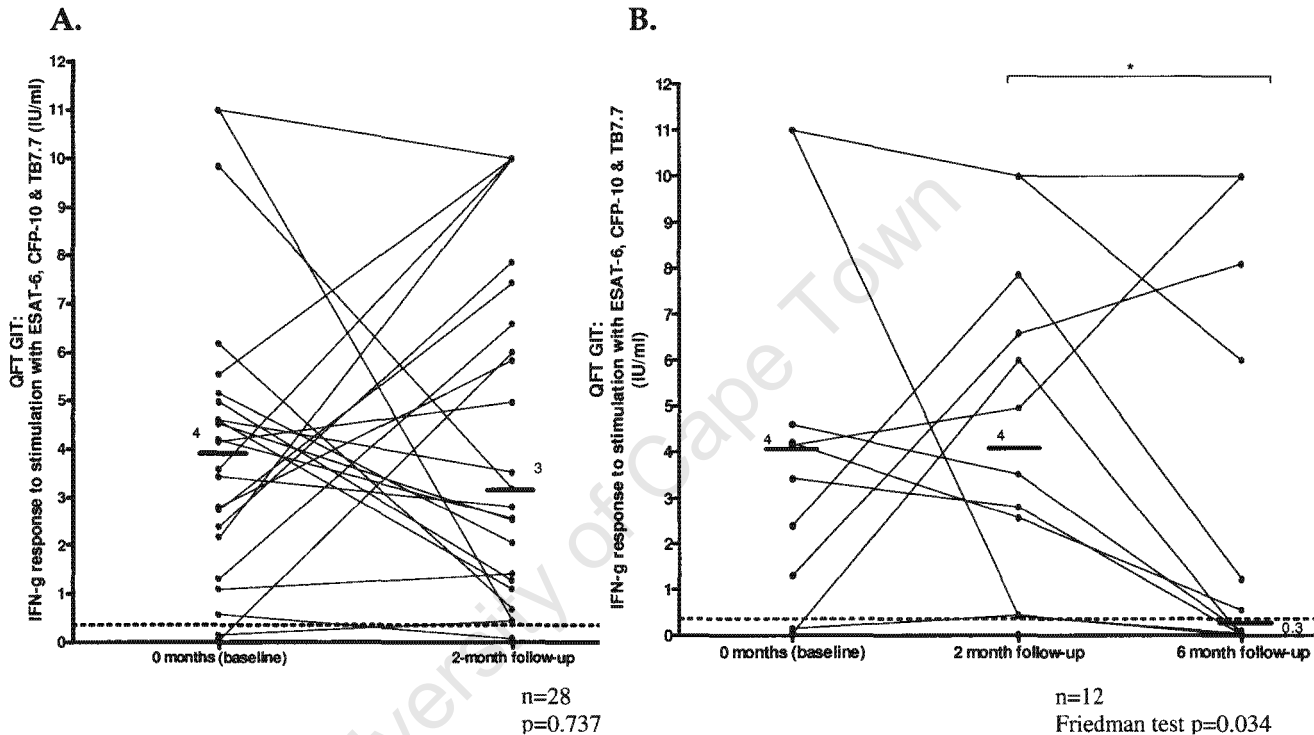


Figure 2.7. Quantitative antigen-specific IFN-gamma responses, in active TB patients on a six-month course of anti-TB therapy, to RD-1 antigens: (A) QFT-GIT (ESAT-6, CFP-10, TB7.7) results at 0 months (baseline) and at 2 months post-treatment initiation [$n=28$], and (B) 0 months (baseline), 2 months post-treatment initiation and at treatment completion (6 months) [$n=12$]. The medians for the QFT-GIT assay are indicated as horizontal solid lines and the cut-points (0.35 IU/ml) are indicated as horizontal dotted lines.

Assessing the results of the subset of patients who returned for both the 2- and 6-month follow-up (figure 2.7 B) revealed that there was no change in the median IFN- γ levels, which remained above the cut-point for positivity of 0.35 IU/ml, at baseline vs. 2-month follow-up (4 vs. 4, respectively; $n=12$; p -value=0.850). However at treatment-completion (6-month follow-up) there

was a significant decrease in the median IFN- γ levels to below the cut-point for positivity of 0.35 I/ml (n=12; Friedman test p-value=0.034).

2.6.2. Evaluation of the risk factors for change in the RD1 ELISPOT (T SPOT TB) and QFT-GIT results vs. culture and smear results

Although there were no significant changes in the median spot counts (T SPOT TB) and QFT levels (QFT-GIT), as shown in the previous figures, the changes in the RD1 responses over the course of treatment were not affected by confounding factors such as a history of previous TB, smoking, HIV infection, other diseases (for example, diabetes, hypertension and epilepsy) or the use of chronic medication.

An evaluation of the possible risk factors for change (confounders) in the RD-1 (T SPOT TB and QFT-GIT) responses at baseline vs. 2-month follow-up, along with the corresponding changes in culture and smear results, are shown in tables 2.5 and 2.6.

Table 2.5. Risk factors for change in the RD1 ELISPOT (T SPOT TB) results vs. culture and smear results

Characteristic	Change in T SPOT result N (%)	No change in T SPOT result N (%)	P-value	Change in culture result N (%)	No change in culture result N (%)	P-value	Change in smear result N (%)	No change in smear result N (%)	P-value
Sex			0.375			0.332			0.341
Male	2 (18.2)	10 (32.3)		8 (36.4)	4 (22.2)		4 (23.5)	8 (34.8)	
Female	9 (81.8)	21 (67.8)		14 (63.6)	14 (77.8)		13 (76.5)	15 (65.2)	
Race			0.272			0.130			0.388
Black African	8 (72.7)	27 (87.1)		20 (90.9)	13 (72.2)		13 (76.5)	20 (87)	
Coloured	3 (27.3)	4 (12.9)		2 (9.1)	5 (27.8)		4 (23.5)	3 (13)	
Previous TB			0.268			0.604			0.601
Yes	6 (54.5)	11 (35.5)		8 (36.4)	8 (44.4)		6 (35.3)	10 (43.5)	
No	5 (45.5)	20 (64.5)		14 (63.6)	10 (55.6)		11 (64.7)	13 (56.5)	
Current smoker			0.105			0.436			0.896
Yes	4 (36.4)	20 (64.5)		12 (54.5)	12 (66.7)		10 (58.8)	14 (60.9)	
No	7 (63.6)	11 (35.5)		10 (45.5)	6 (33.3)		7(41.2)	9 (39.1)	
HIV status			0.103			0.379			0.704
HIV infected	5 (45.5)	6 (20)		6 (28.6)	3 (16.7)		3 (17.6)	6 (27.3)	
HIV uninfected	6 (54.5)	24 (80)		15 (71.4)	15 (83.3)		14 (82.4)	16 (72.7)	
Other diseases			0.558			0.832			0.624
Yes	0 (0)	4 (12.9)		2 (9.1)	2 (11.1)		1 (5.9)	3 (13)	
No	11 (100)	27 (87.1)		20 (90.0)	16 (88.9)		16 (94.1)	20 (87)	
On chronic medication			0.955			0.433			0.738
Yes	1 (9.1)	3 (9.7)		1 (4.5)	2 (11.1)		1 (5.9)	2 (8.7)	
No	10 (90.9)	28 (90.3)		21 (95.5)	6 (88.9)		16 (94.1)	21 (91.3)	

Table 2.6. Risk factors for change in the QFT GIT results vs. culture and smear results

Characteristic	Change in QFT result N (%)	No change in QFT result N (%)	P-value	Change in culture result N (%)	No change in culture result N (%)	P-value	Change in smear result N (%)	No change in smear result N (%)	P-value
Sex			0.602			0.659			0.597
Male	3 (50.0)	18 (81.8)		10 (76.9)	9 (69.2)		11 (78.6)	9 (69.2)	
Female	3 (50.0)	4 (18.2)		3 (23.1)	4 (30.8)		3 (21.4)	4 (30.8)	
Race			0.523			0.430			0.577
Black African	2 (66.7)	21 (84.0)		11 (84.6)	10 (76.9)		11 (50.0)	11 (50.0)	
Coloured	1 (33.3)	4 (16.0)		2 (15.4)	3 (23.1)		11 (50.0)	11 (50.0)	
Previous TB			0.936			0.701			0.782
Yes	2 (66.7)	11 (44.0)		6 (46.2)	5 (38.5)		5 (35.7)	7 (53.8)	
No	1 (33.3)	14 (56.0)		7 (53.8)	8 (61.5)		9 (64.3)	6 (46.2)	
Current smoker			0.322			0.443			0.756
Yes	3 (23.1)	15 (1.0)		8 (61.5)	9 (69.2)		9 (64.3)	9(69.2)	
No	10 (76.9)	0 (0.0)		5 (38.5)	4 (30.8)		5(35.7)	4 (30.8)	
HIV status			0.589			0.354			0.699
HIV infected	0 (0)	4 (16.7)		0 (0)	2 (15.4)		1 (7.1)	2 (16.7)	
HIV uninfected	3 (100.0)	20 (83.3)		12 (100.0)	11 (84.6)		13 (92.9)	10 (83.3)	
Other diseases			0.534			0.565			0.411
Yes	0 (0)	2 (8.0)		0 (0)	2 (15.4)		1 (7.1)	1 (7.7)	
No	3 (100)	23 (92.0)		13 (100)	11 (84.6)		13 (92.9)	12 (92.3)	
On chronic medication			0.750			0.458			0.411
Yes	1 (33.3)	2 (8.0)		1 (7.7)	2 (15.4)		1 (7.1)	1 (7.7)	
No	2 (66.6)	23 (92.0)		12 (92.3)	11 (84.6)		13 (92.9)	12 (92.3)	

2.6.2.1. Changes at 2 months

Please refer to table 2.7. The differences between the changes in T SPOT results vs. changes in culture and smear status from baseline to 2-months were statistically significant (McNemar test p-values of 0.011 and 0.023, respectively), and the differences between the changes in QFT GIT results vs. changes in culture and smear status from baseline to 2-months were

statistically significant (McNemar test p-values of 0.035 and 0.054, respectively).

Table 2.7. Summary table of the McNemar test results used to assess whether there was agreement between the changes in IGRA and culture and smear changes (conversions/reversions) in TB patients after 2 months of treatment. Significant McNemar test p-values (≤ 0.05) denote that there is poor agreement between the changes in the RD1 antigen-specific quantitative T cell responses (T SPOT TB and QFT-GIT) and the changes in culture and smear status at 2 months of treatment.

	McNemar test N of Valid Cases	Exact significance (2- sided)
Changes in T SPOT vs. Changes in Culture from baseline to 2 months	40 [#]	0.011
Changes in T SPOT vs. Changes in Smear from baseline to 2 months	40 [#]	0.023
Changes in QFT-GIT vs. Changes in Culture from baseline to 2 months	26 [¥]	0.035
Changes in QFT-GIT vs. Changes in Smear from baseline to 2 months	27 ^{¥¥}	0.054

[#] Total N=42: no culture result for 2/42; [#] Total N=42: no smear result for 2/42;

[¥] Total N=28: no culture result for 2/28; ^{¥¥} Total N=28: no smear result for 1/28.

2.6.2.2. Changes from 0 to 6 months

The T SPOT TB findings at treatment completion (6 months) were similar to the trends displayed at 2-month follow-up. However, there was agreement between the changes in the QFT-GIT results and changes in culture and smear status at treatment completion (6 months). Please refer to table 2.8 below, which shows that the difference between the changes in QFT-GIT results vs. changes in culture and smear status from 2 months to 6 months, and baseline to 6 months, were not statistically significant.

Table 2.8. Summary table of the McNemar test results used to assess whether there was agreement between the changes (conversion/reversion) in QFT-GIT results and culture and smear results in TB patients at treatment completion (6 months).

	McNemar test N of Valid Cases	Exact significance (2- sided)
Changes in QFT-GIT vs. Changes in Culture from baseline to 6 months	10	1.000
Changes in QFT-GIT vs. Changes in Smear from baseline to 6 months	11	0.125

2.6.3. The frequency and magnitude of PPD and HBHA ELISPOT responses at serial time points during anti-TB treatment

2.6.3.1. PPD

The median PPD spot counts for baseline (0 months) versus 2-month follow-up (figure 2.8 A) remained above the cut-point for positivity, and were not significantly different (86 vs. 88, respectively; $n=42$; $p=0.530$). As shown in figure 2.8 B, the median spot counts at baseline vs. 2-month follow-up vs. 6-month follow-up remained above the cut-point for positivity and there was a trend of increasing median spot counts (34, 93 and 118, respectively). However, the difference between these median spot counts was not statistically significant; $n=17$; Friedman test p -value=0.101).

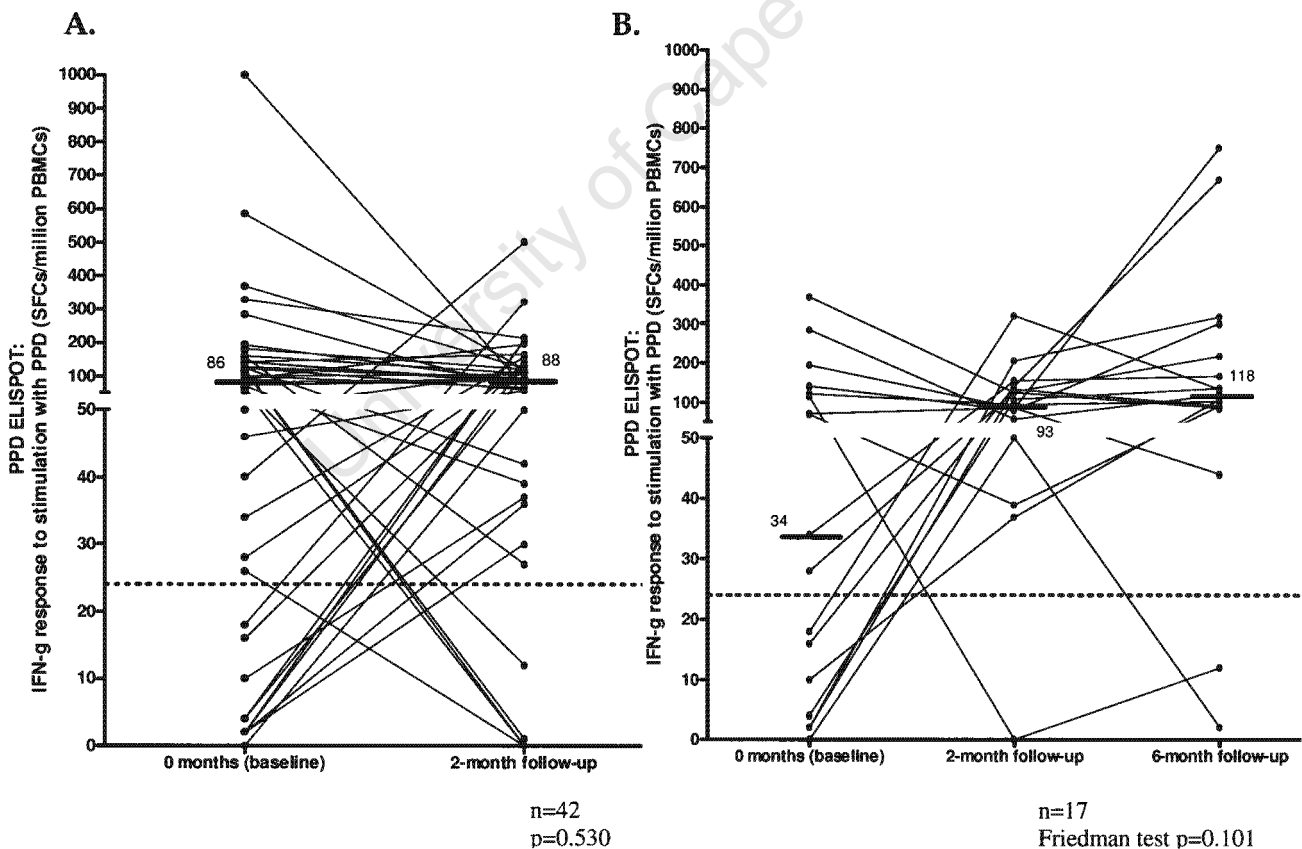


Figure 2.8. Quantitative antigen-specific IFN-gamma responses, in active TB patients on a six-month course of anti-TB therapy, to PPD antigen: (A) PPD ELISPOT results at 0 months (baseline) and at 2 months post-treatment initiation [$n=42$], and (B) 0 months (baseline), 2 months post-treatment initiation and at treatment completion (6 months) [$n=17$]. The medians for the PPD ELISPOT assay are indicated as horizontal solid lines and the cut-points (24 SFCs/million PBMCs) are indicated as horizontal dotted lines.

2.6.3.2. Heparin-binding haemagglutinin (HBHA)

The median HBHA spot counts for baseline (0 months) versus 2-month follow-up (figure 2.9 A) remained below the cut-point for positivity, and were not significantly different (6 vs. 10, respectively; $n=21$; $p=0.755$). As shown in figure 2.9 B, the median spot counts at baseline vs. 2-month follow-up vs. 6-month follow-up remained below the cut-point for positivity (6 vs. 10 vs. 10, respectively) and there was no significant change in the median spot counts ($n=13$; Friedman test p -value= 0.430).

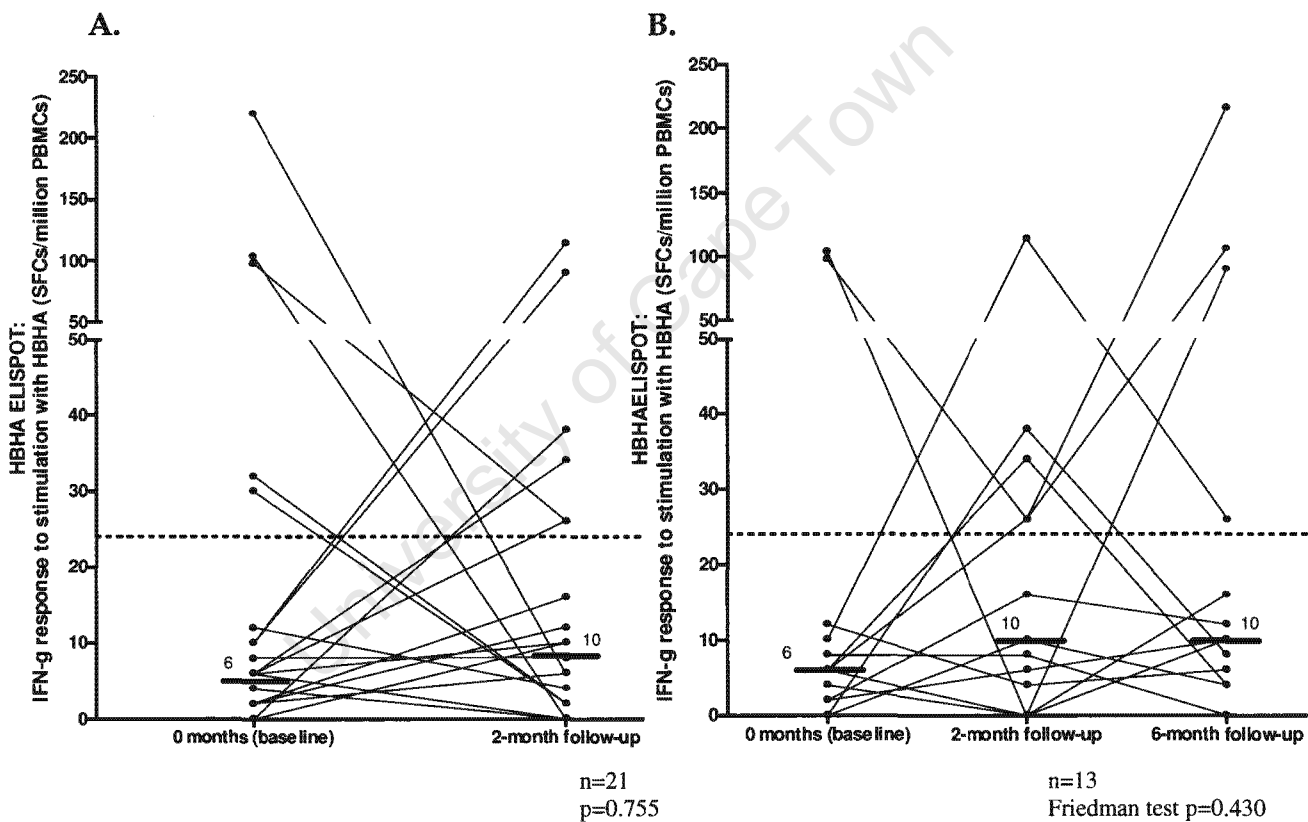


Figure 2.9. Quantitative antigen-specific IFN-gamma responses, in active TB patients on a six-month course of anti-TB therapy, to HBHA antigen: (A) HBHA ELISPOT results at 0 months (baseline) and at 2 months post-treatment initiation [$n=21$], and (B) 0 months (baseline), 2 months post-treatment initiation and at treatment completion (6 months) [$n=13$]. The medians for the PPD ELISPOT assay are indicated as horizontal solid lines and the cut-points (24 SFCs/ million PBMCs) are indicated as horizontal dotted lines.

2.7. DISCUSSION

Overall, our results do not support the use of IGRAs as an early surrogate marker for treatment response in patients on anti-TB treatment. There was poor agreement between the changes in antigen-specific peripheral quantitative T cell responses and changes in sputum smear and culture status at 2 months and at treatment completion (6 months), irrespective of the use of a range of antigens (RD1, PPD and HBHA).

As previously mentioned (please refer to chapter 1, section 1.5.5) existing TB treatment-related study data are conflicting. Several studies have provided data that support the hypothesis that anti-TB treatment leads to a decreased antigen burden, and in turn, a diminished frequency of circulating antigen-specific T lymphocytes (Carrara et al 2004; Dheda et al 2007; Lalvani et al 2001b; Pathan et al 2001; Ribeiro et al 2009). Paradoxically, in our study, the median T SPOT TB and PPD ELISPOT spot counts remained above the cut-point for positivity (24 SFCs/million PBMCs). Furthermore, although there was no significant difference between the median spot counts over the course of anti-TB treatment, there was an increased frequency of circulating antigen-specific T lymphocytes from baseline to 2 months, and at treatment completion at 6 months. How do we explain this increase in median spot counts despite the fact that these patients are on anti-TB treatment? An increased frequency of circulating antigen-specific T lymphocytes could be an indication of re-infection or a high burden of disease with slow decay in T cell responses. There may be a genetic patient-specific differential effect of the variability in pattern recognition receptors for immunogenic RD-1 epitopes, and perhaps an effect of CD4⁺ regulatory T cells (Dosanjh et al 2008; Lalvani et al 2001a), thus resulting in higher or lower T cell counts in some active TB patients.

Our findings are consistent with those of several previous studies, which demonstrated persistently high or positive RD-1-specific test results, even after treatment completion, using pre-commercial RD-1 based IGRAs

(Ferrand et al 2005; Ulrichs et al 2000a; Ulrichs et al 2000b). Furthermore, available data suggest that serial IGRA conversion rates may be much higher in high-burden settings compared with low-burden settings (Pai & O'Brien 2007). However, there are limited data from high burden settings. A study of LTBI-treated Health-care workers (HCWs) in India found that at 6-month follow-up, the subjects had persistently positive IGRA responses (Pai et al 2006a). Furthermore, only one study evaluated serial IGRA responses over 18 months (Pai et al 2006b). However, prospective studies are required in HCWs from high-burden settings, to determine the predictive value of IGRAs for active disease and for the evaluation of serial treatment-related responses over the course of anti-TB treatment. A treatment-related study in another high burden setting (The Gambia), showed that the levels of IFN- γ production in response to PPD (n=7) remained above the cut-point for positivity and unchanged throughout the study (6 months of treatment and follow-up at 9 months) (Turner et al 2000). Serial frequencies of antigen-specific T cell treatment-related responses (in-house ELISPOT assay) in Ugandan active TB patients were also persistently high 3-12 months after treatment completion (Goletti et al 2008). The median PPD ELISPOT responses also remained above the cut-point for positivity in our study. Furthermore, we found that the median T SPOT TB and PPD ELISPOT spot counts were quite similar (T-SPOT TB and PPD ELISPOT median SFCs/million PBMCs at baseline vs. 2-month follow up were 86 vs. 98 and 86 vs. 88, respectively).

The significant decrease in QFT-GIT responses at 6-month follow-up suggests that this test may be a helpful treatment-related response indicator closer to the end of treatment completion. However, it is unclear why this decrease was not seen at 6-months when using the T-SPOT.TB and PPD ELISPOT assays. Besides the two assays having different properties (QFT measures IFN- γ responses in whole blood, whereas T-SPOT TB and PPD ELISPOT measures responses in PBMCs), this difference could perhaps have been due to Type I error (attrition bias/loss-to follow-up). Furthermore, we cannot exclude the possibility that TB 7.7. (QFT-GIT-specific antigen) may be

driving immunosuppressive regulatory T cells or inhibitory pathways (Dhedha et al 2009c; Guyot-Revol et al 2006).

As outlined in the literature review (p. 31), and in contrast to RD-1 antigens, it has been found that cells from individuals with LTBI release IFN- γ in response to HBHA, whereas cells from definite TB patients do not (Temmerman et al 2004). However, in our study HBHA appeared to have poor discriminatory value. Further studies are needed to explain these findings, because the regulatory T cell profile, which may be involved in modulating HBHA responses, may be different in high burden countries and when compared with RD-1 antigen responses.

Investigations using genomic, proteomic and suspension array technologies are required to find new biomarkers that may be useful for tracking disease activity (Dhedha et al 2010b; Walzl et al 2008). Indeed, an essential role of IFN- γ in response to infection with *M.tb* is the activation of macrophages. In delayed hypersensitivity reactions to tuberculin PPD, IFN- γ has been found to induce downstream chemokines such as IFN- γ inducible protein (IP-10 or CXCL10), monokine-inducible protein (MIP or CXCL9), and monocyte chemo-attractant protein 2 (MCP-2) (Kaplan et al 1987). Also, studies have reported the presence of these IFN- γ inducible chemokines in the plasma (Ruhwald et al 2007) and pleural effusions (Okamoto et al 2005) of TB patients. These markers require further evaluation in serial time-point studies in active TB patients. Clearly, IGRAs do not fulfil this requirement. Further studies using different biomarkers, and alternative antigens, are required to clarify the issue.

The rapid diagnosis of active pulmonary cases is important for TB control and there is a great need for novel immunotherapeutic interventions. However, evaluating these interventions is problematic, because it takes several years to complete treatment evaluation studies (Perkins et al 2006). Thus, it would be beneficial if a rapid proxy marker could be used as a surrogate marker of mycobacterial burden, to predict whether or not a

patient is likely to relapse after treatment. However, quantitative T cell assays are unlikely to fulfil the promise created by earlier studies.

A limitation of the study is that, due to quantitative chest X-ray scores being unavailable at the time of analysis, we were unable to assess whether the extent of disease (disease chronicity) accounted for some of the changes observed. Nevertheless, irrespective of specific antigens (RD1, PPD, HBHA), culture remains one of the best treatment monitoring tools. Larger longitudinal studies with long-term patient follow-up and using different antigens are now required to determine the utility of various antigen-specific IGRAs for detecting whether relapse will occur after completion of anti-TB therapy.

University of Cape Town

CHAPTER 3:

Delayed Enumeration of TB-specific T Cell Responses and the Evaluation of T Cell Xtend

3.1. INTRODUCTION

During the initial stages (recruitment of the first 100/500 subjects), it became evident that a major drawback of the enzyme-linked immunospot (ELISPOT) interferon gamma release assay (T-SPOT[®].TB) is that sample processing must be started within 8 hours of the blood-draw. This imposes logistical limitations when sample transport takes longer than expected or dispatch to the laboratory is delayed (not uncommon for peripheral clinics or when patients are seen in the afternoon), and may increase labour costs if a technician is required to process samples after normal working hours. Furthermore, batching of samples over 48 to 72 hours is not possible. The manufacturer of T-SPOT[®].TB (Oxford Immunotec Ltd.) recently introduced a proprietary reagent, T-Cell Xtend, which has been designed to allow for the delayed processing of blood samples without a reduction in test accuracy. According to the manufacturer's package insert, T-Cell Xtend[™] is an antibody complex directed against specific cell surface antigens on human blood cells. This proprietary antibody complex enables the cross-linking of red blood cells with selected white cells. The selected cells form a pellet when applied to density gradient centrifugation, thus allowing the removal of cells that may confound ELISPOT T helper cell 1 (Th1) responses [T-Cell Xtend[™] mechanism of action, Oxford Immunotec Ltd. 2009]. Flexibility in sample processing may improve test utility of the quantitative antigen-specific T-cell assay (T-SPOT[®].TB). Thus, it would be advantageous if blood could be processed the day after it is drawn without compromising assay performance. Moreover, there are limited data comparing TB-specific ELISPOT responses in a clinical setting using blood processed at different

times (Meier et al 2005). The current study was thus designed and incorporated for the remainder of the T-SPOT®.TB evaluation.

The main purpose of this component of the study was to investigate the effect of delayed processing on test accuracy in a clinical setting. Accordingly, blood samples were processed immediately (day 0) and at different times after receipt of the sample [~ 24 hours (day 1) or ~ 32 hours (day 2)] *with* and *without* the addition of T-Cell Xtend™. We hypothesised that delayed sample processing of blood for use in the T SPOT TB assay impacts upon test accuracy of the assay, and that the use of T-Cell Xtend™ allows for the delayed processing of blood for up to 32 hours for use in the T SPOT TB assay without reducing test accuracy.

University of Cape Town

3.2. AIM

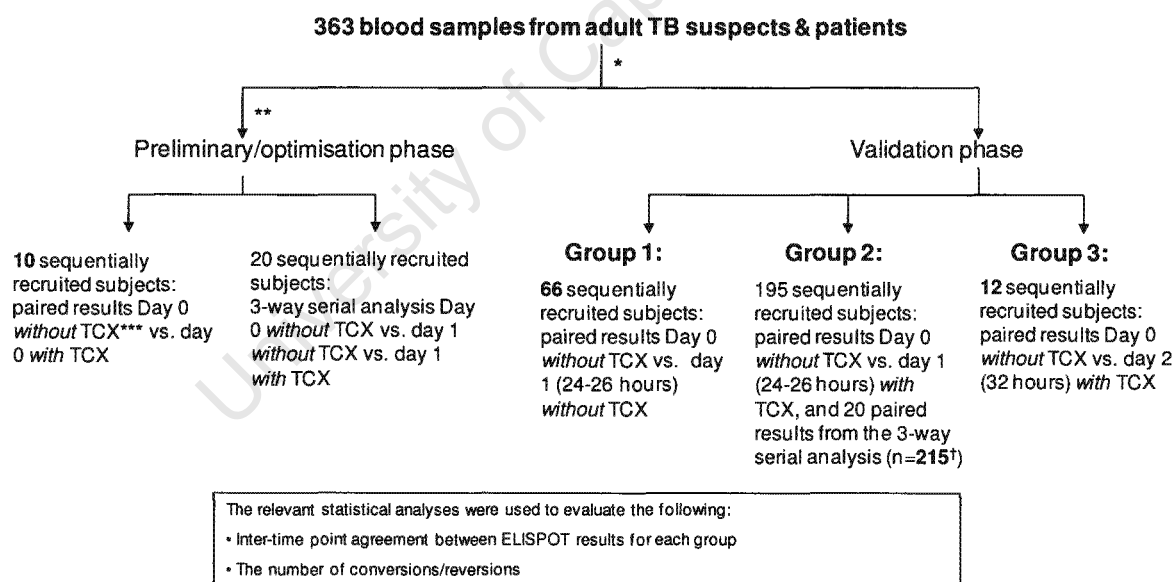
To investigate whether delayed sample processing *with* and *without* the use of T-Cell Xtend™, a proprietary reagent, impacts upon test accuracy of the T-SPOT.TB assay.

University of Cape Town

3.3. MATERIALS AND METHODS

3.3.1. Clinical recruitment

The subjects for the current study were recruited as part of the SAIGRA prospective cohort parent study (please refer to chapter 2 for the sample recruitment site details). After ~10ml blood was assigned for use in the treatment-related study (chapter 2) and various unrelated ongoing studies, the remainder of the sample was available for use in the current study, in which blood samples from 363 sequentially recruited adult TB suspects and patients on treatment, from Cape Town, South Africa, were processed at two study sites, namely, the Lung Infection and Immunity Unit (main study site) and Biotec Laboratories South Africa Ltd. The study flow diagram is shown in figure 3.1.



- * 303 evaluable, paired T SPOT results, and 60 patient samples excluded [19 indeterminate ELISPOT results, 18 inadequate PBMC yield (either for day 0, 1 or 2), 23 other (4 development error, 3 incubator malfunction, 16 administrative errors on case record forms)].
- ** Due to logistical and sample constraints, the study was conducted in a step-wise fashion by conducting a preliminary/optimisation phase in a small group of sequentially recruited subjects, followed by a validation phase in a larger number of subjects who were sequentially recruited for each of the three main study protocol groups
- *** TCX: T-Cell *Xtend*
- † For group 2, 159/215 samples were processed at the main study site (Lung Infection and Immunity Unit), and 56/215 samples were processed at another site (Biotec Laboratories South Africa Ltd, Cape Town). When these 56 samples were excluded from the analysis the conclusions remained unchanged. Thus, analysis at a different site did not impact on the findings presented. In any event both technicians were experienced and trained.

Figure 3.1. Study flow diagram for the investigation of the delayed enumeration of TB-specific T Cell responses and the evaluation of T Cell Xtend. The study was

conducted using two phases, namely, a preliminary/optimisation phase and a validation phase.

Consecutive unselected patients were recruited and there was equal distribution of the sub-groups analysed with respect to clinical background or HIV status. Ethical approval was obtained from the University of Cape Town Health Sciences Faculty Research Ethics Committee and the Committee for Human Research, University of Stellenbosch, Tygerberg. All subjects signed informed consent prior to entering the study.

3.3.2. Laboratory methods

Blood (~20ml) was drawn from all participants into lithium-heparin tubes (BD Europe) and transported to the laboratory within 3-4 hours where peripheral blood mononuclear cells (PBMCs) were isolated from ~10ml blood for the treatment-related study (chapter 1) and other unrelated studies. The remainder of the sample (~5-10ml) was used for the current study, in which blood was processed at different times (as shown in figure 3.1, p.77) for use in the T-SPOT[®].TB assay. For all samples, the blood was stored at room temperature (18-25°C) during transport and was assayed within 8 hours of venipuncture (ie. on the day of blood collection as recommended by the manufacturer of the T-SPOT.TB assay).

3.3.2.1. Isolation of peripheral blood mononuclear cells:

The blood sample for use in the current study (5ml) was transferred from the lithium-heparin tube (BD Bioscience, South Africa) to a 15ml conical centrifuge tube, directly diluted 1:1 with 1x phosphate buffered saline (ie. excluding the initial centrifugation step, as described in chapter 2, in which the robust white cloudy layer of PBMCs was first obtained, and layered on

top of 3-4 ml Ficoll-Paque™. Thereafter, the steps were conducted as previously described (please refer to chapter 2, pp.50-53).

3.3.2.2. The use of T Cell Xtend

For the samples that were used to evaluate the effect of T-Cell Xtend, and according to the manufacturer's instructions (T-cell Xtend™ [package insert] 2008), 25µl of T-Cell Xtend solution was added per 1ml of whole blood for 20 minutes immediately prior to the isolation of the PBMCs for use in the T SPOT TB assay. Please refer to figure 3.2 below.

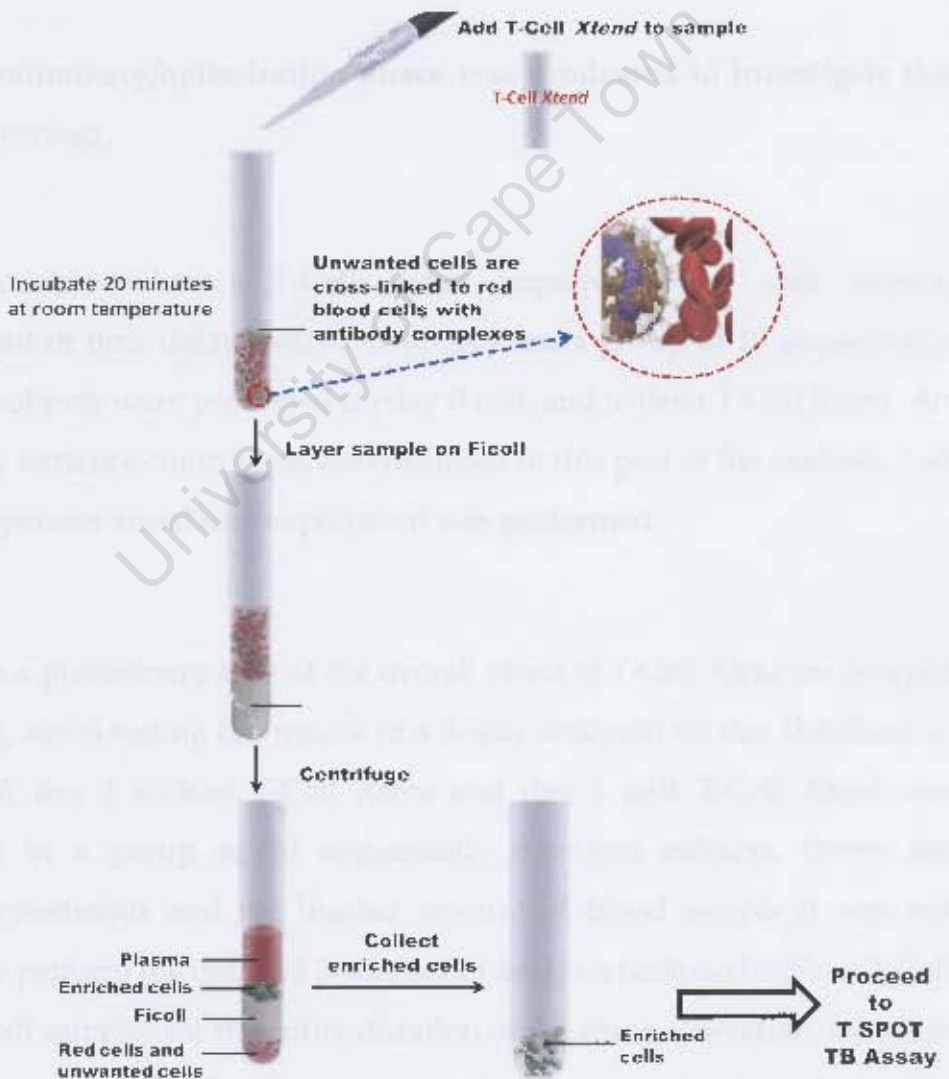


Figure 3.2. Simplified flow diagram showing how T-Cell Xtend should be incorporated into the T SPOT TB assay protocol for use with whole blood.

3.3.2.3. The ELISPOT (T-SPOT.TB) assay

The T-SPOT.TB assay was conducted according to the manufacturer's instructions (T-SPOT®.TB [package insert] 2008), as previously described (chapter 1). However, the well layout was as follows: panel A (ESAT-6), panel B (CFP-10), positive control (PHA) and negative control (AIM-V medium). Thus, the T SPOT TB assay was conducted by seeding the PBMCs at a final concentration of 250,000 PBMCs per well (ie. 100ul of the 2.5×10^6 cells/ml cell suspension into each well), and adding 50 μ l of the relevant antigens and controls to the T SPOT wells (according to the above-mentioned well layout). The antigen-specific T cell responses were scored and the test results were interpreted accordingly (as previously described in chapter 1).

3.3.3. A preliminary/optimisation phase was conducted to investigate the following:

1) To evaluate whether T-Cell *Xtend* impacted upon spot counts, independent of time delay, paired samples from a group of 10 sequentially recruited subjects were processed on day 0 *with* and *without* T-Cell *Xtend*. An intra-assay variance control was also included in this part of the analysis, and a within-operator variability experiment was performed.

2) To gain a preliminary idea of the overall effect of T-Cell *Xtend* on delayed processing, serial testing (by means of a 3-way analysis) on day 0 *without* T-Cell *Xtend*, day 1 *without* T-Cell *Xtend* and day 1 *with* T-Cell *Xtend* was conducted in a group of 20 sequentially recruited subjects. Given the logistical constraints and the limited amount of blood sample it was not possible to perform the detailed 3-way serial analysis (*with* and *without* T-Cell *Xtend*) on all samples for the entire duration of the study. Therefore, after this optimisation phase only 2-way comparisons were performed in the validation phase of the study.

3.3.4. For the validation phase the cohort was divided into three study protocol groups:

Group 1) To evaluate whether delayed processing at ~ 24 hours (independent of T-Cell Xtend) impacted upon the results, paired samples, from 66 sequentially recruited subjects, were processed *without* T-Cell Xtend on day 0 and day 1.

Group 2) To evaluate whether delayed processing ~ 24 hours (with the addition of T-Cell Xtend) impacted upon the results, paired samples from 195 sequentially recruited subjects were processed on day 0 *without* T-Cell Xtend and day 1 *with* T-Cell Xtend. Also, the day 0 without T-Cell Xtend vs. day 1 with T-Cell Xtend results for the 20 subjects that formed part of the optimisation phase (3-way analysis) were included in group 2. Hence, there were a total of 215 evaluable results for this part of the analysis.

Group 3) To assess whether processing of blood could be delayed for up to 32 hours, paired samples (from a group of 12 sequentially recruited subjects), were processed on day 0 *without* T-Cell Xtend and on day 2 *with* T-Cell Xtend.

The baseline (day 0) sample was processed immediately upon arrival at the laboratory. The samples for delayed processing [a delay of either ~24 hours after receipt (day 1) or ~32 hours after receipt (day 2)] were stored at room temperature in an upright position in a dark drawer and processed in the 3 main study protocol groups, at the relevant time-point, with or without the addition of T-Cell Xtend. The groups were sequentially recruited to answer each specific study question.

3.3.5. Definition of conversions and reversions

Conversions and reversions were defined in 2 ways:

(i) Based on data from van Zyl-Smit and colleagues (van Zyl-Smit et al 2009), conversions were defined as an increase in interferon gamma (IFN- γ) response from below 6 spots to above 9 spots (greater than or equal to 3 spots on either side of the manufacturer's cut-point of 24 SFCs/million PBMCs), and reversions were defined as a decrease in IFN-gamma response from above 9 spots to below 6 spots.

(ii) The conventional method, as used by the manufacturer, defines an IGRA conversion as a change from a negative to positive result relative to the cut-point, and a reversion as a change from a positive to negative result relative to the cut-point) (Pai & O'Brien 2007). Method (i) was used for the analysis of the current study. However, method (ii) was also taken into account.

3.3.6. Statistical Analyses

Analysis was performed using the Wilcoxon matched pairs test when comparing two non-parametric data sets. Fischer's exact testing was used to determine the significance levels when comparing concordance and the Friedman test was applied for comparison of results when more than two data sets were involved. A detailed comparison was required to evaluate whether the use of T-Cell Xtend enabled processing without reducing test accuracy. Taking into account that there were 66 subjects in group 1 and 215 subjects in group 2, the number of conversions and reversions across the groups were compared using the Wilcoxon Signed Ranks test (Mann-Whitney U test).

3.4. RESULTS

3.4.1. Clinical and demographic information

The participants were predominantly male (236/363; 65%) and the median age was 33 (range 18-83) years. HIV testing was performed in 85% (307/363) of participants. Of those who were tested, 78% (240/307) were HIV negative. Thirty percent of the participants were patients receiving anti-TB treatment and the remainder were unselected TB suspects. The ELISPOT results for 60/363 (17%) were unavailable: 19 were indeterminate, 18 had an inadequate PBMC yield (either for day 0, 1 or 2) so the assay could not be performed, 4 failed due to plate development errors, 3 were invalid due to an incubator malfunction and there were irreconcilable administrative errors on the case record forms for 16 subjects. Thus, a total of 303/363 (83%) subjects yielded evaluable ELISPOT results and could therefore be included in the analysis (please refer to the study flow diagram, figure 3.1 on p.77).

3.4.2. OPTIMISATION PHASE:

3.4.2.1. T-Cell *Xtend* independent of delay did not impact on test results at baseline (day 0)

The median spot counts for day 0 *with* T-Cell *Xtend* vs. day 0 *without* T-cell *Xtend* were not significantly different (46 vs.49; $p=0.94$; $n=10$, figure 3.3) and there were no conversions or reversions (changes across the cut-point).

An intra-assay variance control was included which showed that intra-test variability was minimal (figure 3.4), and a within-operator variability experiment also showed that variability was minimal. Please refer to the raw data in table 3.1.

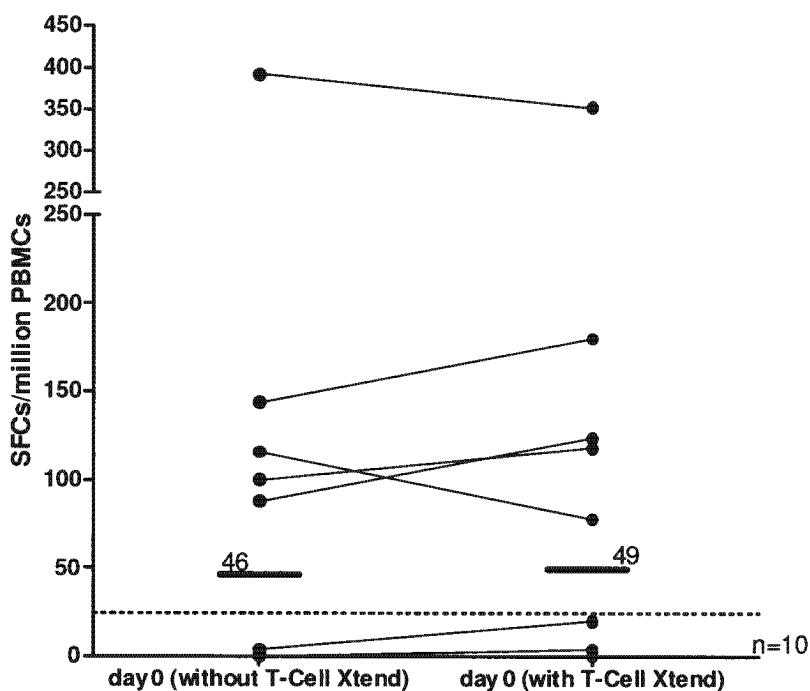


Figure 3.3. Summated response to ESAT-6 and CFP-10: day 0 *without* T-Cell Xtend vs. day 0 *with* T-Cell Xtend. The medians are indicated as horizontal solid lines and the cut-point (24 SFCs/million PBMCs) is indicated as a horizontal dotted line.

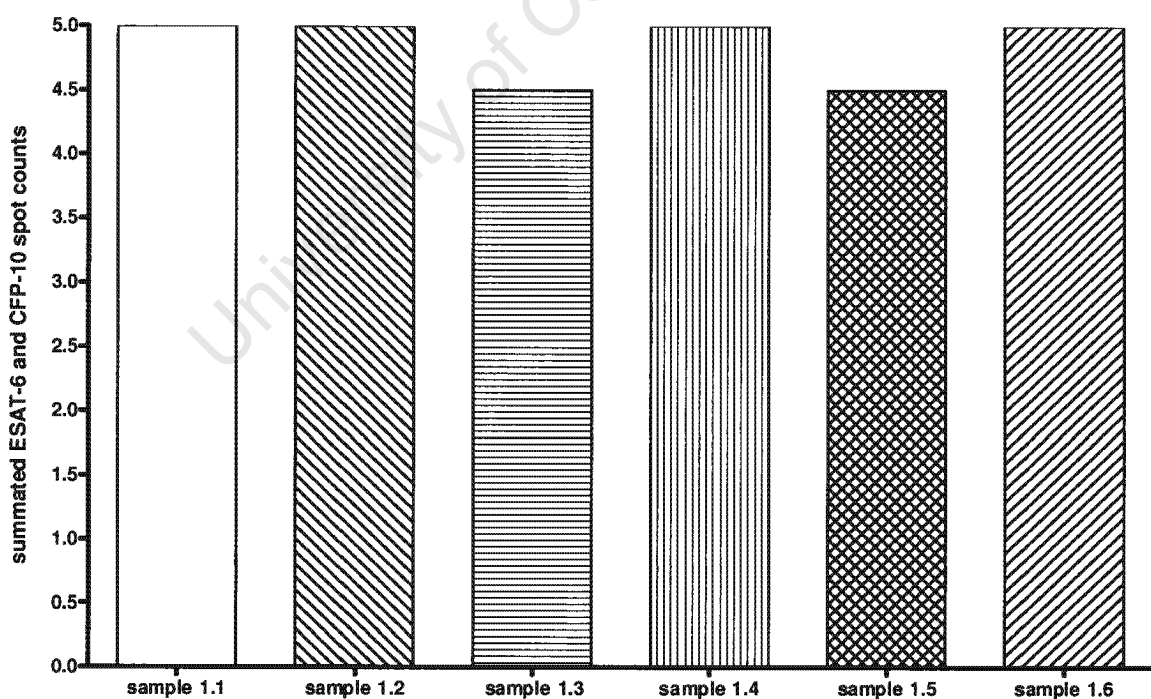


Figure 3.4. One sample was split into 6 (1.1-1.6), processed in parallel by the study operator, and seeded at a final concentration of 250 000 PBMCs per well. Intra-test variability was found to be minimal [average summated spot count =4.8 (values ranging from 4.5 to 5)].

Table 3.1. A within-operator variability experiment showed that variability was minimal.

	Technician 1	Technician 2	Technician 3
ESAT-6 spot count (in duplicate)	19 and 16 Average: 18	19 and 17 Average: 18	18 and 23 Average: 21
CFP-10 spot count (in duplicate)	17 and 11 Average: 14	16 and 14 Average: 15	19 and 18 Average: 18
Positive control spot count (in duplicate)	Too numerous to count	Too numerous to count	Too numerous to count
Negative control spot count (in duplicate)	0	0	0

3.4.2.2. Three-way serial analysis:

There were no significant differences between the median spot counts for day 0 *without* T-Cell Xtend vs. day 1 *without* T-Cell Xtend vs. day 1 *with* T-Cell Xtend (70, 66 and 56, respectively; n=20; Friedman statistic= 0.46; p-value= 0.80, figure 3.5).

One subject reverted across the cut-point on day 1 *without* T-Cell Xtend, but with the addition of T-Cell Xtend this reversion did not occur (table 3.2 box A1 and A2, p.87).

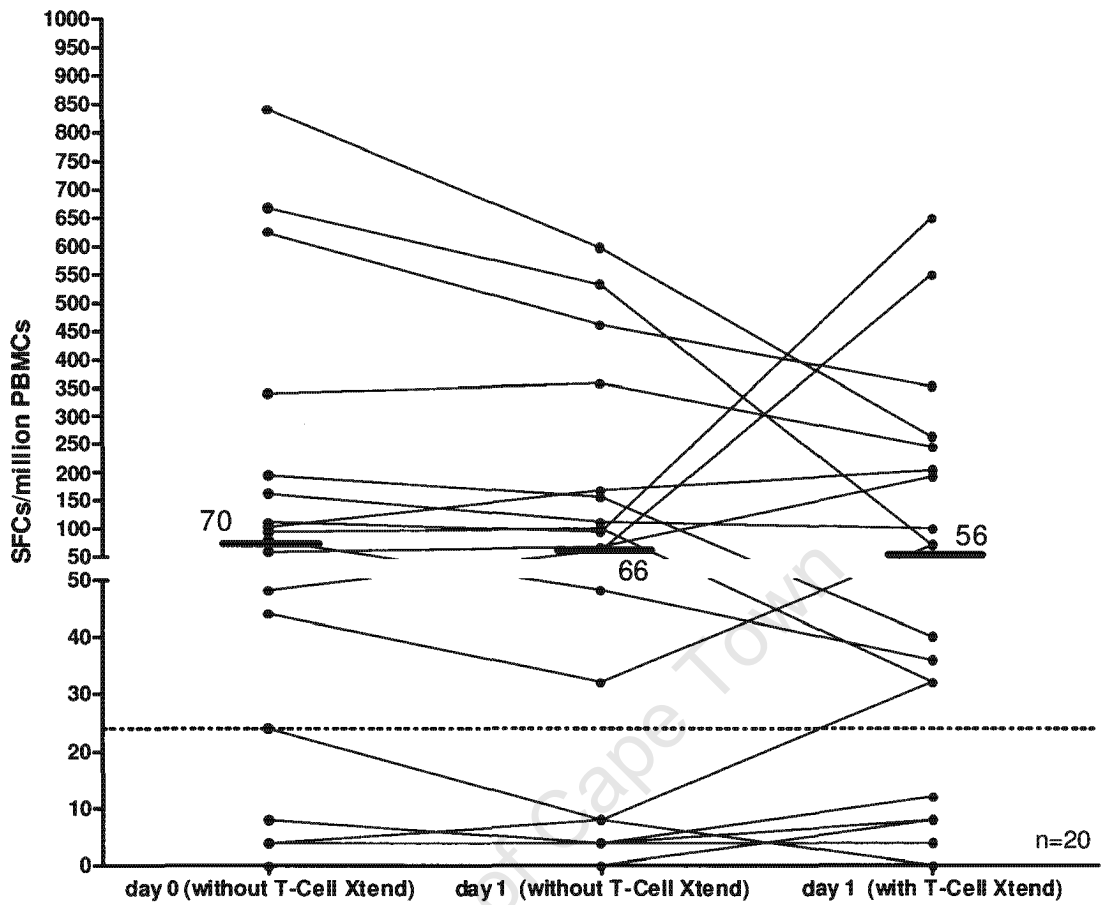


Figure 3.5. Summated response to ESAT-6 and CFP-10 on day 0 *without* T-Cell Xtend vs. day 1 *without* T-Cell Xtend vs. day 1 *with* T-Cell Xtend (n=20). The medians are indicated as horizontal solid lines and the cut-point (24 SFCs/million PBMCs) is indicated as a horizontal dotted line.

Table 3.2: Agreement between serial T-SPOT®.TB results *with* and *without* T-Cell Xtend [agreement= the sum of those who remained T-SPOT positive (≥ 24 SFCs/million PBMCs) and those who remained T-SPOT negative (< 24 SFCs/million PBMCs) divided by the total sample size].

A. *	A1. Day 1 without T-Cell Xtend		A2. Day 1 with T-Cell Xtend		
Day 0 without T-Cell Xtend		T-SPOT +	T-SPOT -	T-SPOT +	T-SPOT -
	T-SPOT +	13	1	14	0
	T-SPOT -	0	6	0	6

B. **	Day 1 without T-Cell Xtend		
Day 0 without T-Cell Xtend		T-SPOT +	T-SPOT -
	T-SPOT +	49	1
	T-SPOT -	2	14

C. ***	Day 1 with T-Cell Xtend		
Day 0 without T-Cell Xtend		T-SPOT +	T-SPOT -
	T-SPOT +	142	3
	T-SPOT -	3	67

D. ****	Day 2 with T-Cell Xtend		
Day 0 without T-Cell Xtend		T-SPOT +	T-SPOT -
	T-SPOT +	10	1
	T-SPOT -	0	1

* Box A1 and A2 = the agreement between results for the 3-way analysis of the same sample (day 0 *without* T-Cell Xtend, day 1 *without* T-Cell Xtend and day 1 *with* T-Cell Xtend; n=20).

** Box B = the agreement between paired T-SPOT results for day 0 and day 1 (both *without* T-Cell Xtend; n=66)

*** Box C = the agreement between paired T-SPOT results for day 0 *without* T-Cell Xtend and day 1 *with* T-Cell Xtend. Box C excludes the 3 converters/reverters who fall within the borderline zone; hence for the purpose of calculating inter-time-point agreement, the corrected sample size total is n=215 minus 3=212.

**** Box D = the agreement between paired T-SPOT results for day 0 *without* T-Cell Xtend and day 2 *with* T-Cell Xtend; n=12.

3.4.3. VALIDATION PHASE

3.4.3.1. Delayed processing (~24 hours) *without* T-Cell Xtend resulted in significant changes in ELISPOT results

The median spot counts on day 1 *without* T-Cell Xtend were found to be significantly higher than day 0 *without* T-Cell Xtend (114 vs.100; $p=0.03$; $n=66$; figure 3.6 A). Inter-time-point agreement between the results was 95.45 % (table 3.2 box B, p.87), and 4.54% subjects showed conversion or reversion across the cut-point (figure 3.6 B). These results were similar when the HIV positive patients were excluded from the analysis (figure 3.7).

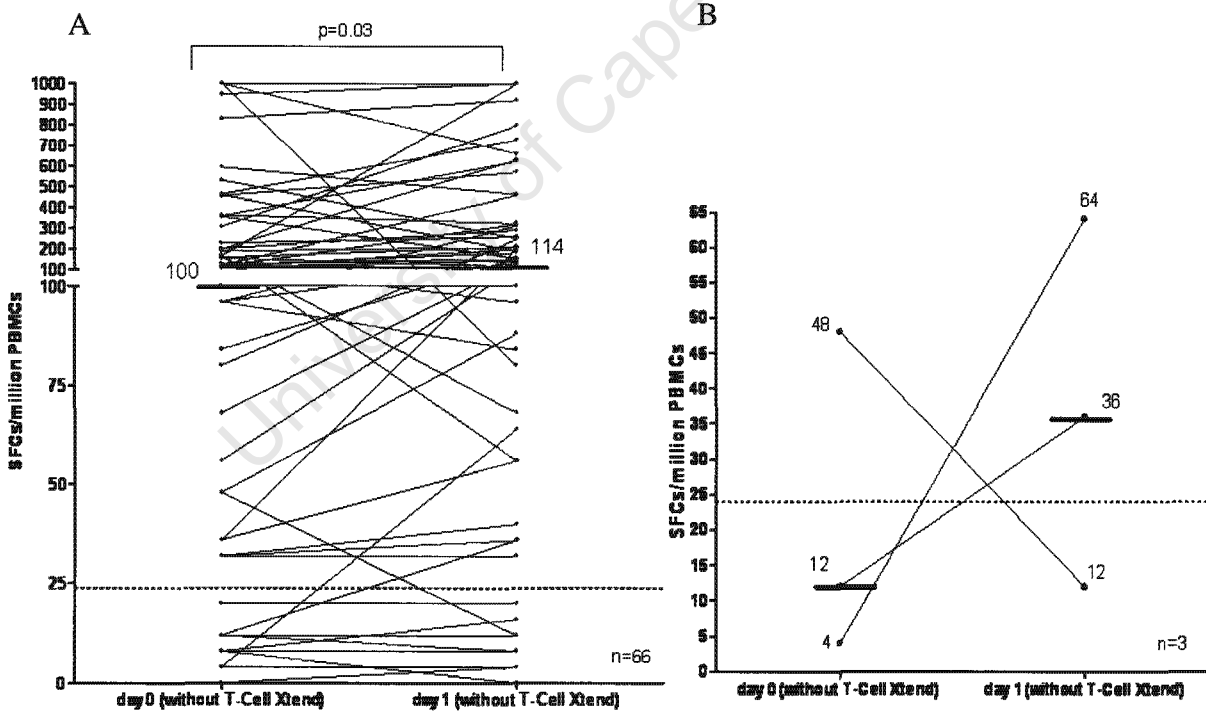
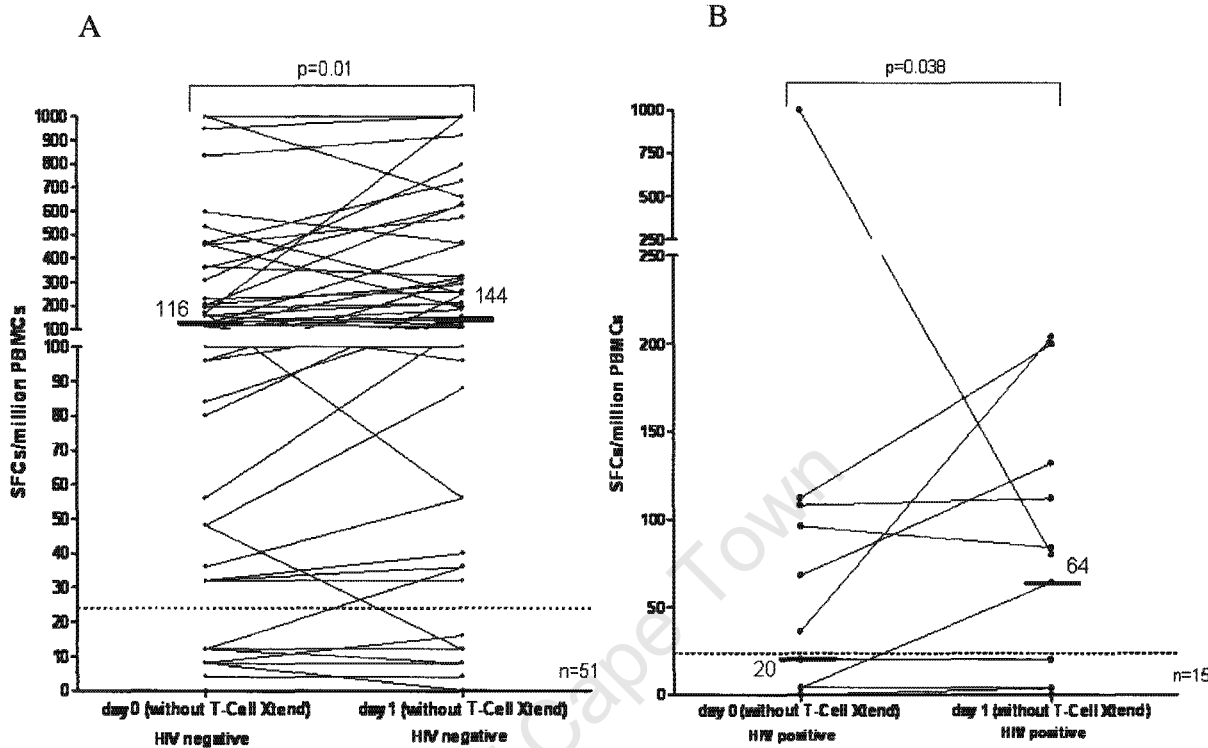


Figure 3.6. Summated response to ESAT-6 and CFP-10 on day 0 vs. day 1 (both *without* T-Cell Xtend). A line graph of each individual's summated response is shown in figure A ($n=66$). For the whole group, 2 conversions and 1 reversion occurred. None of the conversions/reversions fall within the 'borderline zone' (van

Zyl-Smit et al 2009) and were thus all considered to be true conversions/reversions (B). The medians are indicated as horizontal solid lines and the cut-point (24 SFCs/million PBMCs) is indicated as a horizontal dotted line.



Figures 3.7. Summated response to ESAT-6 and CFP-10 on day 0 vs. day 1 (both *without T-Cell Xtend*): response of the HIV negative subjects only (n=51; A) and the HIV positive group (n=15; B). The medians are indicated as horizontal solid lines and the cut-point (24 SFCs/million PBMCs) is indicated as a horizontal dotted line.

3.4.3.2. Delayed processing (~24 hours) with T-Cell Xtend maintained a high level of inter-time-point agreement, but the number of conversions/reversions was not significantly reduced

The median spot counts were the same at day 0 *without T-Cell Xtend* vs. day 1 *with T-Cell Xtend* (56 vs 56; n=215; p= 0.42; figure 3.8 A). When processing was delayed for ~24 hours *with* the addition of T-Cell Xtend, a total of 4 subjects crossed the cut-off from positive to negative (reverters), but 2 of these fall within the 'borderline zone' and were thus not considered to be true reverters. A total of 5 subjects crossed the cut-off from negative to

positive (converters), but 1/5 fell within the 'borderline zone' and was thus not considered to be a true converter. Thus, agreement between the results was 97.17% (table 3.2 box C, p.87) and the conversion/ reversion rate was 2.83% (the summated IFN- γ responses of the 4 true converters and 2 true reverters are shown in figure 3.8 B).

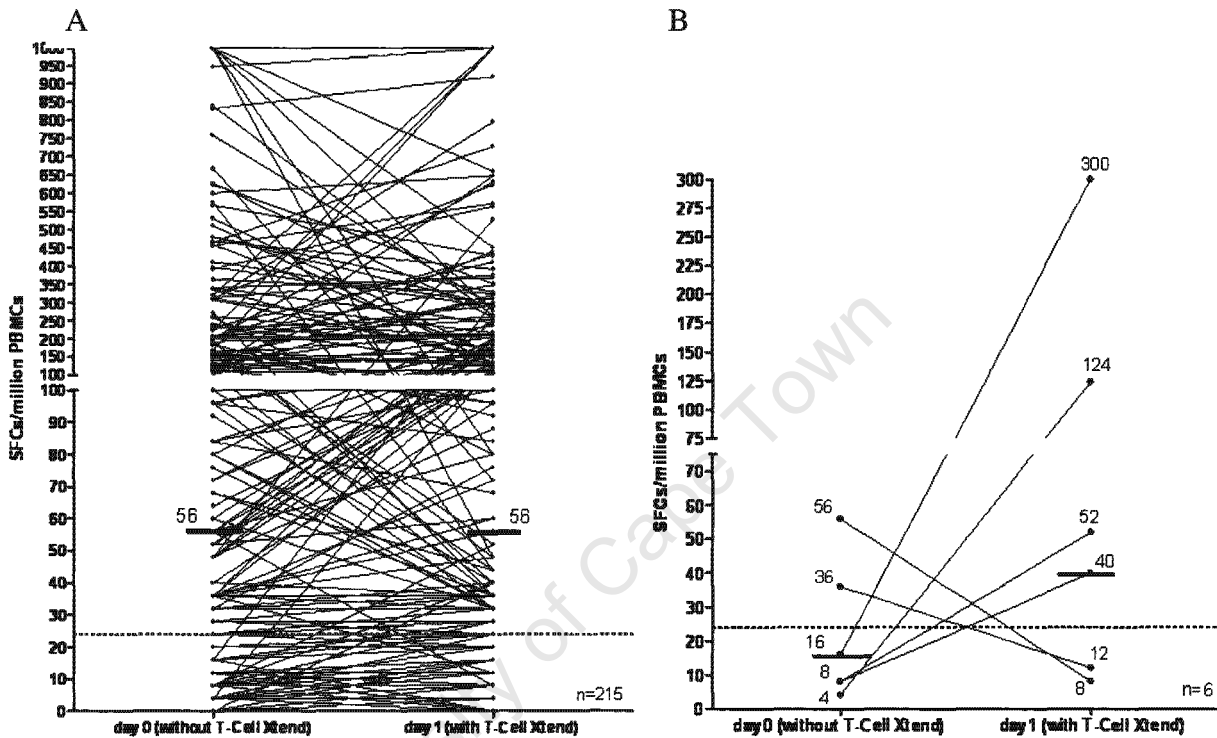


Figure 3.8. Summated response to ESAT-6 and CFP-10 on day 0 *without* T-Cell Xtend vs. day 1 *with* T-Cell Xtend. A line graph of each individual's summated response is shown in figure A (n=215). For the whole group, a total of 9 conversions/reversions occurred. However, 3 of the 9 conversions/reversions fall within the 'borderline zone' (van Zyl-Smit et al 2009). Hence, only 6 (4 conversions and 2 revertions) were considered to be true conversions/reversions (B). The medians are indicated as horizontal solid lines and the cut-point (24 SFCs/ million PBMCs) is indicated as a horizontal dotted line.

The difference between the number of conversions/reversions for group 1 (day 0 *without* T-Cell Xtend and day 1 *without* T-Cell Xtend) vs. group 2 (day 0 *without* T-Cell Xtend vs. day 1 *with* T-Cell Xtend) was not statistically significant (Wilcoxon Signed Ranks test 2-tailed p-value=0.809). These results

were similar when the HIV positive patients were excluded from the analysis (figure 3.9).

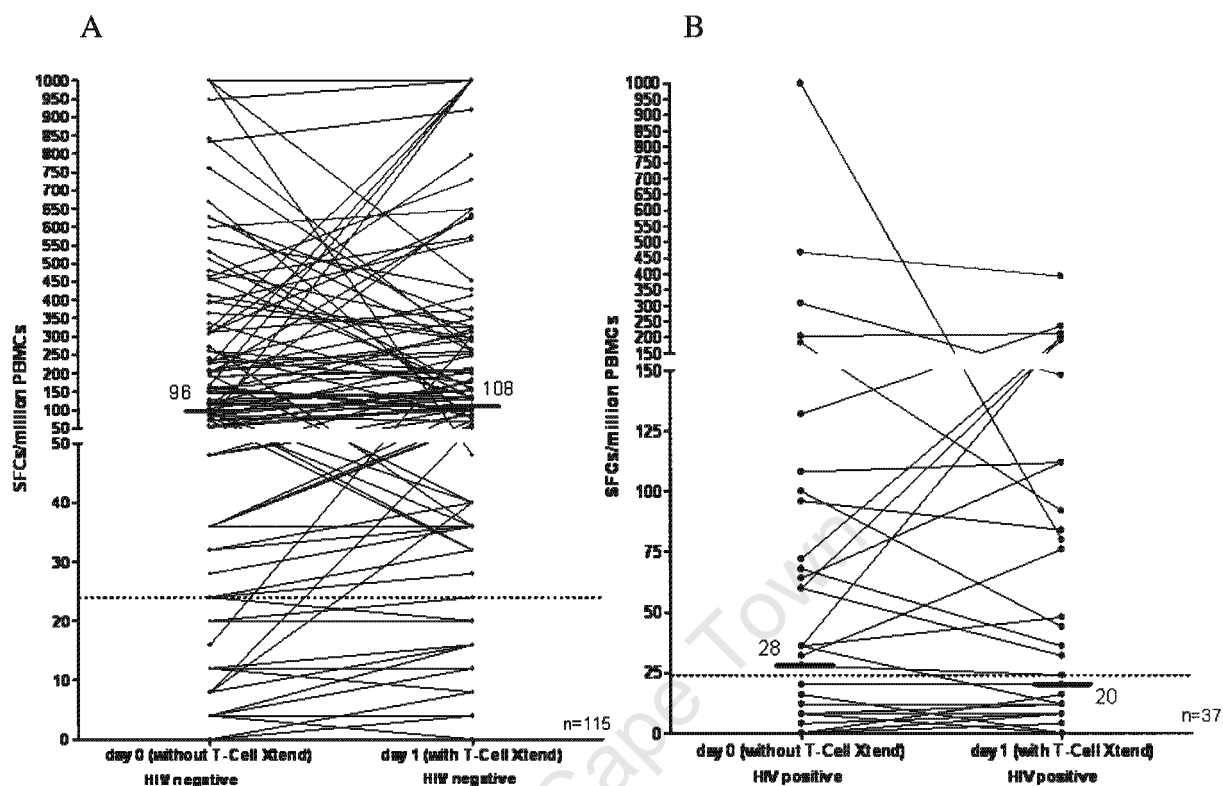


Figure 3.9. Summated response to ESAT-6 and CFP-10 on day 0 *without* T-Cell Xtend vs. day 1 *with* T-Cell Xtend: response of the subjects who had a negative HIV test result (n=115/159; A) and the HIV positive group (n=37/159; B). HIV results for 7/159 subjects were unknown. The medians are indicated as horizontal solid lines and the cut-point (24 SFCs/million PBMCs) is indicated as a horizontal dotted line.

3.4.3.3. Delayed processing for up to 32 hours *with* T-Cell Xtend resulted in high inter-time-point agreement, but the number of conversions/reversions was not significantly reduced

The median spot counts were not significantly different in samples processed on day 0 *without* T-Cell Xtend vs. day 2 *with* T-Cell Xtend (96 and 106; n=12; figure 3.10). Inter time-point agreement between the results was 92% (table 3.2 box D, p.87) and only one subject showed reversion across the cut-point.

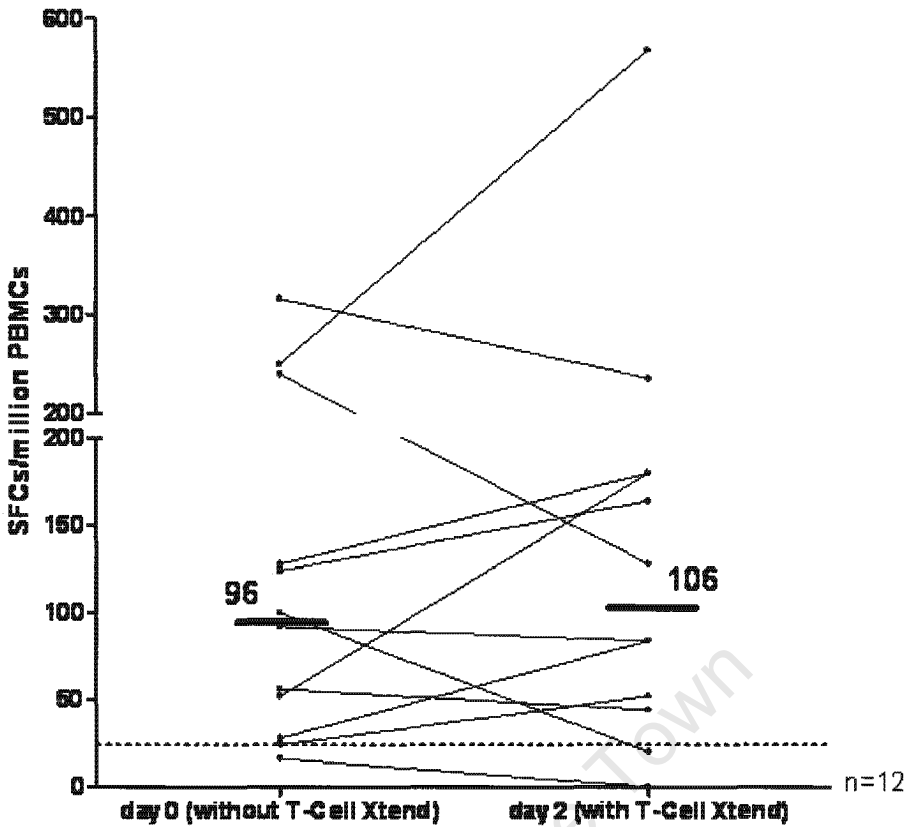


Figure 3.10. Summated response to ESAT-6 and CFP-10 on day 0 *without* T-Cell Xtend vs. day 2 *with* T-Cell Xtend (n=12). The medians are indicated as horizontal solid lines and the cut-point (24 SFCs/ million PBMCs) is indicated as a horizontal dotted line.

3.4.4. Individually scored RD1 antigen-specific spot counts

For all groups, a similar pattern was seen when the data was analysed using the individually scored RD1 antigen-specific spot counts compared to the summated antigen-specific spot counts (figures 3.11 and 3.12).

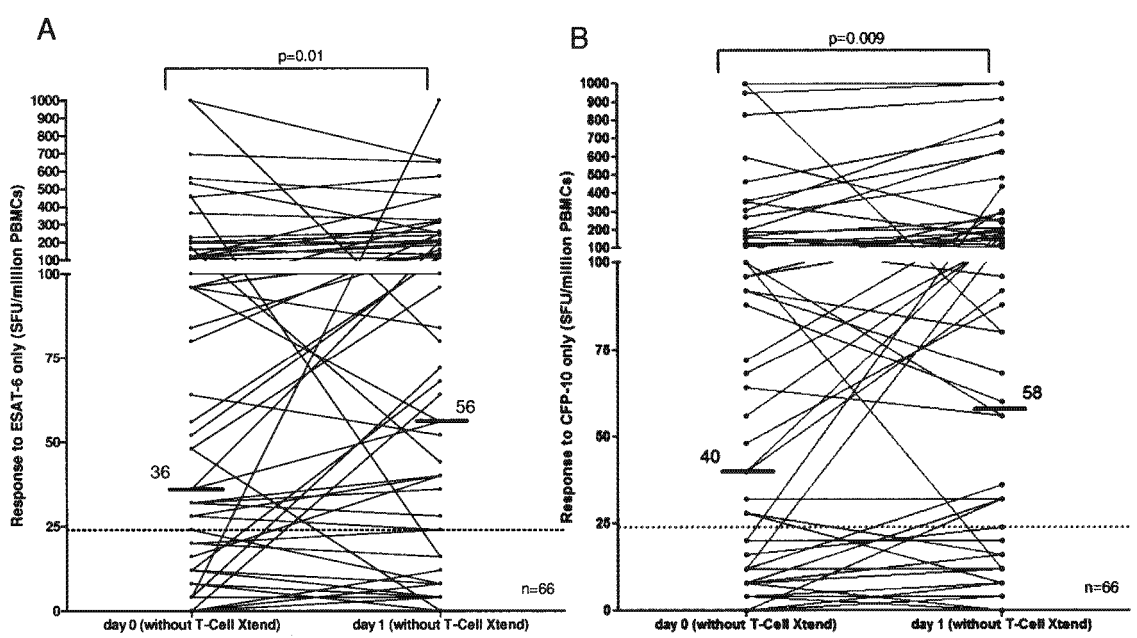


Figure 3.11. Group 1 (delayed processing at ~ 24 hours, independent of T-Cell Xtend): The median SFCs/million PBMCs for the summated RD1 antigen responses were 100 vs. 114, respectively, $n=66$, $p=0.03$ (as shown previously). A similar trend was seen when the results were displayed using (A) the individually scored SFCs/million PBMCs for ESAT-6, and (B) the individually scored SFCs/million PBMCs for CFP-10

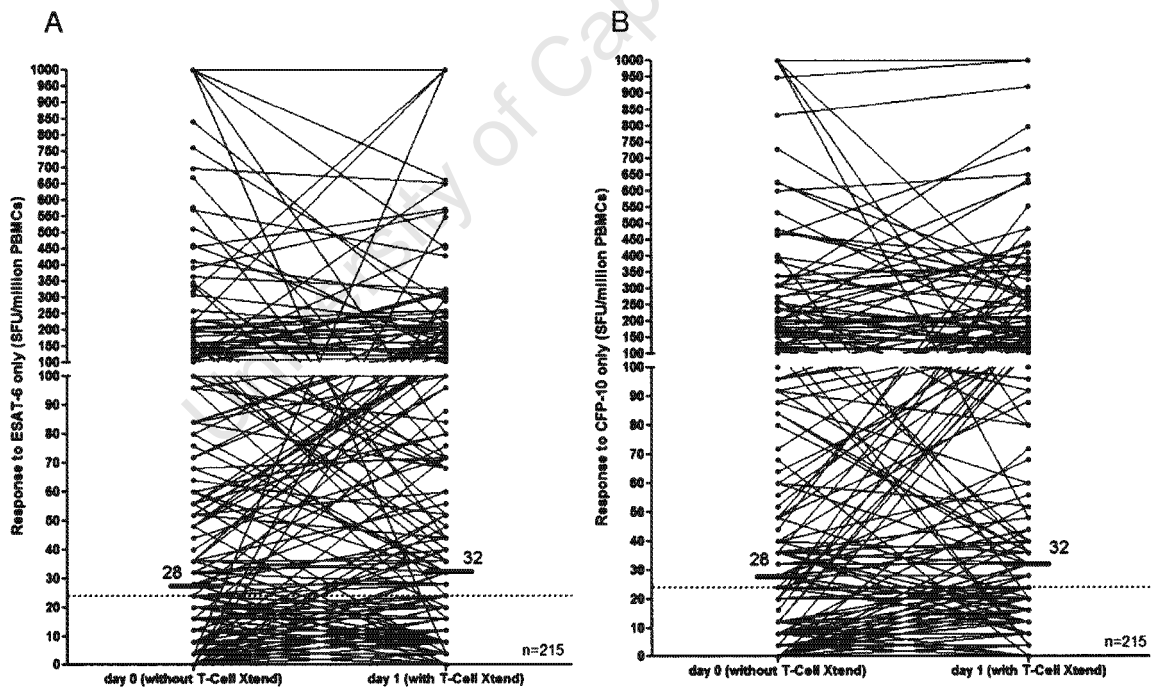


Figure 3.12. Group 2 (delayed processing ~ 24 hours, with the addition of T-Cell Xtend): There were no significant differences between the median SFCs/million PBMCs for the summated RD1 responses (56 vs. 56, respectively, $n=215$, $p=0.42$ (as shown previously), and a similar pattern was seen when the when the results were displayed using (A) the individually scored SFCs/million PBMCs for ESAT-6, and (B) the individually scored SFCs/million PBMCs for CFP-10.

3.5. DISCUSSION

The main aim of this study was to investigate whether delayed sample processing *with* and *without* the use of T-Cell *Xtend* impacted upon the accuracy of the T-SPOT[®].*TB* assay. This study is of practical relevance, because if samples could be batched over a period of 1-2 days, the utility of the assay would be improved and costs reduced through avoidance of after-hours sample processing.

During the analysis phase of the study, the SFCs for each antigen were individually scored, and then compared to the summated spot count. Thus, we are certain that the overall principal findings are not affected by using the summated SFC scores. Other studies have also used summated RD1 values to perform their analyses. For example, Bosshard and colleagues (Respiratory Medicine, 2009) reported SFC values as [highest SFC value of ESAT-6 or CFP-10 antigen - SFC value of negative control] (Bosshard et al 2009) and there are several others (Dheda et al 2009c; Lalvani et al 2001a). The main advantage of this approach, given the large amount of data and graphs, is that it is less confusing and improves readability and understanding of the data. Using a combined antigen-specific readout (i.e. highest spot count) did not impact on the conclusions compared to using the SFCs for each antigen. It was shown that splitting the data by antigen made little difference.

When analysing the summated results, at first glance, the addition of T-Cell *Xtend* appeared to maintain a high level of accuracy when processing was delayed. However, from a clinical point of view, a categorical change in the result (the number of conversions and reversions) is a more meaningful measure of the effect of T-Cell *Xtend*. There was a high agreement between ELISPOT results despite a processing delay of over 24 hours and the use of T-Cell *Xtend* appeared to reduce the amount of conversions/reversions from

4.54% to 2.83%. However, this reduction was not statistically significant. This may be due to type II error and using a larger sample size may have demonstrated a significant difference. Nevertheless, in the serial analysis using the same sample (day 0 *without* T-Cell Xtend vs. day 1 *without* T-Cell Xtend vs. day 1 *with* T-Cell Xtend), even though the median spot counts were not significantly different, with the addition of T-Cell Xtend, all reversions were eliminated.

Conversions and reversions have been defined in this study in accordance with recent findings, which show that an increase in IFN- γ response from below 6 spots to above 9 spots (3 spots on either side of the cut-point) accounts for +/- 95% of the within-subject variability for the T-SPOT.TB assay, and values within this 'borderline zone' are not considered to be true conversions/reversions (van Zyl-Smit et al 2009). This evidence-based definition is more robust compared to the conventional definition (IGRA conversion= change from a negative to positive result relative to the cut-point and reversion= change from a positive to negative result relative to the cut-point) (Pai & O'Brien 2007). Using the evidence-based definition, although there were 9 conversions/reversions (figure 3.8 A), 3 of them were within the 'borderline zone'. Thus, there were only 6 true conversions/reversions (figure 3.8 B) and the conversion/reversion rate was 2.83%. Nevertheless, whichever definition is used (conventional vs. evidence-based), the addition of T-Cell Xtend did not significantly reduce the number conversions/reversions (namely, 4.55% vs. 2.83% as defined by van Zyl-Smit et al (van Zyl-Smit et al 2009) or 4.55% vs. 4.19% as defined by the conventional definition).

An important question that needs to be answered, though, is what is the exact mechanism of action of T-Cell Xtend and how do we explain an increase in spot counts despite storage of blood under hypoxic conditions, which would be expected to reduce cellular viability? The effect of delayed

processing on T cell counts is complex and poorly understood. Furthermore, given the proprietary nature of the product, the exact mechanism by which T-Cell *Xtend* works remains unclear. Delayed processing may reduce the frequency of the T cell marker CD127 (Higgins et al 2005), reduce plasma IL-7 levels (Read et al 2006), and increase the frequency of granulocytes that have T cell inhibitory properties (McKenna et al 2008). Indeed, recently released information from Oxford Immunotec Ltd. has confirmed that the selected white cells that are removed by T-Cell *Xtend* are in fact granulocytes (Oxford Immunotec Ltd 2009).

It is also possible that other immunosuppressive cells or pathways (T regulatory cells or cells producing IL-10, TGF- β , IL-4 and IL-9) are impacted upon by delayed processing. There may be a patient-specific differential effect of these factors as delayed processing may result in higher T cell counts in some patients, whilst in others the counts are reduced. Further studies are needed to clarify this issue. Nevertheless, delayed processing without T-Cell *Xtend*, but not with it, paradoxically resulted in significantly higher spot counts (100 vs. 114 in the whole group and 116 vs. 144 SFCs/million PBMCs in HIV uninfected patients). Although this may be related to sample size, we hypothesise that, in addition to granulocytes, potentially immunostimulatory cells, for example Th1 regulatory cells (Koch et al 2009), may drive responses during delayed processing. Further studies are now required to delineate these pathways, which appear, based on our results, to be independent of HIV status. The effect of a greater processing delay on spot counts, and alternative methods to preserve cellular integrity and function are also required to further improve the utility of the assay. A limitation of the study is that, due to resource constraints and limited sample volume, a small sample number was used for the day 2 analysis and the assays could not simultaneously be conducted in the absence and the presence of T-Cell *Xtend*. Furthermore, these data pertain to samples obtained in a high burden setting and the results may thus not be applicable to other settings.

Clinical Utility of the Clearview-TB®-ELISA Lipoarabinomannan Assay Using Urine Samples from HIV-infected tuberculosis Patients

4.1. INTRODUCTION

The clinical utility of the Clearview-TB®-ELISA (Inverness Medical Innovations, USA), a standardised and commercially available lipoarabinomannan (LAM) antigen-detection assay designed for TB diagnostic testing, requires clarification. As previously mentioned, currently used tools such as smear microscopy have a very low sensitivity and culture testing takes several weeks (Pai et al 2006c). Moreover, newer technologies such as the T cell assays are not useful as rule-in tests for the diagnosis of active TB in adults (Dheda et al 2009a) and molecular assays such as NAAT are not widely available in high burden settings (Pai et al 2006c). Thus, there is an ongoing search for a rapid point-of-care (POC) test (Pai et al 2009).

Urine is a sterile and easily obtainable biological fluid that can be assayed. Hence, this test option is appealing, especially if it can be used for diagnosing TB in patients who are sputum-scarce, and even children. Furthermore, a dipstick-friendly prototype of the test has also been developed and is currently undergoing validation testing for use as a POC test.

Although it is known that LAM may be useful in HIV-infected patients (Lawn et al 2009; Shah et al 2009) the precise sub-groups that may benefit from this test in a high burden primary care setting remains unknown. Most TB cases present to and are diagnosed in primary care facilities. Existing data about the performance outcomes of the urine MTB ELISA Test® (Chemogen) in a primary care setting is limited (Reither et al 2009) and urgent clarification about the utility of the urine Clearview® LAM ELISA is required. Furthermore, there is currently no published data on LAM-related outcomes

stratified by CD4 count in unselected TB suspects from a primary care setting. Previous studies that have stratified the outcome of the LAM test result by CD4 count are limited (Lawn et al 2009; Shah et al 2009). Thus, the aim of the study was to evaluate performance outcomes using the Clearview-TB® LAM ELISA on urine samples from TB suspects recruited at primary care facilities in a high burden setting, and to determine the diagnostic accuracy of urine LAM in HIV-infected patients stratified by CD4 T cell categories.

University of Cape Town

4.2. HYPOTHESIS AND AIMS:

Hypothesis:

The Clearview[®] LAM ELISA may be a useful rapid tool for tuberculosis diagnosis in HIV-infected TB suspects attending primary care facilities in a high burden setting.

Aims:

1. To evaluate performance outcomes of the Clearview[®] LAM ELISA when using urine samples in HIV-infected and uninfected patients with suspected TB.
2. To determine the diagnostic accuracy of urine LAM in HIV-infected patients stratified by CD4 T cell categories.

4.3. MATERIALS AND METHODS

4.3.1. Participants and study design

Five-hundred TB suspects were consecutively recruited from primary care facilities (as mentioned in Chapters 2 and 3), which operate within the Cape Town Metro Region (South Africa). Clinical, physical and demographic information was recorded for each subject on detailed case record forms. All subjects signed informed consent and the study was approved by the University of Cape Town Human Research Ethics Committee. After appropriate counselling an HIV test was conducted and for the participants who were HIV-infected, a CD4 count was also determined. Blood, saliva, sputum and urine samples were collected from each subject and transported to the Lung Infection and Immunity Unit within 3-4 hours for further testing. For the current study, the urine samples were required for LAM detection testing. LAM detection testing was also conducted on the sputum samples. However, only the urine data is reported in this dissertation, please refer to our published manuscript for the full comprehensive study (Dheda et al 2010a).

Two sputum samples were collected for routine fluorescence smear microscopy after centrifugation, and these samples underwent culture testing for *M.tb* (BACTEC MGIT 960, BD Diagnostics, USA). Standard chest radiography and a urine dipstick test (UriCHECK 9, RapiMed Diagnostics, South Africa) were conducted to assess for proteinuria and leukocytosis. Based on the clinical, microbiological and radiological findings, and the overall impression of the attending physician, each patient was allocated to one of four diagnostic categories (outlined in chapter 2, pp.50-53) by the study investigator, who was blinded to the LAM results. In order to avoid incorporation bias, the LAM ELISA results were not taken into account when determining the final TB status. Results of patients with an uncertain

diagnosis were considered as indeterminate and were thus excluded from the analysis. TB treatment was at the discretion of the attending physician.

4.3.2. Laboratory methods:

4.3.2.1. Detection of LAM in urine samples using the Clearview-TB®-ELISA

The urine samples, which were collected in sterile containers, were processed according to the manufacturer's instructions (Clearview TB ELISA [Package Insert]. Inverness Medical Innovations 2009). Briefly, a 1.5ml aliquot of each unprocessed urine specimen was collected and stored at -80°C. A further 8-10ml of the remainder of the urine was heated to 95-100°C for half an hour in a water bath, cooled to room temperature and then centrifuged at 3000rpm for 15 minutes. After centrifugation, 2x1.5ml aliquots of the supernatants were collected and frozen at -80° prior to batching for LAM-ELISA testing. The supernatants were thawed within 2-3 weeks of storage, and further processed according to the manufacturer's instructions. Briefly, the samples, along with the LAM antigen detection kit reagents were left to equilibrate to room temperature. The samples and controls (1 positive control and 1 negative control) were carefully mixed by inverting the tubes several times without creating air bubbles. 100µl of the positive control, negative control and patient samples were added into the appropriately labelled duplicate wells of the Clearview TB® LAM-ELISA kit plate. A new pipette tip was used for each sample. The plate was then sealed with the self-adhesive film and incubated at 20-25°C for 60 minutes. Immediately after the 60 minute incubation, the adhesive film was removed and the contents of the wells were decanted by shaking into a collection basin and then firmly tapping the plate on a paper towel several times to remove any residual contents from the wells. The first manual wash steps was performed with a pre-prepared wash solution by carefully adding 250µl of the solution to each well and vigorously shaking the solution from the wells into a collection basin prior to firmly tapping the plate on a paper towel to remove residual liquid, and

repeating 3-4 times. After the first wash step, 100µl horse radish peroxidase (HRP) conjugate was added to each well in the same order and at the same rate that the samples were added. The plate was promptly covered with self-adhesive sealing tape and incubated at 20-25°C for 60 minutes. After incubation the self-adhesive tape was removed and the HRP conjugate decanted into a collection basin. Another wash step was performed (as described above). After this second wash step, 100µl of chromogenic substrate solution, tetramethylybenzidine (TMB), was added to each well in the same order and at the same rate that the samples were added. The plate was covered with adhesive film and incubated at 20-25°C for 15 minutes. After the 15 minute incubation, the adhesive tape was removed and 100µl stop solution (1 molar sulfuric acid) was added in the same order and at the same rate that the TMB was added. The plate was shaken gently to mix the reagents and read immediately after development (Anthos Labtec HT3 ELISA plate reader set on 450nm). Duplicate positive and negative controls were performed with each run, and the results were determined as the average of duplicate optical density (OD) readings for each sample minus [the average negative control OD reading plus 0.1]. Please refer to figure 4.1 for a summary flow diagram of the procedure.

4.3.2.2. Interpretation of the results

A cut-off value of zero was used for negative and positive results. A result was considered as negative when the average sample OD was less than the cut-off value, and positive when the average sample OD was greater than or equal to the cut-off value.

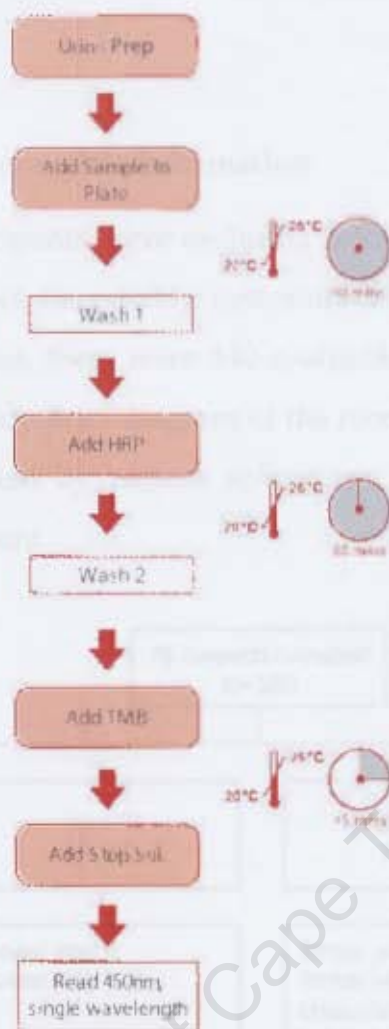


Figure 4.1. Flow diagram illustrating the steps of the Clearview-TB®-LAM ELISA procedure.

4.3.3. Statistical methods

The sample size calculations for the current study were based on the assumption that 30% of the 500 TB suspects would have definite TB, and thus the resulting numbers would be sufficient to estimate the Clearview TB® ELISA test sensitivity and specificity with a +/- 4% precision. The sensitivity, specificity, negative and positive predictive values, along with the 95% confidence intervals (CI), was calculated. Stata 1C version 10 (Stata Corp, Texas, USA) was used for Chi-square analyses. In addition, multivariable, logistic regression analysis was conducted using culture-positive TB as the reference standard to determine the incremental value of urine LAM over sputum smear alone.

4.4. RESULTS

4.4.1. Clinical and demographic information

Sixty (12%) of the participants were excluded from further analysis because their TB status could not be reliably determined (see TB case definitions, chapter 2, pp.47-48). Thus, there were 440 evaluable subjects. Please refer to figure 4.2, which is a study flow diagram of the recruitment that includes the sample numbers stratified by patient subgroups, smear microscopy, HIV status and CD4 T cell count.

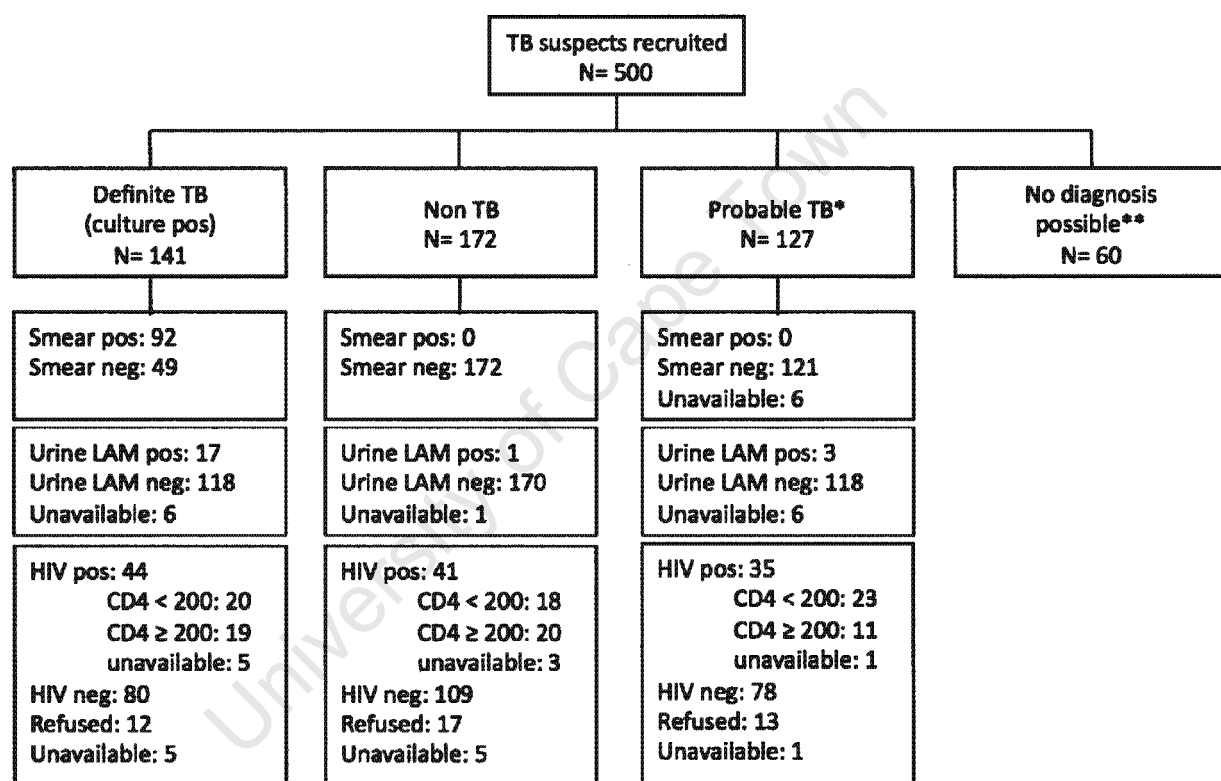


Figure 4.2. Flow chart of patients recruited to the study stratified by patient subgroup, smear microscopy, HIV status, and CD4 T cell count.

The clinical and demographic information of the 440 evaluable TB suspects is shown in table 4.1. The participants were predominantly male (291/440; 66.1%) and the median age was 41 (range 18-84) years. HIV results were available for 90% (398/440) of participants, because 42 refused an HIV test and there were unknown results/no results for 11 subjects. Of those who were tested for HIV and had a result, 31% (123/398) were found to be HIV-

infected. However, only 111/123 (90%) of these infected subjects had available results for CD4 count. The median CD4 count in this group was 177 cells/mm³.

Table 4.1. Clinical and demographic information of the 440 evaluable TB suspects stratified by HIV status.

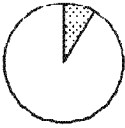

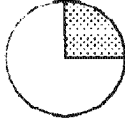
	N (%)	HIV Positive** (%)	HIV Negative** (%)	P-value
Total TB suspects included	440 (100)	120 (27.3)	267 (60.7)	
Age, mean (SD)	41 (12)	38 (10)	42 (13)	0.0006
Sex				
Male	291(66.1)	61 (21)	195 (67)	<0.001
Female	149 (33.9)	59 (39.6)	72 (48.3)	
Race				
Black	314 (71.4)	97 (30.9)	178 (56.7)	0.015
Mixed ancestry (Coloured)	118 (26.8)	22 (18.6)	82 (69.5)	
Caucasian	8 (1.8)	1 (12.5)	7 (87.5)	
Weight in Kg, mean (SD) (n=410)*	61.2 (11.9)	58.3 (10.1)	62.0 (12.2)	0.005
Previous TB				
Yes	161 (36.6)	46 (28.6)	101 (62.7)	0.924
No	279 (63.4)	74 (26.5)	166 (59.5)	
Current Smoker (n= 437)*				
Yes	252 (57.3)	55 (21.8)	170 (67.5)	0.001
No	185 (42.0)	65 (35.1)	95 (51.4)	
Urine protein (n= 384)*				
+	32 (7.3)	13 (40.6)	18 (56.3)	0.362
++	15 (3.4)	4 (26.7)	10 (66.7)	
+++	3 (0.7)	0 (0)	3 (100)	
negative	334 (75.9)	91 (27.2)	210 (62.9)	
CD4 count, median (cells/mm ³) (n=111)*	-	177	-	-

* Irreconcilable ambiguity on the request form precluding processing of sample, or patient consented but did not turn up for the test.

** Excludes 42 (9.5%) patients who refused HIV testing and 11 (2.5%) patients who had no data.

4.4.2. Sensitivity, specificity of urine LAM versus smear microscopy in different categories of TB suspects, and the association of urine LAM with HIV status and urine protein

The sensitivity and specificity of urine LAM alone, smear microscopy alone, and then urine LAM in combination with smear microscopy is shown in figure 4.3.

Definite and non TB groups only	All		HIV Negative		HIV Positive		HIV positive CD4 <200 cells/ μ l	
	Sens. (95%CI)	Spec. (95% CI)	Sens. (95%CI)	Spec. (95% CI)	Sens. (95%CI)	Spec. (95% CI)	Sens. (95%CI)	Spec. (95% CI)
Sputum smear	65 (57, 72)	100 (98, 100)	72* (61, 81)	99 (95, 100)	49* (35, 63)	100 (91, 100)	37** (19, 59)	100 (82, 100)
Urine LAM	13 (8, 19)	99 (97, 100)	6# (3, 14)	99 (95, 100)	21# (11, 35)	100 (91, 100)	37 (19, 59)	95 (84, 99)
Sputum smear and/or urine LAM	68 (60, 75)	99 (97,100)	72 (61, 81)	100 (97,100)	58 (43, 72)	100 (91,100)	53** (32, 73)	100 (83,100)
	PPV (95%CI)	NPV (95% CI)	PPV (95%CI)	NPV (95% CI)	PPV (95%CI)	NPV (95% CI)	PPV (95%CI)	NPV (95% CI)
Sputum smear	100 (95,100)	78 (72,83)	98 (91,100)	83 (76,88)	100 (85,100)	65 (53,76)	100 (65,100)	60 (42,75)
Urine LAM	94 (74,99)	59 (53,64)	83 (44,97)	59 (52,66)	100 (70,100)	55 (43,65)	78 (45,94)	76 (63,86)
Sputum smear and/or urine LAM	99 (94,100)	79 (73,84)	100 (94,100)	83 (76,88)	100 (87,100)	69 (57,80)	100 (72,100)	67 (48,81)
% of sputum smear neg definite TB cases detected by urine LAM	8.6 		0		17.6 		25.4 	

*p=0.001; #p=<0.001; **p=0.07

Pie chart segments represent percentages of patients that were smear positive (shaded) smear negative (non-shaded) and urine LAM positive but smear negative (speckled).

Figure 4.3. The sensitivity, specificity (including 95% confidence intervals (%); top panel of table) and positive and negative predictive value (middle panel of table) of smear-microscopy alone, urine LAM alone, and a combination of urine LAM and smear-microscopy (using culture-positivity as a reference standard). The bottom panel of the table shows the test sensitivity in smear negative culture positive TB patients. For sensitivity calculations the definite TB group (n=141) was used. Specificity calculations were performed using the non-TB group (n=172) as a reference.

In the unselected TB patients, HIV co-infected patients and those who had a CD4 count <200 cells/mm³, smear microscopy had a sensitivity of 65%, 49% and 37%, respectively. By contrast, there were comparative sensitivities for urine LAM in the same groups (13%, 21% and 37%, respectively). Whilst the sensitivity of smear microscopy decreased significantly with increasing

immune paresis (HIV-infected vs. HIV-uninfected and CD4<200 vs. CD4≥200), the sensitivity of urine LAM increased significantly (Figure 2; p<0.003). Except for patients who had a CD4 count <200 cells/mm³, smear microscopy was significantly more sensitive than urine LAM in all of the other groups where sensitivities were equivalent. However, in this group of patients with the CD4 count <200 cells/mm³ the urine LAM assay and smear microscopy appeared to identify different patients. In this group of TB patients with advanced HIV a combination of urine LAM and smear microscopy showed a sensitivity of 53% compared to the lower sensitivity (37%) for smear-microscopy alone. However, this additive value of urine LAM did not reach statistical significance (p=0.07). Comparative multivariable Receiver Operating Characteristic (ROC) curve analysis could not be performed because of the smaller number of HIV-infected patients in the sub-group with a CD4 count <200 cells/mm³. Collectively, 25% of HIV-infected smear negative TB patients with a CD4 count < 200 cells/mm³ had a positive urine LAM test. In the smear negative culture positive group, a significantly greater percentage of patients with CD4 count <200 cells/mm³ were urine LAM positive compared to those with CD4 count ≥200 cells/mm³ (p = 0.003).

Using the non-TB group as a reference, the specificity of urine LAM and smear microscopy in the different subgroups is shown in figure 4.3. There was high specificity for urine LAM (95 to 100%) in all subgroups but specificity was comparatively lowest in the advanced HIV (CD4<200 cells/mm³) subgroup. However, this was not statistically different from other subgroups).

As shown in figure 4.3, urine LAM positivity was significantly higher in HIV-infected subjects versus those not infected with HIV (21% vs. 6%, respectively; p<0.001) and in those with a CD4 count <200 cells/mm³ versus ≥200 cells/mm³ (37% vs. 0%, respectively; p=0.003). In the univariable analysis, the positive urine LAM result was directly associated with HIV

positivity (odds ratio=4.75; p=0.002), but inversely associated with low weight (odds ratio=0.94; p=0.01) and previous TB (odds ratio=0.09; p=0.02). In contrast, in the multivariable analysis, there was an independent association between LAM positivity and HIV-infected status (odds ratio=3.2; p=0.04) and LAM positivity and lack of previous TB (odds ratio=0.09; p=0.02).

In the culture positive HIV-infected TB patients (n=36) there was no significant difference in the LAM positivity between the proportion of patients who had proteinuria and those who did not (6/24 vs. 2/12).

University of Cape Town

4.5. DISCUSSION

Due to the poor sensitivities (13% and 21%, respectively) of the Clearview TB® LAM ELISA in unselected HIV-infected and uninfected suspects from a high burden primary care setting, urine LAM seems to have limited clinical utility. However, because the specificity of the assay in the diagnostically challenging smear negative/culture positive group (n=19) with a CD4 count <200 cells/mm³ was high (95% specificity and 37% sensitivity), urine LAM may possibly be deemed as a reasonable rule-in test for active TB within this sub-group. Thus, even though the sensitivity in this sub-group is low, the high specificity, reasonable cost of the kit, rapid generation of results within a day (potentially even faster if the LAM urine strip can be validated and commercialised as a POC test), the lack of alternative rapid test options and the continuous diagnostic conundrum presented by this particular sub-group of patients, makes urine LAM an attractive test option. Moreover, established PCR-based TB tests such as NAAT have a high specificity and a low sensitivity (~30%) within a similar range when used clinically as rule-in tests for extra-pulmonary TB (Alvarado-Esquivel et al 2009).

Other studies have shown that urine LAM sensitivity in smear negative TB patients varied between 28%-56% when using the pre-commercial version (Lawn et al 2009; Reither et al 2009; Shah et al 2009). However, as previously mentioned, urine LAM outcomes stratified by CD4 count were not available in two of these studies, and specificity was suboptimal in both (Mutetwa et al 2009; Reither et al 2009). Thus, there are no existing data about urine LAM outcomes in specific CD4 categories in patients who present to primary care clinics with suspected TB. Given the availability of rapid HIV testing, and POC CD4 tests in the near-future, if additional studies confirm our findings there is potential to develop an algorithm where patients who usually present the existing clinical TB diagnostic conundrum (smear negative, HIV positive and a CD4 count <200 cells per mm³) may be ideal candidates for the urine LAM assay (especially the POC version). Our work has identified this

specific sub-group that should now be focused on in future studies in order to clarify the diagnostic utility of the urine LAM assay in these patients.

The high specificity of the urine LAM assay, even though it was lower in patients with advanced HIV, was similar to that of two other studies (Lawn et al 2009; Shah et al 2009). The first study was conducted in hospitalised TB patients in a high HIV prevalence setting (Shah et al 2009) and the second study involved individuals being screened for TB prior to antiretroviral therapy (Lawn et al 2009). Similarly, the high specificity in these studies also contrasts with the poorer specificity (~88%) in other studies (Daley et al 2009; Mutetwa et al 2009; Reither et al 2009). This phenomenon may possibly be explained by undiagnosed occult TB disease, which in turn may even justify the lower specificity in the patients with advanced HIV in our study. Furthermore, possible detection of antigen from LTBI subjects, the presence of environmental pathogens or contamination by non-bacterial species (for example, *Candida*) may also be plausible explanations. The non-bacterial species situation may be possible given our findings that several organisms, including *Candida* species, cross-react with the polyclonal LAM antibodies within the assay (Dheda et al 2010a). However, in our study urine was collected at the clinic in sterile containers, but the collection methods used in the other studies have never been clearly described.

As previously mentioned, the urine Clearview® LAM ELISA used in this study is the current commercially-available version. Although the polyclonal antibody used in this ELISA is the same as the one used in the pre-commercial MTB LAM ELISA, there are differences in the manufacturing technology and protocols used. It is unlikely that these differences make the different versions incomparable, but this possibility cannot be dismissed given that several technical factors may impact on ELISA test performance (Sittampalam et al 1996).

A limitation of this study is the low number of HIV-infected definite TB cases who were smear negative and had a CD4 count <200 cells/mm³ (n=19).

However, this is the first study to stratify LAM-related outcomes by CD4 count in unselected TB suspects from a primary care setting. Although urine LAM used in conjunction with smear microscopy may be more sensitive than smear microscopy alone in patients with low CD4 counts, the differences between the two were not statistically significant (ie. urine LAM + smear microscopy vs. only smear microscopy; $p=0.07$). However, this could be due to type II error and the low sample numbers in the low CD4 subgroup. Larger studies are required to recruit a sufficient number of patients with smear negative definite TB and HIV-infection (with a low CD4 count) in order to achieve reliable performance outcome estimates. However, given that it was not possible to clearly evaluate the outcomes in this particular sub-group, our findings can be regarded as preliminary.

University of Cape Town

CHAPTER 5

Diagnostic Utility of the Xpert® MTB/RIF Assay using Bronchoalveolar Lavage Fluid

5.1. INTRODUCTION

One of the many problems that clinicians are faced with when working with tuberculosis cases is the diagnosis of sputum-scarce and smear-negative TB, because the yield of existing rapid diagnostic tools is low, and it is challenging to obtain representative biological samples.

As outlined earlier (please refer to chapter 1, section 1.7), existing NAAT platforms for the detection of *M.tb* are technically demanding and labour-intensive (Vanechoutte & Van Eldere 1997), and problems of sensitivity remain. NAATs have a sensitivity of ~40% in smear negative pulmonary TB) (Ling et al 2008). Thus, a more sensitive, user-friendly test option, such as the Xpert® MTB/RIF Assay (Cepheid 2009), would be very useful.

Helb and colleagues performed the first analysis of the Cepheid Gene Xpert System's MTB/RIF assay. They evaluated the diagnostic utility of the system using sputum samples and showed that it was highly sensitive and simple-to-use (Helb et al 2009). However, in sputum-scarce and smear negative patients representative samples still need to be obtained in order to conduct diagnostic tests. To our knowledge, there are currently no published data evaluating the diagnostic utility of the Xpert® MTB/RIF assay using BAL fluid.

5.2. HYPOTHESIS AND AIM

Hypothesis

The Xpert® MTB/RIF assay, when using bronchoalveolar lavage fluid, may be a useful rapid tool for tuberculosis diagnosis in sputum-scarce or smear negative patients.

Aim:

To evaluate the diagnostic utility of the Xpert® MTB/RIF Assay in a group of pulmonary TB suspects, who are unable to expectorate sputum (sputum-scarce) or who are smear negative, using bronchoalveolar lavage fluid.

University of Cape Town

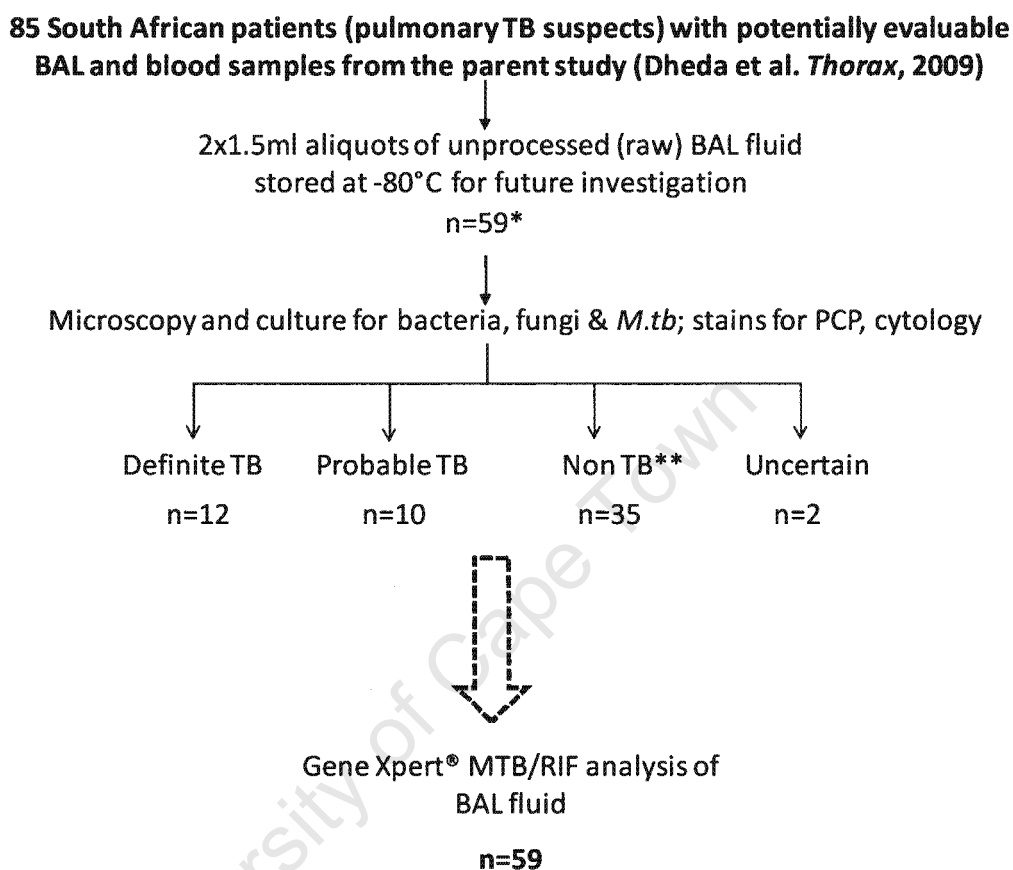
5.3. MATERIALS AND METHODS

5.3.1. Participants and study design

The subjects for the study were recruited as part of a larger, ongoing parent study, which involved the investigation of antigen-specific interferon gamma (IFN- γ) responses to the RD-1 antigens ESAT-6 and CFP-10 (T-SPOT.TB and QuantiFERON-TB-Gold-In-Tube), HBHA and PPD, using BAL cells from 91 consecutively recruited South African patients (Groote Schuur Hospital, Cape Town) with suspected pulmonary TB who were unable to expectorate sputum (sputum-scarce) or who were smear negative (Dheda et al 2009c). Six patients were excluded from the parent study analysis, thus evaluable samples were obtained for 85 patients. Furthermore, unprocessed (raw) BAL fluid stored at -80°C was only available from 59 of the subjects. Thus, the present study group comprised 59 pulmonary TB suspects who were unable to expectorate sputum (sputum-scarce) or who were smear-negative. Archived samples from this group of patients were used. All samples were processed at the Lung Infection and Immunity Unit Laboratory (Groote Schuur Hospital, H 47 Old Main Building, Observatory, Cape Town).

Ethical approval was obtained from the University of Cape Town (UCT) Health Sciences Faculty Research Ethics Committee. All subjects provided informed consent. Detailed clinical and demographic data (including patient history, testing for HIV infection, and chest X-ray) was recorded, and bronchoscopy was performed, under local anaesthesia and conscious sedation, by an experienced pulmonologist. Approximately 150–300 ml of sterile saline was used to lavage a radiologically involved lung segment, and the aspirate (100–150 ml) was collected in a sterile glass bottle. Aliquots of BAL fluid were sent for routine smear microscopy, Gram and fungal staining, cell differential count, cytology for malignant cells and *Pneumocystis jirovecii*, bacterial and fungal culture, and culture for *M.tb*

(MGIT 960). When appropriate, 3–5 trans-bronchial biopsies were performed. The study flow diagram is shown in figure 5.1.



*26/85 unprocessed BAL fluid not stored due to limited availability of BAL fluid (the investigator was blinded to patient details, thus there was no systematic distribution of the groups analysed with respect to clinical background).

** 1 AIP, 1 anthrosilicosis, 2 asthma, 2 bronchiectasis, 2 carcinoma, 1 cryptococcus, 5 bacterial infection, 1 infection/Lymphoid interstitial pneumonia (LIP), 1 LIP, 1 metastatic breast carcinoma, 1 non small cell lung cancer, 1 small cell lung cancer, 1 NSIP (non-specific interstitial pneumonia/fibrosis (NSIP), 1 Pneumocystic Carinii Pneumonia (PCP), 1 Pneumonia, 1 probable eosinophil pneumonia, 1 pulmonary oedema, 1 respiratory bronchiolitis-associated interstitial lung disease (RBILD), 1 Staphylococcus infection/LIP, 1 renal cell carcinoma, 1 sarcoidosis, 2 squamous cell carcinoma, 1 chronic obstructive pulmonary disease (COPD), 4 non TB with no cause identified.

Figure 5.1. Study flow diagram for the investigation of the diagnostic utility of the Xpert® MTB/RIF assay using bronchoalveolar lavage fluid

5.3.2. Laboratory methods:

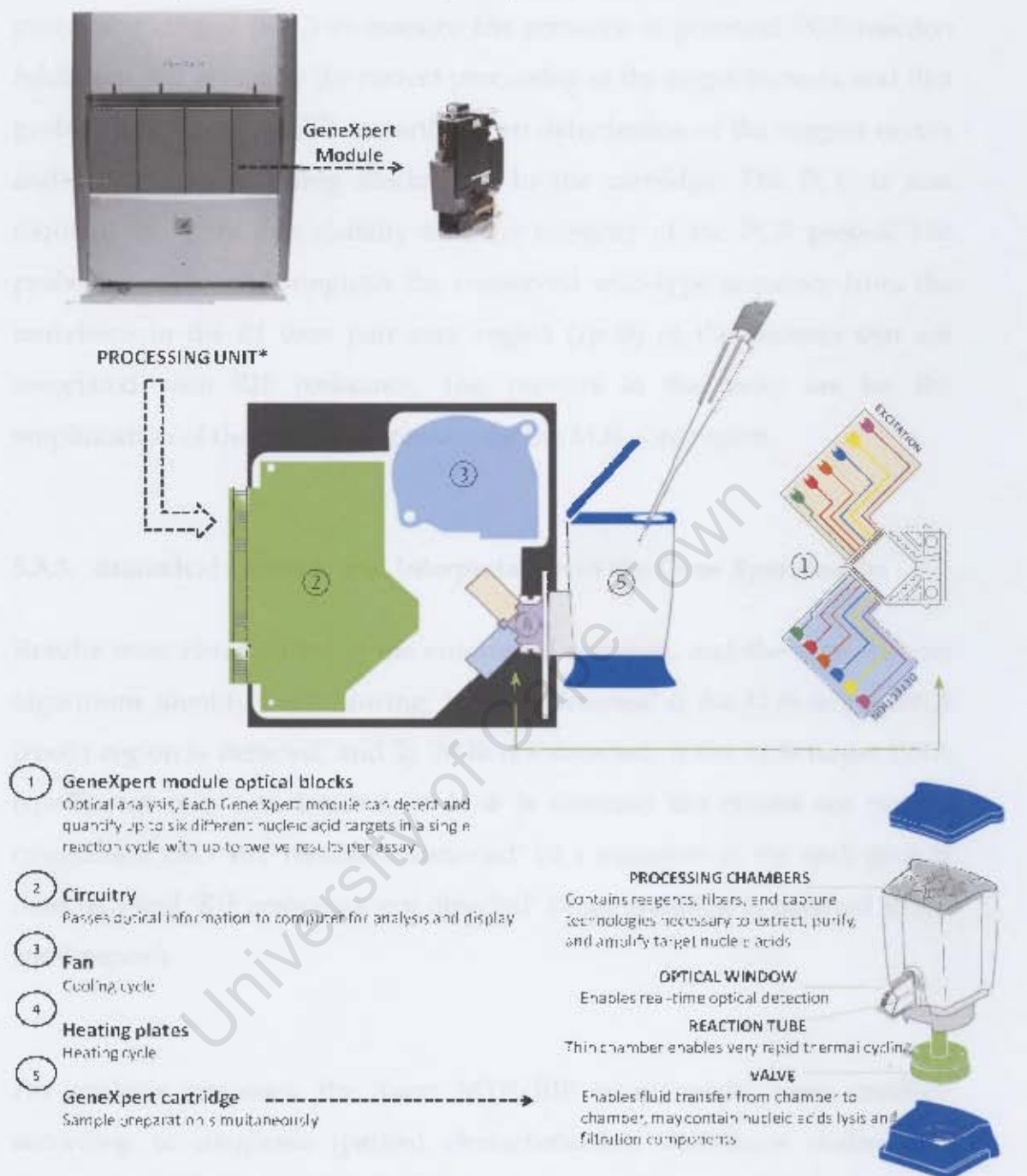
The Xpert MTB/RIF test was conducted according to the manufacturer's instructions (Xpert® MTB/RIF [Package Insert] 2009). Briefly, the frozen, unprocessed samples were thawed and immediately processed. 1ml of BAL fluid was added to 2mL of the Xpert MTB/RIF Sample Reagent (SR), which contains sodium hydroxide and isopropanol. The BAL/SR mixture was vigorously shaken and then incubated at room temperature for a total of fifteen minutes with a second shake occurring at 10 minutes. 2mL of the digested mixture was transferred to the Xpert MTB/RIF cartridge. The automated steps of the procedure were initiated by placing the loaded assay cartridge into the GeneXpert instrument module and then selecting the "*M. tuberculosis* Xpert-Rif" automated detection test option from the included software. The test was started within 30 minutes of adding the sample to the cartridge.

5.3.2.1. The Gene Xpert Dx System: Principle of the procedure

The Gene Xpert Dx system is a fully integrated, automated system for sample processing, nucleic acid amplification, and detection of the target sequences (all in a single hands-free step) in simple and complex specimens using a combination of real-time PCR and reverse transcriptase PCR. The system consists of the Gene Xpert instrument, which needs to be attached to a personal computer and a barcode scanner.

The pre-loaded software for running the Gene Xpert assay (different import assay definition files are available, for example the Xpert MTB/RIF assay) and viewing the results is also required. Single-use disposable Gene Xpert cartridges (containing the PCR reagents for the PCR process) for use with the Gene Xpert system are self-contained, thus eliminating cross-contamination

between the samples. Please refer to figure 5.2, which is a flow diagram of the main components of the Gene Xpert system and their function.



* A four-site GeneXpert System can be configured with 1, 2, 3 or 4 modules. For higher throughput applications, a 16-site system can be configured with 4, 8, 12, or 16 modules. Systems can be easily upgraded with additional modules.

Figure 5.2. Simplified flow diagram showing the main components of the Gene Xpert system, and the function of each component.

The Xpert MTB/RIF assay includes reagents for the detection of *M.tb* and rifampicin resistance, as well as the following internal controls: i) a sample processing control (SPC) to monitor the presence of potential PCR-reaction inhibitors and to ensure the correct processing of the target bacteria, and ii) a probe check control (PCC) to verify when dehydration of the reagent occurs and the PCR tube filling mechanism in the cartridge. The PCC is also required to verify dye stability and the integrity of the PCR probes. The probes are able to distinguish the conserved wild-type sequence from the mutations in the 81 base pair core region (*rpoB*) of the bacteria that are associated with RIF resistance. The primers in the assay are for the amplification of the *rpoB* gene containing the *M.tb* core region.

5.3.3. Statistical methods and interpretation of the Gene Xpert results

Results were obtained using the automated software, and the data analysis algorithms identify the following: 1) '*M.tb* detected' if the *M.tb* target DNA (*rpoB*) region is detected, and 2) '*M.tb* not detected' if the *M.tb* target DNA (*rpoB*) region is not detected. If *M.tb* is detected the results are further categorised into 'RIF resistance detected' (if a mutation in the *rpoB* gene is detected) and 'RIF resistance not detected' (if no mutation is detected in the *rpoB* region).

For analysis purposes, the Xpert MTB/RIF assay results were stratified according to diagnosis (patient characterisation definitions outlined in chapter 1). Confirmed culture positivity and or/biopsy proven TB was used as the reference standard. The sensitivity, specificity, positive and negative predictive values of the Xpert™ MTB/RIF assay were then calculated using the relevant formulae (referred to later).

5.4. RESULTS

The clinical and demographic characteristics of the participants is shown in table 5.1.

Table 5.1. Demographic and clinical characteristics of TB suspects and patients who had an unprocessed (raw) BAL fluid sample stored at -80°C for further analysis (n=59)

Characteristics	No. (% of total)
Median Age, years (range)	44 (18-79)
Gender	
Male	30 (51)
Female	29 (49)
Ethnic origin	
Black African	25 (42)
Caucasian	2 (3)
Coloured	32 (54)
BCG vaccinated	
No BCG vaccination	5 (9)
Former BCG vaccination	12 (20)
BCG vaccination status not known	42 (71)
M. tuberculosis BAL culture result	
Positive	5 (9)
Negative	41 (69)
No result *	13 (22)
Previous TB	23 (39)
Known TB contact	9 (15)
Current smoker	17 (29)
HIV status	
HIV-infected	21 (36)
HIV uninfected	31 (52)
Refused test	4 (7)
Unknown/no result	3 (5)

* If the culture result was not available, the diagnosis was based on histology in keeping with TB (caseous necrosis or acid-fast bacilli with or without granuloma formation)

There was an almost-equal distribution of males and females (51% and 49%, respectively), and the population was predominantly Black African (54%). A total of 21/59 (36%) of the subjects were HIV-infected and 23/59 (39%) had a

known history of previous TB. The BCG vaccination status was unknown for the majority of the group (42/59; 71%).

Please refer to table 5.2, which displays the breakdown of the Gene Xpert results stratified by confirmed diagnoses. The detailed 2x2 (contingency) table is also shown (table 5.3).

Table 5.2. Gene Xpert results stratified by confirmed diagnoses. There were no indeterminate Xpert MTB/RIF results. Hence, all of the results were evaluable (n=59).

	DefiniteTB	Probable TB	Non TB	Uncertain	Total
MTB/RIF Xpert assay					
Positive	7*	1	1**	0	9
Negative	5	9	34	2	50
Total	12	10	35	2	59

* RIF resistance was detected on the Gene Xpert system in the sample of 1/7 definite TB cases.

** 1 Non TB patient was diagnosed with LIP. However, the gene Xpert assay detected RIF resistant *M.tb* in the patient's sample

(Total *M.tb* RIF resistance detected=2/59)

Table 5.3. 2x2 tables of Gene Xpert results stratified by confirmed diagnoses

A.	N=59		Definite TB (n=12)	
			Yes	No
	MTB/RIF Xpert	<i>M.tb</i> detected	7 (TP)	2 (FP)
		<i>M.tb</i> not detected	5 (FN)	45 (TN)
B.	N=59		Probable TB (n=10)	
			Yes	No
	MTB/RIF Xpert	<i>M.tb</i> detected	1 (TP)	8 (FP)
		<i>M.tb</i> not detected	9 (FN)	41 (TP)
C.	N=59		Definite + Probable TB (n=22)	
			Yes	No
	MTB/RIF Xpert	<i>M.tb</i> detected	8 (TP)	1 (FP)
		<i>M.tb</i> not detected	14 (FN)	36 (TP)
D.	N=59		Non TB (n=35)	
			Yes	No
	MTB/RIF Xpert	<i>M.tb</i> detected	1 (FP)	8 (TP)
		<i>M.tb</i> not detected	34 (TN)	16 (FN)

Key: TP=True positives, TN=True negatives, FP=False positives, FN=False negatives.

Sensitivity measures the proportion of actual positives which are correctly identified as such. Thus, this would be calculated using the definite TB group (sensitivity= TP/(TP+FN).

Specificity measures the proportion of negatives which are correctly identified as such. Thus, this would be calculated using the non TB group (specificity=TN/(TN+FP).

Positive predictive value (PPV) or precision rate, or post-test probability of disease, is the proportion of patients with positive test results who are correctly diagnosed: PPV=TP/(TP+FP).

Negative predictive value (NPV) is the proportion of patients with negative test results who are correctly diagnosed: NPV= TN/(TN+FN).

The results revealed a sensitivity of 58% (7/12) in patients with definite TB (see table 5.4 below). The remaining 5/12 of these definite TB patients, who were found to be Gene Xpert negative (*M.tb* not detected), had the following HIV test results: 1 was HIV-infected, 2 were HIV-uninfected and the HIV results were unknown for 2 patients. Furthermore, only 1 had a known history of previous TB. However, all had positive QFT-GIT results, suggesting LTBI.

Table 5.4. Sensitivity, Specificity, Positive and Negative Predictive Values for the evaluation of the diagnostic utility of the Xpert MTB/RIF assay using BAL fluid.

Sample type	Sensitivity in Definite TB (culture- or biopsy-proven)	Positive Predictive Value	Negative Predictive Value	Sensitivity in Smear +ve Culture +ve	Sensitivity in Smear-ve Culture +ve	Sensitivity in Clinical TB (Definite + Probable TB)	Specificity in Non-TB	Sensitivity of smear	Specificity of smear
BAL fluid	58% (7/12)	78% (7/9)	90% (45/50)	100% (3/3)	N/A*	36% (8/22)	97% (34/35)	42% (5/12)	100% (35/35)

* Not applicable: n=1

The specificity of the Xpert MTB/RIF assay was high (97%, 34/35). The positive and negative predictive values were 78% and 90%, respectively. The smear sensitivity in the same cohort was 42% (5/12). However, the difference in the sensitivity of the Xpert MTB/RIF assay compared to the sensitivity of smear microscopy was not statistically significant (Fischer's exact p-value=0.684).

5.5. DISCUSSION

The Xpert MTB/RIF Assay offers a promising technical opportunity to bridge the gap in time-to-diagnosis of TB suspects, with the added bonus of simultaneously detecting drug resistance (Helb et al 2009; Pai & Ling 2008).

In this preliminary study using BAL fluid, the Xpert MTB/RIF Assay appears to perform better than smear microscopy (sensitivity of 58% vs. 42%, respectively). However, why did Xpert did not detect *M.tb* in the BAL fluid of the 5/12 definite TB patients? It is known that the Xpert® MTB/RIF assay was originally optimised for use with sputum, and previous studies have reported that the assay has a sensitivity of 90% when using sputum samples from definite TB subjects (C Boehme et al. 2010, NEJM In Press). It should thus be noted that these data using BAL fluid are preliminary, using stored samples, and, moreover, reflect results based on using unprocessed BAL fluid only. Further studies are being conducted using more patients to evaluate the difference between using unprocessed BAL fluid and a centrifuged pellet. We expect the latter to have a higher sensitivity but no data are as yet available. The study by Helb et al. included analytic tests of *M.tb* DNA demonstrating a limit of detection (LOD) of 4.5 genomes per reaction. Furthermore, experiments using sputum spiked with known numbers of *M.tb* colony forming units (CFU) predicted a clinical LOD of 131 CFU/mL. (Helb et al 2009) Thus, our future Gene Xpert investigations will also include a component where the BAL fluid will be spiked with *M.tb* to determine the LOD (threshold of sensitivity) of the Xpert® MTB/RIF assay. Furthermore, we aim to conduct experiments to determine the optimal buffer to sample ratios of different biological fluids.

Although specificity was excellent at 97%, the one subject (1/35) in the non-TB group who had a positive Gene Xpert result (*M.tb* detected), had initially been diagnosed with lymphoid interstitial pneumonia (LIP) [Table 5.2]. Moreover, the *M.tb* in this subject's sample was detected as being RIF resistant on the Xpert assay. Hence, it is in cases such as these where the use

of a rapid novel diagnostic assay, such as Xpert MTB/RIF, would prove useful.

As outlined in the literature review (chapter 1, section 1.7), using various in-house PCR assays, several studies have demonstrated that PCR is more sensitive than smear microscopy in detecting *M.tb* in the BAL fluid of sputum-scarce or smear negative patients with active pulmonary TB (Chen et al 2002; Liam et al 1998; Tan et al 1999; Tueller et al 2005). The main concern in developing countries is the cost and feasibility of new molecular technologies. Although in-house PCR assays are less expensive, there is a risk that the assay has been poorly standardised and is thus unreliable. On the other hand, besides the advantage of obtaining a result in under 2 hours, the Gene Xpert system is a controlled, sensitive and reliable automated system that eliminates the possibility of cross-contamination of samples through the use of the sealed cartridges. Extensive field evaluations in different settings are underway and it remains to be seen whether this promising new rapid NAAT test will survive the rigors of field evaluation, especially in the developing world.

IGRAS

In contrast to low burden settings, there was poor agreement between the changes in RD1 antigen-specific quantitative T cell responses and culture conversion/reversion in TB patients followed-up after 2 months of anti-TB treatment. However, no meaningful conclusions could be made about the responses at 6 months due to the low sample number. The QFT-GIT test appeared to be a helpful treatment-related response indicator closer to the end of treatment completion. HBHA had no discriminatory value.

Although delayed processing (~24 hours), independent of T-Cell *Xtend*, resulted in significant changes in the median spot counts, there was a high agreement between spot counts when samples were processed immediately and after the delay. However, although the use of T-Cell *Xtend* appeared to reduce the number of conversions/reversions this reduction was not found to be statistically significant. Larger studies in different geographical settings and with 3-way parallel sample analyses are now required to clarify the efficacy of T-cell *Xtend* at 32 hours.

Clearview[®] LAM ELISA Assay

The use of the urine LAM ELISA to diagnose unselected TB suspects from a primary care high burden setting does not seem to be useful. It is evident, though, that a targeted approach to using LAM may be useful in those who have advanced HIV infection. However, even in this sub-group, sensitivity is modest. Larger targeted studies, especially in the low CD4 count sub-group in ambulant and hospitalised TB populations from different geographical settings are now required.

Xpert® MTB/RIF Assay

The Xpert MTB/RIF assay appears to be a promising rule-in test, with a specificity of 97%, when using BAL fluid from patients with suspected TB. However, although the sensitivity (almost 60%) is higher than that of smear microscopy, the Xpert MTB/RIF assay in this study did not have incremental value over smear (difference between the sensitivities was not statistically significant). It should be emphasized that these are preliminary data, and larger studies evaluating BAL and other biological samples for use with the MTB/RIF Gene Xpert assay are now required.

University of Cape Town

REFERENCES

(Reference style: 'Ann Rev Medicine')

- T-Cell Xtend™ [mechanism of action], Oxford Immunotec Ltd. 2009.
http://www.youtube.com/watch?v=VAsezk74TkE&feature=youtube_gdata,
Retrieved 25/09/2009.
- World Health Organization (2007) "Tuberculosis" Fact sheet N° 104. Retrieved 12
November 2009.
<<http://who.int/mediacentre/factsheets/fs104/en/index.html>>
- World Health Organization (2009). "Epidemiology" . *Global tuberculosis control: epidemiology, strategy, financing*. pp. 6-33. Retrieved 12 November 2009.
<http://who.int/entity/tb/publications/global_report/2009/pdf/chapter1.pdf>
- Aderem A, Underhill DM. 1999. Mechanisms of phagocytosis in macrophages. *Annual review of immunology* 17:593-623
- Aiken AM, Hill PC, Fox A, McAdam KP, Jackson-Sillah D, et al. 2006. Reversion of the ELISPOT test after treatment in Gambian tuberculosis cases. *BMC Infect Dis* 6:66
- Al-Attayah R, Mustafa AS, Abal AT, Madi NM, Andersen P. 2003. Restoration of mycobacterial antigen-induced proliferation and interferon-gamma responses in peripheral blood mononuclear cells of tuberculosis patients upon effective chemotherapy. *FEMS Immunol Med Microbiol* 38:249-56
- Alvarado-Esquivel C, Garcia-Corral N, Carrero-Dominguez D, Enciso-Moreno JA, Gurrola-Morales T, et al. 2009. Molecular analysis of Mycobacterium isolates from extrapulmonary specimens obtained from patients in Mexico. *BMC Clin Pathol* 9:1
- Arend SM, Andersen P, van Meijgaarden KE, Skjot RL, Subronto YW, et al. 2000. Detection of active tuberculosis infection by T cell responses to early-secreted antigenic target 6-kDa protein and culture filtrate protein 10. *J Infect Dis* 181:1850-4
- Baba K, Sornes S, Hoosen AA, Lekabe JM, Mpe MJ, et al. 2008. Evaluation of immune responses in HIV infected patients with pleural tuberculosis

- by the QuantiFERON TB-Gold interferon-gamma assay. *BMC Infect Dis* 8:35
- Barnes PF. 2001. Diagnosing latent tuberculosis infection: The 100-year upgrade. *American Journal of Respiratory & Critical Care Medicine* 163:807-8
- Blakemore R, Story E, Helb D, Kop J, Banada P, et al. 2010. Evaluation of the analytical performance of the Xpert MTB/RIF assay. *J Clin Microbiol* 48:2495-501
- Boehme C, Molokova E, Minja F, Geis S, Loscher T, et al. 2005. Detection of mycobacterial lipoarabinomannan with an antigen-capture ELISA in unprocessed urine of Tanzanian patients with suspected tuberculosis. *Trans R Soc Trop Med Hyg* 99:893-900
- Boehme CC, Nabeta P, Henostroza G, Raqib R, Rahim Z, et al. 2007. Operational feasibility of using loop-mediated isothermal amplification for diagnosis of pulmonary tuberculosis in microscopy centers of developing countries. *J Clin Microbiol* 45:1936-40
- Bosshard V, Roux-Lombard P, Perneger T, Metzger M, Vivien R, et al. 2009. Do results of the T-SPOT.TB interferon-gamma release assay change after treatment of tuberculosis? *Respir Med* 103:30-4
- Brennan PJ, Nikaido H. 1995. The envelope of mycobacteria. *Annual review of biochemistry* 64:29-63
- Brock I, Munk ME, Kok-Jensen A, Andersen P. 2001. Performance of whole blood IFN-gamma test for tuberculosis diagnosis based on PPD or the specific antigens ESAT-6 and CFP-10. *Int J Tuberc Lung Dis* 5:462-7
- Brunello F, Favari F, Fontana R. 1999. Comparison of the MB/BacT and BACTEC 460 TB systems for recovery of mycobacteria from various clinical specimens. *J Clin Microbiol* 37:1206-9
- Cardona PJ, Soto CY, Martin C, Giquel B, Agusti G, et al. 2006. Neutral-red reaction is related to virulence and cell wall methyl-branched lipids in *Mycobacterium tuberculosis*. *Microbes and infection / Institut Pasteur* 8:183-90

- Carrara S, Vincenti D, Petrosillo N, Amicosante M, Girardi E, Goletti D. 2004. Use of a T cell-based assay for monitoring efficacy of antituberculosis therapy. *Clin Infect Dis* 38:754-6
- Cashmore TJ, Peter JG, van Zyl-Smit RN, Semple PL, Maredza A, et al. 2010. Feasibility and diagnostic utility of antigen-specific interferon-gamma responses for rapid immunodiagnosis of tuberculosis using induced sputum. *PLoS ONE* 5:e10389
- Caviedes L, Moore DA. 2007. Introducing MODS: a low-cost, low-tech tool for high-performance detection of tuberculosis and multidrug resistant tuberculosis. *Indian J Med Microbiol* 25:87-8
- Cepheid. 2009.
- Chan J, Fan XD, Hunter SW, Brennan PJ, Bloom BR. 1991. Lipoarabinomannan, a possible virulence factor involved in persistence of *Mycobacterium tuberculosis* within macrophages. *Infect Immun* 59:1755-61
- Chapman AL, Munkanta M, Wilkinson KA, Pathan AA, Ewer K, et al. 2002. Rapid detection of active and latent tuberculosis infection in HIV-positive individuals by enumeration of *Mycobacterium tuberculosis*-specific T cells. *AIDS* 16:2285-93
- Chegou NN, Walzl G, Bolliger CT, Diacon AH, van den Heuvel MM. 2008. Evaluation of adapted whole-blood interferon-gamma release assays for the diagnosis of pleural tuberculosis. *Respiration* 76:131-8
- Chen NH, Liu YC, Tsao TC, Wu TL, Hsieh MJ, et al. 2002. Combined bronchoalveolar lavage and polymerase chain reaction in the diagnosis of pulmonary tuberculosis in smear-negative patients. *Int J Tuberc Lung Dis* 6:350-5
- Clark-Curtiss JE, Haydel SE. 2003. Molecular genetics of *Mycobacterium tuberculosis* pathogenesis. *Annual review of microbiology* 57:517-49
- Clark SA, Martin SL, Pozniak A, Steel A, Ward B, et al. 2007. Tuberculosis antigen-specific immune responses can be detected using enzyme-linked immunospot technology in human immunodeficiency virus (HIV)-1 patients with advanced disease. *Clinical and experimental immunology* 150:238-44

- Clearview TB ELISA [Package Insert]. Inverness Medical Innovations, Waltham, Massachusetts, USA. 2009.
- Comstock GW. 1978. Tuberculosis in twins: a re-analysis of the Prophit survey. *Am Rev Respir Dis* 117:621-4
- Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, et al. 2003. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. *Archives of internal medicine* 163:1009-21
- Cowley D, Govender D, February B, Wolfe M, Steyn L, et al. 2008. Recent and rapid emergence of W-Beijing strains of *Mycobacterium tuberculosis* in Cape Town, South Africa. *Clin Infect Dis* 47:1252-9
- Daffe M, Draper P. 1998. The envelope layers of mycobacteria with reference to their pathogenicity. *Adv Microb Physiol* 39:131-203
- Daley P, Michael JS, Hmar P, Latha A, Chordia P, et al. 2009. Blinded evaluation of commercial urinary lipoarabinomannan for active tuberculosis: a pilot study. *Int J Tuberc Lung Dis* 13:989-95
- Daley P, Thomas S, Pai M. 2007. Nucleic acid amplification tests for the diagnosis of tuberculous lymphadenitis: a systematic review. *Int J Tuberc Lung Dis* 11:1166-76
- Das S, Paramasivan CN, Lowrie DB, Prabhakar R, Narayanan PR. 1995. IS6110 restriction fragment length polymorphism typing of clinical isolates of *Mycobacterium tuberculosis* from patients with pulmonary tuberculosis in Madras, south India. *Tuber Lung Dis* 76:550-4
- Delogu G, Sanguinetti M, Posteraro B, Rocca S, Zanetti S, Fadda G. 2006. The *hbaA* gene of *Mycobacterium tuberculosis* is specifically upregulated in the lungs but not in the spleens of aerogenically infected mice. *Infect Immun* 74:3006-11
- Dheda K, Davids V, Lenders L, Roberts T, Meldau R, et al. 2010a. Clinical utility of a commercial LAM-ELISA assay for TB diagnosis in HIV-infected patients using urine and sputum samples. *PLoS ONE* 5:e9848
- Dheda K, Lampe FC, Johnson MA, Lipman MC. 2004a. Outcome of HIV-associated tuberculosis in the era of highly active antiretroviral therapy. *J Infect Dis* 190:1670-6

- Dheda K, Pooran A, Pai M, Miller RF, Lesley K, et al. 2007. Interpretation of Mycobacterium tuberculosis antigen-specific IFN-gamma release assays (T-SPOT.TB) and factors that may modulate test results. *J Infect*
- Dheda K, Rook G, Zumla A. 2004b. Peripheral T cell interferon-gamma responses and latent tuberculosis. *Am J Respir Crit Care Med* 170:97-8; author reply 8
- Dheda K, Schwander SK, Zhu B, van Zyl-Smit RN, Zhang Y. 2010b. The immunology of tuberculosis: from bench to bedside. *Respirology (Carlton, Vic)* 15:433-50
- Dheda K, Shean K, Zumla A, Badri M, Streicher EM, et al. 2010c. Early treatment outcomes and HIV status of patients with extensively drug-resistant tuberculosis in South Africa: a retrospective cohort study. *Lancet* 375:1798-807
- Dheda K, Smit RZ, Badri M, Pai M. 2009a. T-cell interferon-gamma release assays for the rapid immunodiagnosis of tuberculosis: clinical utility in high-burden vs. low-burden settings. *Current opinion in pulmonary medicine* 15:188-200
- Dheda K, Udhwadia ZF, Huggett JF, Johnson MA, Rook GA. 2005. Utility of the antigen-specific interferon-gamma assay for the management of tuberculosis. *Current opinion in pulmonary medicine* 11:195-202
- Dheda K, Van-Zyl Smit RN, Sechi LA, Badri M, Meldau R, et al. 2009b. Clinical diagnostic utility of IP-10 and LAM antigen levels for the diagnosis of tuberculous pleural effusions in a high burden setting. *PLoS ONE* 4:e4689
- Dheda K, van Zyl-Smit RN, Meldau R, Meldau S, Symons G, et al. 2009c. Quantitative lung T cell responses aid the rapid diagnosis of pulmonary tuberculosis. *Thorax* 64:847-53
- Dheda K, van Zyl-Smit RN, Sechi LA, Badri M, Meldau R, et al. 2009d. Utility of quantitative T-cell responses versus unstimulated interferon- γ for the diagnosis of pleural tuberculosis. *Eur Respir J* 34:1118-26
- Diaz-Infantes MS, Ruiz-Serrano MJ, Martinez-Sanchez L, Ortega A, Bouza E. 2000. Evaluation of the MB/BacT mycobacterium detection system for

susceptibility testing of *Mycobacterium tuberculosis*. *J Clin Microbiol* 38:1988-9

- Diel R, Loddenkemper R, Meywald-Walter K, Gottschalk R, Nienhaus A. 2008. Comparative Performance of Tuberculin Skin Test, QuantiFERON-TB-Gold In Tube Assay, and T-Spot.TB Test in Contact Investigations for Tuberculosis. *Chest*
- Diel R, Loddenkemper R, Nienhaus A. 2010. Evidence-based comparison of commercial interferon-gamma release assays for detecting active TB: a metaanalysis. *Chest* 137:952-68
- Dinnes J, Deeks J, Kunst H, Gibson A, Cummins E, et al. 2007. A systematic review of rapid diagnostic tests for the detection of tuberculosis infection. *Health technology assessment (Winchester, England)* 11:1-314
- Dominguez J, De Souza-Galvao M, Ruiz-Manzano J, Latorre I, Prat C, et al. 2009. T-cell responses to the *Mycobacterium tuberculosis*-specific antigens in active tuberculosis patients at the beginning, during, and after antituberculosis treatment. *Diagnostic microbiology and infectious disease* 63:43-51
- Dominguez J, Ruiz-Manzano J. 2006. [The tuberculin skin test: time for a change?]. *Archivos de bronconeumologia* 42:47-8
- Dormans J, Burger M, Aguilar D, Hernandez-Pando R, Kremer K, et al. 2004. Correlation of virulence, lung pathology, bacterial load and delayed type hypersensitivity responses after infection with different *Mycobacterium tuberculosis* genotypes in a BALB/c mouse model. *Clinical and experimental immunology* 137:460-8
- Dosanjh DP, Hinks TS, Innes JA, Deeks JJ, Pasvol G, et al. 2008. Improved diagnostic evaluation of suspected tuberculosis. *Annals of internal medicine* 148:325-36
- Drobniewski FA, Caws M, Gibson A, Young D. 2003. Modern laboratory diagnosis of tuberculosis. *Lancet Infect Dis* 3:141-7
- Ernst JD. 1998. Macrophage receptors for *Mycobacterium tuberculosis*. *Infect Immun* 66:1277-81
- Ferguson JS, Voelker DR, McCormack FX, Schlesinger LS. 1999. Surfactant protein D binds to *Mycobacterium tuberculosis* bacilli and

- lipoarabinomannan via carbohydrate-lectin interactions resulting in reduced phagocytosis of the bacteria by macrophages. *J Immunol* 163:312-21
- Ferrand RA, Bothamley GH, Whelan A, Dockrell HM. 2005. Interferon-gamma responses to ESAT-6 in tuberculosis patients early into and after anti-tuberculosis treatment. *Int J Tuberc Lung Dis* 9:1034-9
- Ferrara G, Losi M, D'Amico R, Roversi P, Piro R, et al. 2006. Use in routine clinical practice of two commercial blood tests for diagnosis of infection with *Mycobacterium tuberculosis*: a prospective study. *Lancet* 367:1328-34
- Ferrari G, Langen H, Naito M, Pieters J. 1999. A coat protein on phagosomes involved in the intracellular survival of mycobacteria. *Cell* 97:435-47
- Gatfield J, Pieters J. 2000. Essential role for cholesterol in entry of mycobacteria into macrophages. *Science* 288:1647-50
- Gaynor CD, McCormack FX, Voelker DR, McGowan SE, Schlesinger LS. 1995. Pulmonary surfactant protein A mediates enhanced phagocytosis of *Mycobacterium tuberculosis* by a direct interaction with human macrophages. *J Immunol* 155:5343-51
- Golden MP, Vikram HR. 2005. Extrapulmonary tuberculosis: an overview. *Am Fam Physician* 72:1761-8
- Goletti D, Carrara S, Mayanja-Kizza H, Baseke J, Mugerwa MA, et al. 2008. Response to *M. tuberculosis* selected RD1 peptides in Ugandan HIV-infected patients with smear positive pulmonary tuberculosis: a pilot study. *BMC Infect Dis* 8:11
- Goletti D, Carrara S, Vincenti D, Girardi E. 2007. T cell responses to commercial mycobacterium tuberculosis-specific antigens in HIV-infected patients. *Clin Infect Dis* 45:1652-4
- Goletti D, Carrara S, Vincenti D, Saltini C, Rizzi EB, et al. 2006. Accuracy of an immune diagnostic assay based on RD1 selected epitopes for active tuberculosis in a clinical setting: a pilot study. *Clin Microbiol Infect* 12:544-50

- Gordin FM, Slutkin G, Schechter G, Goodman PC, Hopewell PC. 1989. Presumptive diagnosis and treatment of pulmonary tuberculosis based on radiographic findings. *Am Rev Respir Dis* 139:1090-3
- Greco S, Girardi E, Navarra A, Saltini C. 2006. Current evidence on diagnostic accuracy of commercially based nucleic acid amplification tests for the diagnosis of pulmonary tuberculosis. *Thorax* 61:783-90
- Griffith DE, Kerr CM. 1996. Tuberculosis: disease of the past, disease of the present. *J Perianesth Nurs* 11:240-5
- Grzybowski S, Barnett GD, Styblo K. 1975. Contacts of cases of active pulmonary tuberculosis. *Bull Int Union Tuberc* 50:90-106
- Guyot-Revol V, Innes JA, Hackforth S, Hinks T, Lalvani A. 2006. Regulatory T cells are expanded in blood and disease sites in patients with tuberculosis. *Am J Respir Crit Care Med* 173:803-10
- Hamasur B, Bruchfeld J, Haile M, Pawlowski A, Bjorvatn B, et al. 2001. Rapid diagnosis of tuberculosis by detection of mycobacterial lipoarabinomannan in urine. *J Microbiol Methods* 45:41-52
- Harada N, Higuchi K, Yoshiyama T, Kawabe Y, Fujita A, et al. 2008. Comparison of the sensitivity and specificity of two whole blood interferon-gamma assays for *M. tuberculosis* infection. *J Infect* 56:348-53
- Hawgood BJ. 2007. Albert Calmette (1863-1933) and Camille Guerin (1872-1961): the C and G of BCG vaccine. *Journal of medical biography* 15:139-46
- Helb D, Jones M, Story E, Boehme C, Wallace E, et al. 2009. Rapid detection of *Mycobacterium tuberculosis* and rifampin resistance by use of on-demand, near-patient technology. *J Clin Microbiol* 48:229-37
- Hellyer TJ, Fletcher TW, Bates JH, Stead WW, Templeton GL, et al. 1996. Strand displacement amplification and the polymerase chain reaction for monitoring response to treatment in patients with pulmonary tuberculosis. *J Infect Dis* 173:934-41
- Higgins J, Metcalf JA, Stevens RA, Baseler M, Nason MC, et al. 2005. Effects of lymphocyte isolation and timing of processing on detection of

- CD127 expression on T cells in human immunodeficiency virus-infected patients. *Clinical and diagnostic laboratory immunology* 12:228-30
- Hill PC, Fox A, Jeffries DJ, Jackson-Sillah D, Lugos MD, et al. 2005. Quantitative T cell assay reflects infectious load of Mycobacterium tuberculosis in an endemic case contact model. *Clin Infect Dis* 40:273-8
- Hope JC, Villarreal-Ramos B. 2007. Bovine TB and the development of new vaccines. *Comp Immunol Microbiol Infect Dis*
- Houben EN, Nguyen L, Pieters J. 2006. Interaction of pathogenic mycobacteria with the host immune system. *Curr Opin Microbiol* 9:76-85
- Hougardy JM, Schepers K, Place S, Drowart A, Lechevin V, et al. 2007. Heparin-Binding-Hemagglutinin-Induced IFN-gamma Release as a Diagnostic Tool for Latent Tuberculosis. *PLoS ONE* 2:e926
- Houghton RL, Lodes MJ, Dillon DC, Reynolds LD, Day CH, et al. 2002. Use of multiepitope polyproteins in serodiagnosis of active tuberculosis. *Clinical and diagnostic laboratory immunology* 9:883-91
- Iademarco MF, Castro KG. 2003. Epidemiology of tuberculosis. *Semin Respir Infect* 18:225-40
- Jafari C, Thijsen S, Sotgiu G, Goletti D, Benitez JA, et al. 2009. Bronchoalveolar lavage enzyme-linked immunospot for a rapid diagnosis of tuberculosis: a Tuberculosis Network European Trialsgroup study. *Am J Respir Crit Care Med* 180:666-73
- Janeway CA, Jr., Medzhitov R. 2002. Innate immune recognition. *Annual review of immunology* 20:197-216
- Kang YA, Lee HW, Hwang SS, Um SW, Han SK, et al. 2007. Usefulness of whole-blood interferon- γ assay and interferon- γ enzyme-linked immunospot assay in the diagnosis of active pulmonary tuberculosis. *Chest* 132:959-65
- Kaplan G, Luster AD, Hancock G, Cohn ZA. 1987. The expression of a gamma interferon-induced protein (IP-10) in delayed immune responses in human skin. *J Exp Med* 166:1098-108
- Kaul KL. 2001. Molecular detection of Mycobacterium tuberculosis: impact on patient care. *Clin Chem* 47:1553-8

- Knutson KL, Hmama Z, Herrera-Velitz P, Rochford R, Reiner NE. 1998. Lipoarabinomannan of *Mycobacterium tuberculosis* promotes protein tyrosine dephosphorylation and inhibition of mitogen-activated protein kinase in human mononuclear phagocytes. Role of the Src homology 2 containing tyrosine phosphatase 1. *J Biol Chem* 273:645-52
- Kobashi Y, Mouri K, Yagi S, Obase Y, Fukuda M, et al. 2008. Usefulness of the QuantiFERON TB-2G test for the differential diagnosis of pulmonary tuberculosis. *Internal medicine (Tokyo, Japan)* 47:237-43
- Kobashi Y, Obase Y, Fukuda M, Yoshida K, Miyashita N, et al. 2007. Usefulness of QuantiFERON TB-2G, a diagnostic method for latent tuberculosis infection, in a contact investigation of health care workers. *Internal medicine (Tokyo, Japan)* 46:1543-9
- Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ. 2009. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nature immunology* 10:595-602
- Kubica G, Wayne L. 1984. Clinical microbiology. *The Mycobacteria: a Sourcebook, Part A.* pp. 133-75. New York: Marcel Dekker
- Kumar VA, Abul K.; Fausto, Nelson; & Mitchell, Richard N. 2007. *Robbins Basic Pathology* pp. 516-22 Saunders Elsevier.
- Lalvani A. 2007. Diagnosing tuberculosis infection in the 21st century: new tools to tackle an old enemy. *Chest* 131:1898-906
- Lalvani A, Millington KA. 2008. T-cell interferon-gamma release assays: can we do better? *Eur Respir J* 32:1428-30
- Lalvani A, Nagvenkar P, Udawadia Z, Pathan AA, Wilkinson KA, et al. 2001a. Enumeration of T cells specific for RD1-encoded antigens suggests a high prevalence of latent *Mycobacterium tuberculosis* infection in healthy urban Indians. *J Infect Dis* 183:469-77
- Lalvani A, Pathan AA, McShane H, Wilkinson RJ, Latif M, et al. 2001b. Rapid detection of *Mycobacterium tuberculosis* infection by enumeration of antigen-specific T cells. *Am J Respir Crit Care Med* 163:824-8

- Lawn SD, Edwards DJ, Kranzer K, Vogt M, Bekker L-G, Wood R. 2009. Urine lipoarabinomannan assay for tuberculosis screening prior to ART: diagnostic yield and association with immune reconstitution disease.
- Lee RE, Brennan PJ, Besra GS. 1996. Mycobacterium tuberculosis cell envelope. *Curr Top Microbiol Immunol* 215:1-27
- Liam CK, Chen YC, Yap SF, Srinivas P, Poi PJ. 1998. Detection of Mycobacterium tuberculosis in bronchoalveolar lavage from patients with sputum smear-negative pulmonary tuberculosis using a polymerase chain reaction assay. *Respirology (Carlton, Vic)* 3:125-9
- Ling DI, Flores LL, Riley LW, Pai M. 2008. Commercial nucleic-acid amplification tests for diagnosis of pulmonary tuberculosis in respiratory specimens: meta-analysis and meta-regression. *PLoS ONE* 3:e1536
- Lipscomb MF, Masten BJ. 2002. Dendritic cells: immune regulators in health and disease. *Physiological reviews* 82:97-130
- Liu XQ, Dosanjh D, Varia H, Ewer K, Cockle P, et al. 2004. Evaluation of T-cell responses to novel RD1- and RD2-encoded Mycobacterium tuberculosis gene products for specific detection of human tuberculosis infection. *Infect Immun* 72:2574-81
- Lyashchenko K, Colangeli R, Houde M, Al Jahdali H, Menzies D, Gennaro ML. 1998. Heterogeneous antibody responses in tuberculosis. *Infect Immun* 66:3936-40
- Mack U, Migliori GB, Sester M, Rieder HL, Ehlers S, et al. 2009. LTBI: latent tuberculosis infection or lasting immune responses to M. tuberculosis? A TBNET consensus statement. *Eur Respir J* 33:956-73
- Malik AN, Godfrey-Faussett P. 2005. Effects of genetic variability of Mycobacterium tuberculosis strains on the presentation of disease. *The Lancet infectious diseases* 5:174-83
- McKenna KC, Beatty KM, Vicetti Miguel R, Bilonick RA. 2008. Delayed processing of blood increases the frequency of activated CD11b+ CD15+ granulocytes which inhibit T cell function. *J Immunol Methods*
- Meier T, Eulenbruch HP, Wrighton-Smith P, Enders G, Regnath T. 2005. Sensitivity of a new commercial enzyme-linked immunospot assay (T

- SPOT-TB) for diagnosis of tuberculosis in clinical practice. *Eur J Clin Microbiol Infect Dis* 24:529-36
- Menozzi FD, Rouse JH, Alavi M, Laude-Sharp M, Muller J, et al. 1996. Identification of a heparin-binding hemagglutinin present in mycobacteria. *J Exp Med* 184:993-1001
- Menzies D. 2008. Using tests for latent tuberculous infection to diagnose active tuberculosis: can we eat our cake and have it too? *Annals of internal medicine* 148:398-9
- Menzies D, Pai M, Comstock G. 2007. Meta-analysis: new tests for the diagnosis of latent tuberculosis infection: areas of uncertainty and recommendations for research. *Annals of internal medicine* 146:340-54
- Mol CJ, Koethe SM. 2006. QuantiFERON-TB GOLD--An innovation in tuberculosis screening. *Aaohn J* 54:245-7
- Monkongdee P, McCarthy KD, Cain KP, Tasaneeyapan T, Nguyen HD, et al. 2009. Yield of acid-fast smear and mycobacterial culture for tuberculosis diagnosis in people with human immunodeficiency virus. *Am J Respir Crit Care Med* 180:903-8
- Moore DA, Evans CA, Gilman RH, Caviedes L, Coronel J, et al. 2006. Microscopic-observation drug-susceptibility assay for the diagnosis of TB. *N Engl J Med* 355:1539-50
- Moore DA, Roper MH. 2007. Diagnosis of smear-negative tuberculosis in people with HIV/AIDS. *Lancet* 370:1033-4
- Mortier E, Pouchot J, Girard L, Boussougant Y, Vinceneux P. 1996. Assessment of urine analysis for the diagnosis of tuberculosis. *BMJ* 312:27-8
- Mutetwa R, Boehme C, Dimairo M, Mangwanya D, Munyati S, et al. 2009. Diagnostic accuracy of commercial urinary lipoarabinomannandetection in African TB suspects and patients. *The International Journal of Tuberculosis and Lung Disease*
- Nathan C, Shiloh MU. 2000. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proceedings of the National Academy of Sciences of the United States of America* 97:8841-8

- Ng VH, Cox JS, Sousa AO, MacMicking JD, McKinney JD. 2004. Role of KatG catalase-peroxidase in mycobacterial pathogenesis: countering the phagocyte oxidative burst. *Molecular microbiology* 52:1291-302
- Nguyen L, Pieters J. 2006. Battle for survival: interplay between pathogenic mycobacteria and the host immune system. *Discov Med* 6:82-6
- Noss EH, Pai RK, Sellati TJ, Radolf JD, Belisle J, et al. 2001. Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of *Mycobacterium tuberculosis*. *J Immunol* 167:910-8
- Nour EM, Cherian BP, Quantrill SJ. 2007. Diagnostic yield of early morning urine samples in the diagnosis of tuberculosis. *QJM* 100:464-5
- O'Connor TM, Sheehan S, Cryan B, Brennan N, Bredin CP. 2000. The ligase chain reaction as a primary screening tool for the detection of culture positive tuberculosis. *Thorax* 55:955-7
- Okamoto M, Kawabe T, Iwasaki Y, Hara T, Hashimoto N, et al. 2005. Evaluation of interferon-gamma, interferon-gamma-inducing cytokines, and interferon-gamma-inducible chemokines in tuberculous pleural effusions. *J Lab Clin Med* 145:88-93
- Onyebujoh P, Rook GA. 2004. Tuberculosis. *Nat Rev Microbiol* 2:930-2
- Orme I. 2004. Adaptive immunity to mycobacteria. *Curr Opin Microbiol* 7:58-61
- Oxford Immunotec Ltd. 2009. *T-Cell Xtend™ [mechanism of action]*, http://www.youtube.com/watch?v=VAsezk74TkE&feature=youtube_gdata, Retrieved 25/09/2009.
- Pai M, Flores LL, Hubbard A, Riley LW, Colford JM, Jr. 2004. Nucleic acid amplification tests in the diagnosis of tuberculous pleuritis: a systematic review and meta-analysis. *BMC Infect Dis* 4:6
- Pai M, Flores LL, Pai N, Hubbard A, Riley LW, Colford JM, Jr. 2003. Diagnostic accuracy of nucleic acid amplification tests for tuberculous meningitis: a systematic review and meta-analysis. *Lancet Infect Dis* 3:633-43

- Pai M, Gardam M, Haldane D. 2008a. Canadian Tuberculosis Committee: Updated recommendations on interferon gamma release assays for latent tuberculosis infection. *Can Commun Dis Rep* 2008., pp. 1-12
- Pai M, Joshi R, Dogra S, Mendiratta DK, Narang P, et al. 2006a. Persistently elevated T cell interferon-gamma responses after treatment for latent tuberculosis infection among health care workers in India: a preliminary report. *J Occup Med Toxicol* 1:7
- Pai M, Joshi R, Dogra S, Mendiratta DK, Narang P, et al. 2006b. Serial testing of health care workers for tuberculosis using interferon-gamma assay. *Am J Respir Crit Care Med* 174:349-55
- Pai M, Kalantri S, Dheda K. 2006c. New tools and emerging technologies for the diagnosis of tuberculosis: part II. Active tuberculosis and drug resistance. *Expert Rev Mol Diagn* 6:423-32
- Pai M, Kalantri S, Menzies D. 2006d. Discordance between tuberculin skin test and interferon-gamma assays. *Int J Tuberc Lung Dis* 10:942-3
- Pai M, Ling DI. 2008. Rapid diagnosis of extrapulmonary tuberculosis using nucleic acid amplification tests: what is the evidence? *Future microbiology* 3:1-4
- Pai M, Menzies D. 2007a. Interferon-gamma release assays: what is their role in the diagnosis of active tuberculosis? . *Clin Infect Dis* 44:74-7
- Pai M, Menzies D. 2007b. The New IGRA and the Old TST: Making Good Use of Disagreement. *Am J Respir Crit Care Med* 175:529-31
- Pai M, Minion J, Sohn H, Zwerling A, Perkins MD. 2009. Novel and improved technologies for tuberculosis diagnosis: progress and challenges. *Clinics in chest medicine* 30:701-16, viii
- Pai M, O'Brien R. 2007. Serial Testing for Tuberculosis: Can We Make Sense of T Cell Assay Conversions and Reversions? *PLoS Med* 4:e208
- Pai M, Zwerling A, Menzies D. 2008b. Systematic review: T-cell-based assays for the diagnosis of latent tuberculosis infection: an update. *Annals of internal medicine* 149:177-84
- Patel VB, Singh R, Connolly C, Coovadia Y, Peer AK, et al. 2010. Cerebrospinal T Cell Responses Aid the Diagnosis of Tuberculosis

Meningitis in a HIV and TB Endemic Population. *Am J Respir Crit Care Med*

- Pathan AA, Wilkinson KA, Klenerman P, McShane H, Davidson RN, et al. 2001. Direct ex vivo analysis of antigen-specific IFN-gamma-secreting CD4 T cells in Mycobacterium tuberculosis-infected individuals: associations with clinical disease state and effect of treatment. *J Immunol* 167:5217-25
- Pereira Arias-Bouda LM, Nguyen LN, Ho LM, Kuijper S, Jansen HM, Kolk AH. 2000. Development of antigen detection assay for diagnosis of tuberculosis using sputum samples. *J Clin Microbiol* 38:2278-83
- Perkins MD, Roscigno G, Zumla A. 2006. Progress towards improved tuberculosis diagnostics for developing countries. *Lancet* 367:942-3
- Peter J, Green C, Hoelscher M, Mwaba P, Zumla A, Dheda K. 2010. Urine for the diagnosis of tuberculosis: current approaches, clinical applicability, and new developments. *Current opinion in pulmonary medicine* 16:262-70
- Pethe K, Alonso S, Biet F, Delogu G, Brennan MJ, et al. 2001. The heparin-binding haemagglutinin of M. tuberculosis is required for extrapulmonary dissemination. *Nature* 412:190-4
- Pfyffer GE, Cieslak C, Welscher HM, Kissling P, Rusch-Gerdes S. 1997. Rapid detection of mycobacteria in clinical specimens by using the automated BACTEC 9000 MB system and comparison with radiometric and solid-culture systems. *J Clin Microbiol* 35:2229-34
- Piersimoni C, Scarparo C. 2003. Relevance of commercial amplification methods for direct detection of Mycobacterium tuberculosis complex in clinical samples. *J Clin Microbiol* 41:5355-65
- Prescott L, Harley J, Klein D. 2005. *Microbiology 6th edition*. pp. 8t, 674-702 and 882-4. New York: McGraw-Hill.
- QuantIFERON®-TB Gold In-Tube [Package Insert], Cellestis Ltd., Australia. 2007.
- Raby E, Moyo M, Devendra A, Banda J, De Haas P, et al. 2008. The effects of HIV on the sensitivity of a whole blood IFN-gamma release assay in Zambian adults with active tuberculosis. *PLoS ONE* 3:e2489

- Rangaka MX, Diwakar L, Seldon R, van Cutsem G, Meintjes GA, et al. 2007. Clinical, immunological, and epidemiological importance of antituberculosis T cell responses in HIV-infected Africans. *Clin Infect Dis* 44:1639-46
- Rantakokko-Jalava K, Marjamaki M, Marttila H, Makela L, Valtonen V, Viljanen MK. 2001. LCx Mycobacterium tuberculosis assay is valuable with respiratory specimens, but provides little help in the diagnosis of extrapulmonary tuberculosis. *Ann Med* 33:55-62
- Read SW, Rupert A, Stevens R, O'Shea A, Sereti I. 2006. Delayed sample processing leads to marked decreases in measured plasma IL-7 levels. *J Acquir Immune Defic Syndr* 42:511-2
- Reither K, Saathoff E, Jung J, Minja L, Kroidl I, et al. 2009. Low sensitivity of a urine LAM-ELISA in the diagnosis of pulmonary tuberculosis.
- Ribeiro S, Dooley K, Hackman J, Loreda C, Efron A, et al. 2009. T-SPOT.TB responses during treatment of pulmonary tuberculosis. *BMC Infect Dis* 9:23
- Richeldi L, Ewer K, Losi M, Hansell DM, Roversi P, et al. 2004. Early diagnosis of subclinical multidrug-resistant tuberculosis. *Annals of internal medicine* 140:709-13
- Romani L, Puccetti P, Bistoni F. 1997. Interleukin-12 in infectious diseases. *Clinical microbiology reviews* 10:611-36
- Rothel JS, Andersen P. 2005. Diagnosis of latent Mycobacterium tuberculosis infection: is the demise of the Mantoux test imminent? *Expert review of anti-infective therapy* 3:981-93
- Ruhwald M, Bjerregaard-Andersen M, Rabna P, Kofoed K, Eugen-Olsen J, Ravn P. 2007. CXCL10/IP-10 release is induced by incubation of whole blood from tuberculosis patients with ESAT-6, CFP10 and TB7.7. *Microbes and infection / Institut Pasteur* 9:806-12
- Russell DG. 2001. Mycobacterium tuberculosis: here today, and here tomorrow. *Nat Rev Mol Cell Biol* 2:569-77
- Sada E, Brennan PJ, Herrera T, Torres M. 1990. Evaluation of lipoarabinomannan for the serological diagnosis of tuberculosis. *J Clin Microbiol* 28:2587-90

- Sarmiento OL, Weigle KA, Alexander J, Weber DJ, Miller WC. 2003. Assessment by meta-analysis of PCR for diagnosis of smear-negative pulmonary tuberculosis. *J Clin Microbiol* 41:3233-40
- Schlesinger LS. 1993. Macrophage phagocytosis of virulent but not attenuated strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors. *J Immunol* 150:2920-30
- Shah M, Variava E, Holmes CB, Coppin A, Golub JE, et al. 2009. Diagnostic Accuracy of a Urine Lipoarabinomannan Test for Tuberculosis in Hospitalized Patients in a High HIV Prevalence Setting. *J Acquir Immune Defic Syndr*
- Shin AR, Lee KS, Lee JS, Kim SY, Song CH, et al. 2006. *Mycobacterium tuberculosis* HBHA protein reacts strongly with the serum immunoglobulin M of tuberculosis patients. *Clin Vaccine Immunol* 13:869-75
- Sittampalam GS, Smith WC, Miyakawa TW, Smith DR, McMorris C. 1996. Application of experimental design techniques to optimize a competitive ELISA. *J Immunol Methods* 190:151-61
- Smith I. 2003. *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. *Clinical microbiology reviews* 16:463-96
- Steingart KR, Henry M, Ng V, Hopewell PC, Ramsay A, et al. 2006. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. *Lancet Infect Dis* 6:570-81
- T-cell Xtend™ [package insert], Oxford Immunotec Ltd. Oxford, England. 2008.
- T-SPOT®.TB [package insert], Oxford Immunotec Ltd. Oxford, England. 2008.
- Tan YK, Lee AS, Khoo KL, Ong SY, Wong SY, Ong YY. 1999. Rapid mycobacterial tuberculosis detection in bronchoalveolar lavage samples by polymerase chain reaction in patients with upper lobe infiltrates and bronchiectasis. *Ann Acad Med Singapore* 28:205-8
- Temmerman S, Pethe K, Parra M, Alonso S, Rouanet C, et al. 2004. Methylation-dependent T cell immunity to *Mycobacterium tuberculosis* heparin-binding hemagglutinin. *Nat Med* 10:935-41

- Tessema TA, Hamasur B, Bjun G, Svenson S, Bjorvatn B. 2001. Diagnostic evaluation of urinary lipoarabinomannan at an Ethiopian tuberculosis centre. *Scand J Infect Dis* 33:279-84
- Thoen C, Lobue P, de Kantor I. 2006. The importance of *Mycobacterium bovis* as a zoonosis. *Vet Microbiol* 112:339-45
- Thomas MM, Hinks TS, Raghuraman S, Ramalingam N, Ernst M, et al. 2008. Rapid diagnosis of *Mycobacterium tuberculosis* meningitis by enumeration of cerebrospinal fluid antigen-specific T-cells. *Int J Tuberc Lung Dis* 12:651-7
- Todd B. 2006. The QuantiFERON-TB Gold Test: a new blood assay offers a promising alternative in tuberculosis testing. *Am J Nurs* 106:33-4, 7
- Tueller C, Chhajer PN, Buitrago-Tellez C, Frei R, Frey M, Tamm M. 2005. Value of smear and PCR in bronchoalveolar lavage fluid in culture positive pulmonary tuberculosis. *Eur Respir J* 26:767-72
- Turner J, Corrah T, Sabbally S, Whittle H, Dockrell HM. 2000. A longitudinal study of in vitro IFN γ production and cytotoxic T cell responses of tuberculosis patients in the gambia. *Tuber Lung Dis* 80:161-9
- Ulrichs T, Anding P, Porcelli S, Kaufmann SH, Munk ME. 2000a. Increased numbers of ESAT-6- and purified protein derivative-specific gamma interferon-producing cells in subclinical and active tuberculosis infection. *Infect Immun* 68:6073-6
- Ulrichs T, Anding R, Kaufmann SH, Munk ME. 2000b. Numbers of IFN- γ -producing cells against ESAT-6 increase in tuberculosis patients during chemotherapy. *Int J Tuberc Lung Dis* 4:1181-3
- van Zyl-Smit RN, Pai M, Pehrah K, Meldau R, Kieck J, et al. 2009. Within-subject variability and boosting of T-cell interferon-gamma responses after tuberculin skin testing. *Am J Respir Crit Care Med* 180:49-58
- Vaneechoutte M, Van Eldere J. 1997. The possibilities and limitations of nucleic acid amplification technology in diagnostic microbiology. *Journal of medical microbiology* 46:188-94
- Vincenti D, Carrara S, Butera O, Bizzoni F, Casetti R, et al. 2007. Response to region of difference 1 (RD1) epitopes in human immunodeficiency virus (HIV)-infected individuals enrolled with suspected active

tuberculosis: a pilot study. *Clinical and experimental immunology* 150:91-8

- Wagstaff AJ, Zellweger JP. 2006. T-SPOT.TB: an in vitro diagnostic assay measuring T-cell reaction to *Mycobacterium tuberculosis*-specific antigens. *Mol Diagn Ther* 10:57-63; discussion 4-5
- Walzl G, Ronacher K, Djoba Siawayá JF, Dockrell HM. 2008. Biomarkers for TB treatment response: challenges and future strategies. *J Infect* 57:103-9
- Wilkinson KA, Kon OM, Newton SM, Meintjes G, Davidson RN, et al. 2006. Effect of Treatment of Latent Tuberculosis Infection on the T Cell Response to *Mycobacterium tuberculosis* Antigens. *J Infect Dis* 193:354-9
- Wilkinson KA, Wilkinson RJ, Pathan A, Ewer K, Prakash M, et al. 2005. Ex vivo characterization of early secretory antigenic target 6-specific T cells at sites of active disease in pleural tuberculosis. *Clin Infect Dis* 40:184-7
- Wilson D, Nachega J, Morroni C, Chaisson R, Maartens G. 2006. Diagnosing smear-negative tuberculosis using case definitions and treatment response in HIV-infected adults. *Int J Tuberc Lung Dis* 10:31-8
- Xpert® MTB/RIF [Package Insert], Cepheid. Sunnyvale CA, US. 2009.
- Zink A, Haas CJ, Reischl U, Szeimies U, Nerlich AG. 2001. Molecular analysis of skeletal tuberculosis in an ancient Egyptian population. *Journal of medical microbiology* 50:355-66