THE SENSITIVITY AND SPECIFICITY OF PLEURAL FLUID MTB-SPECIFIC ELISPOT ASSAY AMONG ADULTS WITH SUSPECTED TUBERCULOUS PLEURISY COMPARED TO PLEURAL BIOPSY IN MULAGO HOSPITAL

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A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF MEDICINE (INTERNAL MEDICINE) OF MAKERERE UNIVERSITY.

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DECLARATION

I, Rejani Lalitha, hereby declare that the work presented in this dissertation has not been presented for any other degree in any university.

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Part of the study was done by Dr. Kansiime Jackson, a senior house officer at the department of medicine and a research assistant, who collected the initial data and performed the first thirteen pleural biopsies. The Prinicipal investigator trained the research assistant in data collection as well as in performing a pleural biopsy. The PI performed the rest of the thirty eight pleural biopsies, collected the rest of the data, interpreted chest X-rays and laborartoty results, enetered, cleaned and coded data. The PI was also involved in the processing of pleural fluid samples for Elispot assay and performed a few assays. The initial data analysis was done by Dr. Opio who taught the PI basics of data analysis using STATA. The re-analysis was done by the PI and analysis report confirmed by the statistician.

DEDICATION

My beloved husband Kenneth and my daughter Lillian, my parents, in-laws and to all those who matter in my life.

ACKNOWLEDGEMENT

I wish to express my sincere gratitude to the MIND study group which is led by clinicians from Makerere University and the University of California, San Francisco for providing me ELIPSOT kits for this study. Sincere thanks to my supervisors Associate Professor Harriet Mayanja-Kizza, Dr. Worodria William and Dr. Adithya Cattamanchi for the valuable guidance they provided through out the preparation of this dissertation.

I wish to also express my exceptional gratitude to JCRC Immunology lab especially Mr. Isaac Sewanyana and Mr. Steven Mutyala for providing all the help and guidance through out the study.

My sincere thanks also go to Dr. Opio Christopher Kenneth for all the guidance given at all stages of this research and for analyzing the data.

I would like to thank all the members of the Department of Medicine and fellow colleagues for all the assistance they have provided. My special thanks to Dr. Kansiime Jackson who helped me collect part of the data presented in this book.

I would like to thank all the members of the MIND study team especially Dr. Nankya Florence, Dr. Rachael Kyeyune and Patrick Byanyima during the recruitment of study participants.

I wish to extend my sincere gratitude to Makerere University Pathology team led by Dr. Lynnette Tumwine, Mr. Lawrence Osuwat, Mrs. Betty and the staff of NTLP laboratory, Wandegeya for the great work and dedication. My special thanks to Ms. Asiimwe Faridah, for helping me transport samples to the labs and other assistance provided.

Last but not least, my sincere appreciation to all my study patients without whom, this work would not have been possible.

Above all, I thank God for enabling me, for the strength, courage and wisdom He has given me through out my study.

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LIST OF ABBREVIATIONS AND ACRONYMS

ADA – Adenosine Deaminase

AFB – Acid Alcohol Fast Bacilli

BCG - Bacille Calmette–Guérin

CFP-10 – Culture Filtrate Protein-10

DOTS – Directly Observed Treatment Short course

ELISA – Enzyme Linked Immunosorbent Assay

Elispot – Enzyme Linked Immunospot

EPTB – Extra pulmonary Tuberculosis

ESAT-6 – Early Secretory Antigenic Target-6

HIV – Human Immunodeficiency Virus

IFN-γ – Interferon gamma

IGRAs – Interferon Gamma Release Assays

IL – Interleukin

JCRC – Joint Clinical Research Centre

LDH – Lactate Dehydrogenase

LJ – Lowenstein Jensen

MTB – Mycobacterium tuberculosis

NPV – Negative Predictive Value

NTLP – National Tuberculosis and Leprosy Programme

PBS – Pooled Bovine Serum

PE – Pleural effusion

PEMCs – Pleural effusion mononuclear cells

PI – Principal Investigator

PPE – Parapneumonic Effusion

PPV – Positive Predictive Value

PTB – Pulmonary Tuberculosis

TB – Tuberculosis

TNF-α – Tumor Necrosis Factor Alpha

TST – Tuberculin Skin Test

ZN – Ziehl Neelsen

OPERATIONAL DEFINITIONS

1. **Pleural effusion:** This is excess or abnormal accumulation of fluid in the pleural space.
2. **Tuberculous pleural effusion suspect:** A patient with all of the following:
    a. Medical history compatible with TB (at least two of the following symptoms for more than two weeks i.e. cough, unintentional weight loss, loss of appetite, swelling of glands, fevers, drenching night sweats; and chest pain with or without shortness of breath for more than two weeks).
    b. Pleural effusion more than 25% of hemithorax on chest X-ray.
    c. Exudative pleural effusion by Light's criteria.
    d. Pleural fluid lymphocytosis (greater than 50%).
3. **Tuberculous pleural effusion case (confirmed):** Tuberculous pleural effusion suspect with either caseating granuloma on pleural tissue histopathology or positive LJ pleural tissue culture.
4. **Pyothorax:** This is frank pus in the pleural space.
5. **Lights criteria:** Exudates are defined by the presence of at least one of the criteria
    (a) Pleural fluid–serum protein ratio greater than 0.5
    (b) Pleural fluid–serum Lactate Dehydrogenase (LDH) ratio greater than 0.6, and
    (c) Pleural fluid LDH concentration greater than 200 IU/L.
6. **MTB-specific Elispot assay:** An immunological assay that enumerates the T-cell responses specific for secreted antigens of *M. tuberculosis*, ESAT-6 and CFP-10.
7. **Karnofsky performance status:** a score that allows patients to be classified as to their functional impairment (appendix 1).

8. **Sputum smear positive TB case:** This is a patient with at least two sputum smears positive for AFBs or one sputum smear positive for AFBs and chest X-ray consistent with active TB or one sputum smear positive for AFBs plus a positive culture for *Mycobacterium tuberculosis*.
9. **Sputum smear negative TB case:** This is a patient with at least two sputum smears negative for AFBs.
10. **Diagnostic accuracy of Elispot assay:** This is a measure of the extent to which the results of Elispot assay reflects true disease status. The measures include sensitivity, specificity, and positive and negative predictive values.
11. **Sensitivity of MTB-specific Elispot assay:** This is defined as the number of individuals with a positive Elispot assay among the total number of individuals with a positive MTB culture or histopathology of pleural tissue.
12. **Specificity of MTB-specific Elispot assay:** This is defined as the number of individuals with a negative Elispot assay among the total number of individuals with a negative MTB culture or histopathology of pleural tissue.
13. **Positive predictive value of MTB-specific Elispot assay:** This is the number of individuals who have TB by histopathology or culture among those with a positive Elispot test.
14. **Negative predictive value of MTB-specific Elispot assay:** This is the number of individuals who do not have TB by histopathology or culture among those with a negative Elispot test.

ABSTRACT

Introduction and rationale: Pleural effusions are a common reason for admission in Mulago hospital and tuberculosis is the most common infectious cause. The diagnosis of tuberculous pleural effusion with standard analysis of pleural fluid is inaccurate. Pleural tissue histopathology and culture for *Mycobacterium tuberculosis* is considered the gold standard but the procedure is invasive, requires skilled clinicians and does not provide immediate results. To address these limitations, we evaluated whether an MTB-specific Enzyme Linked Immunospot (Elispot) assay performed on pleural fluid mononuclear cells accurate and feasible for diagnosing tubercuous pleurisy.

Objectives: To evaluate the diagnostic accuracy of MTB-specific Elispot assay performed on pleural fluid mononuclear cells among adults with suspected tuberculous pleural effusion at Mulago hospital pulmonology unit.

Methods: This cross-sectional study was conducted in Mulago Hospital, pulmonology unit. The study was done in two phases, initial phase between October 2008 and April 2009 and the latter phase in June 2009. Patients above 18 years with clinical and radiological suspicion of tuberculous pleural effusion following chart review with pleural effusion greater than 25% of hemithorax, and provided written informed consent were consecutively screened. All screened patients underwent diagnostic thoracocentesis. Those with exudative pleural effusion by Light's criteria and lymphocyte predominant effusions were enrolled. Standardized questionnaire was administered and patients underwent pleural biopsy. Biopsy specimens were cultured on Lowenstein-Jensen media and submitted for histopathology. At the time of biopsy, 50 mL of pleural fluid was collected for isolation of pleural fluid mononuclear cell. In the first phase the cells were stored in liquid nitrogen and Elispot run as a batch whereas in the second phase Elispot was run on fresh cells. Also 15 mL of pleural fluid was collected during biopsy for cytology. Elispot assays were performed if the pleural fluid mononuclear cell count was greater than 1000, 000 and viability greater than 67% after storage

or on fresh samples. Characteristics of patients were compared using Fisher's exact test for dichotomous variables and Wilcoxon rank-sum test for continuous variables. Sensitivity and specificity of Elispot were calculated using combined results of pleural tissue culture and histopathology as a reference standard.

Results: Of the 51 patients who underwent pleural biopsy, 25 patients had Elispot done. The rest 26 (51%) patients could not undergo Elispot because of inadequate pleural fluid mononuclear cell yield. Patients with TB and those without had similar characteristics except for a higher axillary temperature among those with TB. The prevalence of TB pleural effusion was 64%. The sensitivity, specificity, positive and negative predictive values, and positive and negative likelihood ratios were 81.3%, 55.6%, 76.5%, 62.5%, 1.83 and 0.34 respectively.

Among the 51 pleural biopsies done, 35 (68.6%) had TB pleural effusion by combined gold standards. Among the 25 Elispot assays done, 16 (64%) had TB by combined gold standards and 13 (52%) had TB by both Elispot and gold standards. Median spot forming cell counts were higher in patients with tuberculous pleural effusion compared to those without for both ESAT-6 (790 vs. 142, p=0.01) and CFP-10 (731 vs. 27, p=0.11).

Conclusions: The diagnostic accuracy of commercial (T-SPOT.TB) Elispot assay may be low when used alone. It may be a clinically useful adjunct test for diagnosing TB pleural effusion.

Recommendations: Larger studies are needed in our setting to determine accurately the diagnostic value of pleural fluid Elispot assay for TB pleurisy. Future studies should carefully consider procedures for collecting, processing and storing pleural fluid mononuclear cells for Elispot assay.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Tuberculosis (TB) is one of the leading causes of morbidity and mortality worldwide, affecting more than 8 million persons annually with 2 to 3 million deaths [(1)] and is a major health problem especially in the developing countries. Tuberculous pleural effusion is the second most common extra-pulmonary manifestation of active *Mycobacterium tuberculosis* (MTB) infection after lymph node TB, accounting for up to 23% of TB cases [(2)] and, 30% of pleural effusions (PEs) in Western Europe[(3)]. Pleural effusion occurs in approximately 5% of patients with TB [(7)]. TB may account for 14% of all pleural exudates and 25% of all pleural effusions in areas with high incidence of TB.

1.2 PROBLEM STATEMENT

Pleural biopsy for culture of MTB and histopathological detection of caseating granulomas are regarded as the gold standard for the diagnosis of tuberculous pleural effusion, with sensitivities of 39–80 and 50–97%, respectively [(4, 5, 6, 7, 8, 9, 10, 11, 12)]. However, pleural biopsy is invasive and neither tissue culture nor histopathologic examinations are routinely available in Uganda. Though easier to collect, examination of pleural fluid by culture or ZN stain has poor sensitivity for tuberculous pleural effusion. [(4, 8, 10, 12, 13)]. Therefore, diagnosis of tuberculous pleural effusion is typically made based on a compatible clinical presentation and the presence of suggestive pleural fluid characteristics including elevated protein levels and lymphocytosis. More recently, MTB-specific T-cell interferon (IFN)-γ release assays (TIGRAs) have been developed for the diagnosis of MTB infection from the peripheral blood [(14)]. While these tests are unable to distinguish between active and latent TB when performed on blood samples, recent studies report high sensitivity and specificity for tuberculous pleural effusion when TIGRAs are performed on pleural fluid [(15, 16)].

1.3 JUSTIFICATION OF THE STUDY

Though tuberculous pleural effusion is a common manifestation of extra-pulmonary TB, diagnosis is challenging in low-income settings such as Uganda. The prolonged incubation time of culture, whether performed on pleural biopsy specimens or pleural fluid, delays time to diagnosis and initiation of effective anti-tuberculous therapy. Examination of pleural fluid for AFB is highly insensitive and biochemical criteria have both poor sensitivity and specificity. We know that in active TB, MTB-specific T-cells clonally expand and are recruited to the site of the infection [17] and that memory T-cells are typically present only in blood and lymphoid tissues. Therefore, evaluating for interferon-γ production by MTB-specific T-cells present in pleural fluid samples using an Elispot-based TIGRA may be highly sensitive and specific for diagnosis of tuberculous pleural effusion. This approach is also rapid and can enable early initiation of anti-tuberulous treatment. No data exist as to the diagnostic accuracy of pleural fluid Elispot in our setting. This study was designed to determine whether pleural fluid mononuclear cells can be reliably sampled from tuberculous pleural effusion suspects admitted to Mulago Hospital and if so, provide data regarding the sensitivity and specificity of the assay in a tuberculosis-endemic setting.

1.4 RESEARCH QUESTION

Can an MTB-specific Elispot assay performed on pleural fluid mononuclear cells improve diagnosis of tuberculous pleural effusion among adults admitted to Mulago hospital pulmonology unit (Ward 4C Pulmonology/TB) over the study period?

1.5 STUDY OBJECTIVES

1.5.1GENERAL OBJECTIVE

To determine the diagnostic accuracy of an MTB-specific Elispot assay performed on pleural fluid mononuclear cells for the diagnosis of tuberculous pleural effusion among adults admitted to Mulago hospital pulmonology unit (Ward 4C Pulmonology/TB) over the study period.

1.5.2 SPECIFIC OBJECTIVES

Primary objectives

1. To determine the sensitivity and specificity of MTB-specific Elispot assay on pleural fluid mononuclear cells among clinically suspected TB pleural effusion, with pleural tissue culture and histopathology as the gold standard over the study period.
2. To determine the PPV, NPV and likelihood ratios of MTB-specific Elispot assay on pleural fluid mononuclear cells in suspected TB pleural effusion, with pleural tissue culture and histopathology as the gold standard over the study period.

1.6 DEFINITION OF GOLD STANDARDS FOR DIAGNOSIS OF TB PLEURAL EFFUSION

1. MTB tissue culture: This was done on a solid culture media which is egg-based, the Löwenstein-Jensen (LJ) media. Mycobacterial culture colonies on LJ media do not produce significant amounts of pigment; have a buff-color and smooth surface appearance after 6-8 weeks.
2. Pleural biopsy tissue histopathology - Tissue histology after staining with Haematoxylin and Eosin (H&E) was taken to be suggestive of tuberculosis only if evidenced by presence of granulomatous inflammation with epithelioid cells, Langerhans type giant cells, and caseation necrosis. Tissue staining by ZN stain was taken positive for AFBs if pink staining rods were seen.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Burden of Tuberculosis

About a third of the world's population is infected with *Mycobacterium tuberculosis* [23]. Tuberculous pleural effusion occurs in approximately 5% of patients with *Mycobacterium tuberculosis* infection. Among communicable diseases, TB is among the top ten killer diseases worldwide, killing nearly 2 million people each year. There was an estimated 8.8 million new TB cases in 2005, 7.4 million in Asia and sub-Saharan Africa. A total of 1.6 million people have died of TB, including 195 000 patients infected with HIV [1] (13% of TB cases have co-existent HIV infection). Most cases (5-6 million) occur in people aged 15-49 years, with significant socio-economic impact. Sub-Saharan Africa presents with the highest incidence rate (around 300/100,000 population per year [24]. Even though TB has declined steadily in Western Europe and North America, the global TB burden appears on the rise, especially in the former Soviet Union, Eastern Europe, and Africa. In Uganda, a total of 45,216 new sputum smear positive cases were notified in 2006 and the mortality among all TB patients during the same year was high with an estimated 26,042 deaths reported [1]. Tuberculosis primarily causes pulmonary tuberculosis but with HIV/AIDS we now encounter many cases of extra-pulmonary TB, and TB of the pleura is the second commonest manifestations of extra-pulmonary TB after TB adenitis [26, 33].

2.2 Pathobiology of Tuberculous pleural effusion

Tuberculous pleural effusion occurs as a result of TB antigens entering the pleural space, usually through the rupture of a sub pleural caseous focus [9, 26, 28], followed by a local, delayed hypersensitivity reaction mediated by CD4+ T-cells [18, 27]. This process may occur during primary or reactivation TB and may or may not involve viable bacilli entering the pleural space [19]. The presence of mycobacterial antigens in the pleural space elicits an intense immune response, initially by neutrophils and macrophages [20, 21] followed by interferon (IFN)-gamma-producing T-helper cell

(Th) type 1 lymphocytes [18, 22] resulting in a lymphocyte-predominant exudative effusion. IFN-gamma, produced by T-lymphocytes, is capable of activating macrophages, increasing their bactericidal capacity against M.tuberculosis and is involved in granuloma formation.

2.3 Clinical Manifestations of tuberculous pleural effusion

Symptoms of tuberculous pleural effusion which are relevant to clinical diagnosis may develop abruptly or insidiously [9] and include:

1. Chest pain, present in 75%, is the commonest symptom among patients presenting with pleural effusion. It is usually unilateral and pleuritic in nature (worse on inspiration). About 10% of patients may develop bilateral pleural effusion and therefore may present with bilateral chest pain [9].
2. Cough is a non-specific symptom. It occurs in 70% of patients with pleural effusion, and is usually non productive [9].
3. Constitutional symptoms such as fever, weight loss, night sweats, anorexia and malaise may occur in association with tuberculous pleural effusion but may also occur in other pleural infections or pleural malignancy.
4. Dyspnoea is a common but a non-specific symptom.
5. Haemoptysis is a common symptom in pulmonary tuberculosis but unusual in tuberculous pleural effusion.

Patients with risk factors for development of TB include alcohol abuse, bronchogenic carcinoma, diabetes mellitus, silicosis and immunosuppressive states due to immunosuppressants (corticosteroids, cytotoxic agents) and congenital or acquired immunodeficiency diseases. Chest signs may include reduced chest movements with respiration on the side of the effusion, the trachea may be deviated to the opposite side, reduced vocal resonance, stony dull percussion notes up to the level of the effusion, reduced breath sounds or absent breath sounds if the effusion is massive and

reduced tactile fremitus. A pleural friction rub may be heard and inspiratory crackles may be noted just above the level of the fluid.

Tuberculous pleural effusion has a non-specific clinical presentation [34]. Hence the need for a high index of suspicion in patients presenting with unilateral pleural effusion and constitutional symptoms in the absence of chronic medical conditions like nephritic syndrome, cardiac failure or liver cirrhosis.

2.4 Laboratory diagnosis of Tuberculous pleural effusion

Definitive diagnosis of extra-pulmonary tuberculosis (EPTB) is often difficult. An absolute diagnosis of tuberculous pleural effusion requires the demonstration on culture of *Mycobacterium tuberculosis* from pleural tissue together with a compatible clinical picture of the disease. The diagnosis of tuberculous pleural effusion can be difficult because of the low sensitivity of the various diagnostic tools. Conventional diagnostic tests for TB pleurisy include microscopic examination of the pleural fluid for acid-fast bacilli, mycobacterial culture of pleural fluid, sputum or pleural tissue, and histopathological examination of pleural tissue for granulomatous inflammation. These tests have recognized limitations for clinical use when used alone, although, in combination, they have been recognized as the best reference standard for evaluation of the accuracy of novel tests [35].

2.4.1 Chest X-ray

A postero-anterior (PA) view of chest X-ray usually shows a unilateral homogenous opacification if the effusion is unilateral though in about 10% it may be bilateral, and are small to moderate in size usually occupying less than two thirds of a hemithorax [8]. The lung field which is above the level of effusion may show evidence of pulmonary tuberculosis like upper-lobe infiltrates, cavitary infiltrates, and hilar or paratracheal adenopathy [24]. A PA view of chest X-ray is abnormal in the presence of about 200 mL pleural fluid and only 50 mL of pleural fluid is required to produce a detectable posterior costophrenic angle blunting on a lateral chest X-ray. Lateral decubitus films are

occasionally useful as free fluid gravitates to the most dependent part of the chest wall, differentiating between pleural thickening and free fluid. Diagnosis of TB pleurisy by means of radiographic examination of chest is unreliable because no chest X-ray pattern is absolutely typical of TB pleurisy especially with underlying HIV infection [70].

2.4.2 Chest Sonogram

A chest sonogram is useful where effusions are very small. A complex septated pattern in the sonographic appearance is a useful predictor of tuberculosis in lymphocyte-rich exudative pleural effusions [72].

2.4.3 Thoracocentesis and pleural fluid analysis

The pleural fluid of TB pleurisy has a characteristic yellow-citrine color. Proteins are elevated and usually range from 0.3 to 300 g/dl, normal being 1-2 g/dl. The pH of pleural fluid in TB is usually below 7.25, the normal pleural fluid pH being 7.60 to 7.64 which is similar to that of plasma. White blood cell counts are elevated in the pleural fluid in TB pleural effusion and are predominantly lymphocytic, though this finding is not specific for TB pleurisy as there are many other causes of lymphocyte predominant pleural effusion like malignancies, other inflammatory pleural diseases like sarcoidosis, rheumatoid arthritis etc. Combined data from two series [37] show that 90 (94%) of 96 exudative pleural effusions consisting of more than 50 percent lymphocytes were due to cancer or tuberculosis. In these series, 90 (78%) of 116 tuberculous pleural effusions contained more than 50 percent lymphocytes [37, 38]. The level of glucose in pleural fluid correlates with pH; therefore glucose levels can be used as an alternative to measuring pH. The normal pleural fluid glucose is similar to that of plasma. The presence of a low pleural-fluid glucose concentration (less than 40 mg per deciliter) indicates that the patient probably has a complicated parapneumonic [39] or a malignant effusion [40, 41] – refer to appendix 3 for classes of parapneumonic effusion. TB pleurisy rarely causes a low glucose pleural effusion [42]. Therefore, the presence of glucose less than 40 mg/dl

together with other abnormal findings in pleural fluid can be used to make a diagnosis of complicated parapneumonic effusions.

Direct examination of pleural fluid by Ziehl Neelsen staining requires bacillary densities of 10,000/mL and, therefore, detects acid-fast bacilli (AFB) in less than 5% of TB pleurisy cases [35, 43], due to the paucibacillary nature of the disease. LJ Cultures for acid fast bacilli are positive in only 20 to 30% of pleural fluid samples and are limited by the lengthy delay in obtaining results: up to 8 weeks if solid culture media are used.

All pleural fluid should be classified as exudates or transudates. Exudates are defined by Light's criteria (appendix 2).

For indications, contraindications and complications of thoracocentesis refer to appendix 4.

2.4.4 Light's criteria for evaluation of exudative pleural effusion

By Light's criteria, exudates are defined by the presence of at least one of the following criteria:

(1) Pleural fluid–serum protein ratio greater than 0.5

(2) Pleural fluid–serum LDH ratio greater than 0.6, and

(3) Pleural fluid LDH concentration greater than 200 IU/L.

The sensitivity and specificity of Light's criteria for exudates are 98% and 83% respectively [44]. Light's criteria correctly identifies almost 100% of pleural exudates but misdiagnoses 10–30% of transudates [45, 46, 47, 48, 49, 50]. According to Isabirye (Uganda, 2002), tuberculous pleural effusion was the commonest cause of exudative pleural effusion among patients in Mulago Hospital contributing to 66.7% of all patients with exudative pleural effusions [51].

2.4.5 Sputum examination in tuberculous pleural effusion

Sputum smears are rarely positive in primary cases of TB and cultures are positive in primary cases in only 25-33% of patients. In contrast, sputum smear is positive in 50% and culture in 60% of reactivation TB [73]. Tuberculous pleural effusion is considered a primary infection.

2.4.6 Novel tests for diagnosing tuberculous pleural effusion

Conventional diagnostic tests, such as microscopic examination of the pleural fluid, biochemical tests, culture of pleural fluid, sputum or pleural tissue, and histopathological examination of pleural tissue, have known limitations [52]. The newer tests and biomarkers for diagnosis of TB pleurisy are the nonspecific inflammatory markers (Adenosine Deaminase (ADA) activity, neopterin, leptin, lysozyme, fibronectin, interferon gamma measurement, Interleukin-2, Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-1β, complement activation, CD4+ T cell count), specific immune response markers (T-cell response to Early Secretary Antigenic Target-6 (ESAT-6) and Culture Filtrate Protein (CFP-10); serologic/antibody tests), detection of *Mycobacterium tuberculosis* nucleic acid by commercial Nucleic Acid Amplification Tests, and scoring systems based on combination of tests[52].

2.4.6.1 Elispot assay on pleural fluid mononuclear cells

Elispot or Enzyme Linked Immunosorbent Spot is a new specific immunological test developed by Cecil Czerkinsky in 1983 [71]. The Elispot assay is based on, and was developed from a modified version of the ELISA immunoassay. Elispot assays were originally developed to enumerate B cells secreting antigen-specific antibodies, and have subsequently been adapted for various tasks, especially the identification and enumeration of cytokine-producing cells at the single cell level. Simply put, at appropriate conditions the Elispot assay allows visualization of the secretory product of individual activated or responding cells. Each spot that develops in the assay represents a single reactive cell. Thus, the Elispot assay provides both qualitative (type of immune protein) and quantitative (number of responding cells) information.

2.4.6.2 Elispot Procedure: The Elispot assays employ a technique very similar to the sandwich enzyme-linked immunosorbent assay (ELISA) technique. For blood, samples should be processed within eight hours of collection, however, this duration is not known for pleural fluid. Refer to

appendix 5 for Elispot technique at JCRC, Uganda and appendix 6 for the description of the procedure. In Uganda, the Elispot wells can be read at JCRC immunology laboratory using an automated Immunospot Reader®. The response of cultures is considered positive when the test well contains at least six more spots and has twice the number of spots than the control well [54]**.**

Samples are usually processed within eight hours of collection and Elispot done on fresh samples. The yield from frozen pleural fluid samples has not been reported by the previous authors of various studies.

2.4.6.3 Validity of MTB-specific Elispot assay

The recently developed in vitro T-cell based IFN-γ release assays (IGRAs) is used for diagnosis of TB infection. These tests use peripheral blood mononuclear cells (PBMCs), but they can be used with pleural fluid mononuclear cells. When used on peripheral blood they detect TB infection but cannot distinguish between latent and active TB infection unlike their use on pleural fluid which detects active TB infection [54]. The assay detects IFN-γ secreted by mononuclear cells in response to in vitro stimulation with the MTB-specific antigens, Early Secretory Antigenic Target-6 (ESAT-6) and Culture Filtrate Protein-10 (CFP-10) [53]. The genes that encode these antigens are not present in any of the Mycobacterium bovis, Bacille Calmette–Guerin (BCG) strains or certain common nontuberculous (environmental) mycobacteria. Thus, in theory, the test should not cross-react with antigens present due to BCG vaccination [15, 53]. Several studies (appendix 7) have found elevated concentrations of INF-gamma in TB pleural effusions, which are related to increased production at the disease site by effector T cells [55]. In a recent study, the commercial T-SPOT.TB test (Oxford Immunotec Ltd, Oxford, UK) was performed on peripheral blood mononuclear cells and mononuclear cells from pleural fluid from 20 patients clinically suspected to have TB pleuritis and 21 subjects with other diagnoses. The sensitivity of T-SPOT.TB was 90% using the blood assay and

95% for pleural fluid, but the specificity was only 67% for blood and 76% for pleural fluid [54]. This poor specificity of the test on the blood cells may reflect positive reactions due to coincidental latent TB infection, coexisting or transient infection [52]. There are no published data available from Africa to validate the utility of MTB-specific Elispot assay where TB is very common and has a high TST positivity.

A metaanalysis done by Jiang et al in 2007 [55] concluded that IFN-γ determination is a sensitive and specific test for the diagnosis of tuberculous pleural effusion. The measurement of IFN- γ levels in pleural effusions is a useful tool for diagnosing tuberculous pleural effusion. The results of IFN- γ assays should be interpreted in parallel with clinical findings and the results of conventional tests. The sensitivity ranged from 64 to 100% (mean 89; 95% confidence interval [CI] 0.87 to 0.91), while specificity ranged from 86 to 100% (mean 97; 95% CI 0.96 to 0.98) [55, 56]. In a study done by Lawn et al [57], multivariate analysis confirmed that Elispot responses on peripheral blood mononuclear cells had a strong inverse association with a history of recent TB treatment (adjusted OR = 0.06, 95%CI = 0.10–0.40, $p < 0.01$) and they were independent of CD4 cell count and viral load. Among HIV+ individuals who had not received TB treatment both the magnitude and proportion of positive Elispot responses (but not TST) were similar to those of HIV-negative controls. The proportion of positive Elispot responses in patients with advanced HIV infection was independent of CD4 cell count but had a strong inverse association with history of TB treatment [57].

2.4.6.4 Limitations of Elispot assay

Enzyme linked immunospot assays are expensive and require significant infrastructure and staff training for reliable performance. There are no published data in Uganda or Africa to evaluate the diagnostic accuracy of the test.

2.4.6.5 Elispot in the clinical management of Tuberculous pleural effusion

Although Elispot kits are not yet available in Uganda due to the lack of sufficient data on its diagnostic accuracy in our setting, pleural fluid sample processing can be done by trained laboratory technicians at JCRC, Mengo, Uganda. Elispot kits are expensive but the results of the test could be availed in 24 hours and the procedure of thoracocentesis is noninvasive and associated with very few complications compared to a pleural biopsy. They have a very good sensitivity. A positive test in a patient with a high pretest probability is confirmatory of TB infection.

Delayed diagnosis and treatment can increase the risk for dissemination of M. tuberculosis and decrease survival for some subgroups of TB patients [58, 59, 60]. Thus, new technologic developments, which facilitate rapid diagnosis, are needed for successful control of this disease. The major problem with tuberculin skin testing (TST) is cross-reactivity with antigens in other mycobacteria, such as the M. bovis BCG vaccine strain and environmental mycobacterial species. This cross-reactivity leads to false-positive results and decrease PPV, especially in BCG-vaccinated persons and in areas of high incidence of Non Tuberculous Mycobacterium (NTM) disease. In Taiwan in 2001, 2.74% of preschool children were TST positive, whereas active TB developed in only 2.29/100,000 children 5-9 years of age [61]. Use of ESAT-6 and CFP-10, two antigens encoded in the region of difference 1 (RD-1), which distinguishes M. tuberculosis from other mycobacteria, has increased the specificity and PPV of IFN-gamma Elispot assays for infection [62, 63, 64, 65, 66, 67].

2.4.7 Pleural biopsy

Biopsy of pleural tissue for combined histological examination and mycobacterial culture of tissue is the most sensitive of the currently available diagnostic methods, but may still be falsely negative in 15–20% of these cases [68]. Pleural biopsy is invasive, and the yield as well as complication rates are dependent on the skills of the operator because it is technically difficult, particularly in children. Hence, biopsy adds considerable risk and cost to workup compared to the newer rapid

immunological assays. The sensitivity of pleural biopsy in TB pleural effusion has been reported to be in the range of 50-80% [5, 9, 29, 30, 31, 32]. In patients with TB pleural effusions, pleural biopsy reveals granulomas in 50 to 97% of patients. For tissue cultures, the tissue biopsy specimens are minced and homogenized in a sterile homogenizer. A portion of the homogenate is directly inoculated onto a culture media. Culture media most often used for diagnosis are: (1) Solid culture media: egg-based Löwenstein-Jensen, or agar-based Middlebrook 7H10 or 7H11 (growth can take up to 6 weeks) or (2) Liquid culture media (growth in 1-3 weeks) e.g. BACTEC, MGIT [69].

For indications, contraindications and complications of pleural biopsy refer to appendix 9.

2.4.8 Pleural fluid Cytology

Cytology of pleural fluid is not very useful in the diagnosis of TB pleural effusion. However, it is of importance in ruling out other causes of exudative pleural effusion with lymphocyte predominance like malignancies. Cytology is more sensitive than blind biopsy in patients suspected of having serosal metastases. In patients with clinically and/or pathologically proven pleural malignancy, cytological examination of pleural fluid is positive in 71% of cases and pleural biopsy in 45%, presumably because fluid provides a more representative sample.

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study site and setting

The study was carried out at Mulago National Referral Hospital on Ward 4C Pulmonology unit (which includes the TB ward and Pulmonology ward) of Mulago hospital. Mulago hospital is a National Referral hospital located in Kampala, the capital city of Uganda. The Pulmonology unit is located on 4th floor of Lower Mulago Hospital Complex (MHC) in Ward 4C. The unit has a bed capacity of 25 patients and admits 2 to 4 patients daily. Cases admitted include pleural effusions, pneumonia, and others. It consists of 2 nurses for the day, 1 for evening and 1 for night, one intern doctor, Senior House Officers, three physicians and medical students. The unit admits about 3 patients with suspected tuberculous pleural effusion every week.

The study was carried out in two phases; the first phase between October 2008 and April 2009 when Elispot assay was done on frozen and thawed pleural fluid cells and second phase in June 2009 when Elispot assay was done on fresh cells. The study was done in two phases as the pleural fluid cell yields were low when Elispot was done on frozen and thawed samples.

Patients were recruited on consecutive days from Monday to Friday during the study period.

3.2 Study design

This was a cross-sectional pilot study. The initial phase was undertaken during the period October 2008 to April 2009 and the second phase in June 2009, on adult patients with suspected TB pleural effusion admitted to TB ward and Pulmonolgy unit of Mulago hospital, Kampala, Uganda.

3.3 Study population

3.3.1 Target population: All adult patients with pleural effusion in Uganda.

3.3.2 Accessible population: All adult patients admitted to pulmonology unit during the study period were screened for possible tuberculous pleural effusion.

3.3.3 Sample population: All adult patients with suspected tuberculous pleural effusion admitted to Mulago hospital Pulmonology Unit during the study period and who met the eligibility criteria.

3.4 Selection/ eligibility Criteria

3.4.1 Inclusion criteria

1. All adult patients, 18 years and above.
2. Medical history compatible with tuberculous pleural effusion.
3. Pleural effusion by clinical examination, and chest X-ray.
4. Moderate (25-75% of the hemithorax) and large (greater than 75% of the hemithorax) pleural effusions on chest X-ray.
5. Exudative pleural effusion by Light's criteria.
6. Lymphocyte predominant effusion.
7. Written informed consent.

3.4.2 Exclusion criteria

1. All patients on anti-tuberculosis drugs.
2. Bilateral pleural effusion.
3. Complicated parapneumonic effusions (LDH≥1000 IU/L and neutrophil predominance).
4. Pyothorax.
5. Loculated pleural effusion on chest X-ray.
6. Patients with well documented chronic history of heart failure/nephrotic syndrome/liver cirrhosis.
7. Very sick patients (as defined by Karnofsy score of less than 30) – Appendix 1.
8. Contraindications to thoracocentesis or pleural biopsy like mechanical ventilation, uncooperative patient.
9. Withdrawal of consent at any time during the study period.

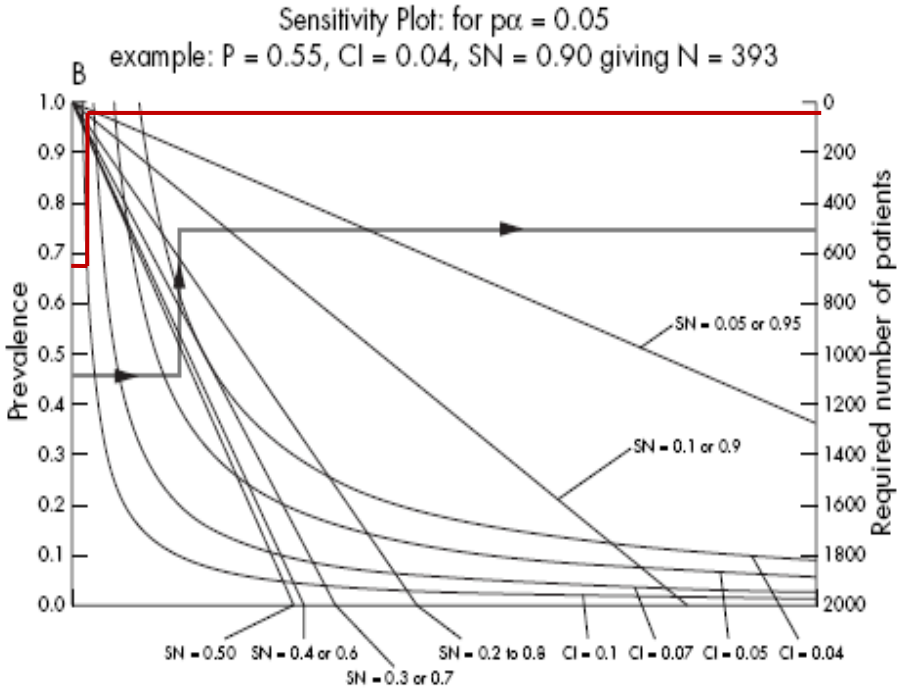
3.5 Sample size estimation

The figure below shows the two by two reporting table for a diagnostic test.

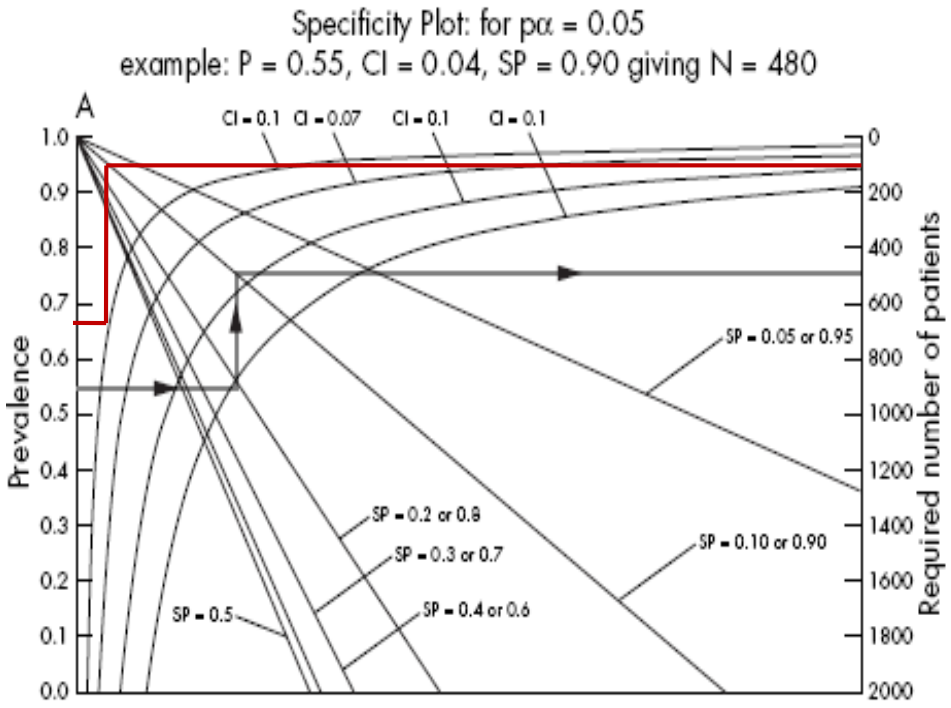
		TB diagnosis by Gold Standard	
		Positive	**Negative**
Elispot result	**Test positive**	**a** True Positive	**b** False positive
	Test negative	**c** False Negative	**d** True Negative

Sensitivity = a/a+c and Specificity = d/d+b

Sensitivity is the proportion of patients with the target disease that have a positive test result. And high sensitivity helps rule out disease. Whereas, specificity is the proportion of patients who do not have the disease and whose test is negative. Using nomograms, the number needed for specificity and sensitivity [74] with a prevalence of about 0.67 from Isabirye's study [51] was as shown below (the red lines indicates the sensitivity and specificity plot respectively):



- The number required for sensitivity of 0.9 with confidence interval of 0.1, and prevalence of 0.67 was 50.



- The number required for specificity of 0.9, confidence interval of 0.1, and prevalence of 0.67 was 100.

So the maximum number needed was 100. But due to lack of adequate funds and time, we recruited 51 patients over a period of eight months for Elispot assay, although only 25 patients had Elispot done. This study was therefore limited by sample size however this was a pilot study.

3.6 Study Protocol

3.6.1 Screening and Recruitment of participants

The Principal Investigator and the research assistant screened patients from TB and pulmonology wards of Mulago Hospital (New Mulago) from Monday to Friday between 10.00 am and 2.00 pm. Patients admitted to pulmonology unit during the study period with a pleural effusion more than 25% of hemithorax on a chest X-ray following chart review were approached and details of the study given. The investigator explained the study objectives to patients with pleural effusion and responded to any questions by the study participants. If the patient was interested in participating in the study, more information regarding the study and risks/benefits were given. Those willing to participate were requested to sign or provide thumb print on the screening consent form (appendix 15a). Questionnaires were administered and a focused history was taken and a physical examination carried out. A diagnostic thoracocentesis was done according to the standard protocol (appendix 10) and the pleural fluid sample and blood were sent for Light's criteria to screen for eligibility. Pleural fluid total and differential white cell counts were done. On the same day, three sputum containers were provided for early morning sputum samples. An HIV antibobody test (ELISA) was done on all patients after counseling and if positive CD4 counts were done. The Mulago HIV serology and counseling services was utilized for this purpose. Patients with exudative effusion by Light's criteria and lymphocyte predominant pleural effusion were given details of a blind closed pleural biopsy using Abrahm's percutaneous pleural biopsy needle. Patients who were willing to continue participation were requested to provide consent for a pleural biopsy (refer to appendix 15b for a pleural biopsy consent form). Pleural biopsy was then done and pleural fluid sample sent to JCRC for Elispot assay. Pleural fluid samples for Elispot were processed within 4 hours of collection of pleural fluid samples. During the initial phase mononuclear cells from pleural fluid were frozen in liquid nitrogen as Elispot kits were not available at the beginning of the study. In the second phase

Elispot assay was done on fresh samples as freezing and thawing during the initial stage resulted in a low cell yield. Pleural biopsy samples were sent for histopathology and ZN stain to Makerere University Pathology laboratory and for MTB culture at NTLP laboratory, Wandegeya. At the same time pleural fluid was sent to the pathology laboratory for cytology.

3.6.2 Data collection procedure

Consenting patients were each assigned a study number for identification. A focused clinical assessment was done using a standardized pre-tested questionnaire (appendix 14) in which the socio-demographic information, relevant history and physical examination findings were recorded.

3.6.3 Study instruments used for data collection

The Principal investigator (PI) and research assistant collected data using pre-tested standardized questionnaires to get information on demographics, past medical history, drug history, clinical findings, procedures and outcome of the procedures. Thermometers, stethoscope, face mask, examination and sterile gloves and sterile Abram's pleural biopsy set were availed for the study. All laboratory request forms were identified using the study numbers. Results of the laboratory tests were entered in patients' questionnaires.

3.6.4 Laboratory examination procedures

Procedure for Sputum Ziehl-Neelsen (ZN) Stain

Direct smear examinations using the ZN staining were performed on sputum samples from consenting patients to determine AFB sputum smear positive TB patients and the standard procedure for ZN stain was followed (appendix 13).

Procedure for pleural tissue histopathology and Hematoxylin and Eosin (H & E) Staining

At least 4 pleural biopsy specimens were sent to Makerere University Pathology laboratory in 10% Formalin solution. At least 4 tiny pieces of pleural tissue were sent from each patient. Paraffin tissue blocks were made and stained with H & E using the protocol in appendix 8.

Procedure for pleural tissue processing for MTB culture

At least 4 pleural biopsy specimens were obtained from each patient and crushed in sterile normal saline solution to make a suspension using a mortar and pestle. The suspension was then sent to NTLP laboratory for TB culture on LJ medium.

Procedure for pleural fluid MTB-specific Elispot assay

Each patient had 50mLs of pleural fluid sent in 50mLs Falcon® tubes, mixed with 2500IU of standard heparin. Samples were sieved using disposable mesh, centrifuged and mixed with RPMI (bicarbonate buffering system) and then send to JCRC Immunology laboratory for further processing (appendix 5). Pleural fluid mononuclear cells were prepared by Ficoll–Hypaque gradient centrifugation from pleural fluid. During the initial phase of the study, cell viability and counts were assessed before freezing them in liquid nitrogen. Samples were frozen if cell counts were more than 1 million and cell viability above 67%. Samples for MTB-specific Elispot were run as a batch at the end of phase one in April 2009. Cell Viability and counts were rechecked after samples were thawed and incubated over night. MTB-specific Elispot assays were performed using testplates from the commercial T-SPOT.TB test if the samples had adequate cell counts and viability. Briefly, 250,000 PEMCs per well were plated overnight on 96-well plates that had been pre-coated with a mouse anti-human IFN gamma antibody. The cells were left unstimulated (negative control), or were stimulated with phytohaemagglutinin (positive control) or ESAT-6 and CFP-10 peptides in separate wells. Culture of the plates, washing, counterstaining, visualization and analysis of the spots were performed according to the manufacturer's recommendations. The response of stimulated cultures was considered positive when the test well contained at least six more spots and had twice the number of spots than the control well.

3.7 Study variables

Study variables included serum and pleural fluid protein, serum and pleural fluid LDH, pleural fluid total and differential white cell counts, pleural fluid ZN stain, sputum smear ZN stain, HIV serology, CD4 count, Elispot assay on pleural fluid mononuclear cells, pleural biopsy tissue histopathology and ZN stain, pleural tissue LJ culture for MTB, pleural fluid cytology. Other variables included patient demographics, past medical history, drug history, physical examination and chest X-ray.

3.7.1 Measurements

3.7.1.1 Clinical Variables

Socio-demographic characteristics: This included age, sex, highest education level attained.

History of symptoms: Respiratory symptoms like cough, chest pain with or without dyspnoea and other symptoms like fevers, drenching night sweats, unintentional weight loss, loss of appetite, and swelling of glands for 2 or more weeks were obtained.

Past Medical history: Risk factors for TB like HIV, Diabetes Mellitus were assessed and we excluded those with chronic history of heart failure/nephrotic syndrome/liver cirrhosis.

Drug history and history of allergies: This was done to assess for intake of medications like anti-tuberculosis medication, history of allergies to drugs like iodine or lignocaine.

Family social history: We assessed for history of close contact with a person who has had cough for more than a month or use of anti-tuberculosis medication in the last one year. History of cigarette smoking and intake of alcohol was also obtained.

Physical examination

General examination was done to obtain clinical data like axillary temperature, pallor, significant peripheral lympadenopathy and pedal edema. Examination of the respiratory system to assess respiratory rate, location of trachea, and chest percussion notes were recorded and chest auscultation

was done. Other examination included measurement of blood pressure and jugular venous pressure, auscultation of heart sounds and any added sounds.

3.7.1.2 Laboratory Variables

This included a postero-anterior view of chest X-ray, pleural fluid and serum proteins / LDH for Light's criteria, pleural fluid total and differential cell counts, sputum ZN stain, HIV serology (ELISA), CD4 counts, pleural tissue histopathology / ZN stain / MTB tissue culture, pleural fluid cytology, pleural fluid Elispot assay.

CHAPTER FOUR

4.1 Data management

Individual patient records and forms were kept in files after data collection. Raw data was stored in a secure place and locked at the discretion of the principal investigator to ensure safety and confidentiality. A data log sheet was created to follow the progress of the study which was reviewed continuously by the supervisors. Data was checked for completeness everyday. Data was entered into Microsoft Excel 2003 and double entered to prevent data entry errors. Data was entered at the end of the day on a daily basis as the patients were recruited. Data was regularly reviewed, cleaned, completed and organized. Data was backed up to prevent data loss. The database was edited continuously and the accuracy of the data entered was checked by cross checking the print of a data set with a random number of questionnaires picked from among the questionnaires.

4.2 Data analysis

Data was entered into Microsoft Excel 2003 and exported to STATA® version 10.0. Descriptive statistics was generated to describe the baseline characteristic of the study population and sub group analysis of those for whom Elispot was done was done separately. Data was presented in the form of frequency distribution tables. Exploratory inferential statistics were generated to determine statistical difference between the groups using Fisher's exact test for dichotomous variables and Wilcoxon rank-sum test for continuous variables. A p value of less than 0.05 was considered significant. Continuous variables were summarized in numbers, medians and interquartile range and categorical variables in numbers, frequencies and percentages. The primary objectives of the study were answered using two by two tables and sensitivity, specificity, positive predictive value, negative predictive value and the likelihood ratios were calculated. Specifically Stata command "diagt" followed by gold standard variable and the test variable was run to obtain the primary objectives of the study.

4.3 Quality control

To ensure quality of results, a number of measures were undertaken which included:

- The questionnaires were pre-tested and standardized before the study was commenced to ensure internal validity.
- The questionnaire were discussed in detail in English and translated to Luganda and then back translated. The questionnaires were checked and edited for completeness at the end of each interview before the PI or research assistant left the patient bed side.
- The principal investigator trained the research assistant, who was a senior medical doctor, on the study concept and how to administer the questionnaires.
- Competent and experienced laboratory technologists were used to carry out the laboratory procedures and reliable laboratories were used.

4.4 Ethical considerations

Ethical approval to carry out the study was sought from the Department of Medicine of Makerere University, the Faculty of Medicine Research and Ethics Committee, and the Mulago hospital ethics committee. Written/thumbprint informed consents were obtained from all participants before screening and before the pleural biopsy in the language they understood. All benefits and potential risks of the study were clearly explained to the participants before obtaining the recruitment consent. Participants were allowed to withdraw from the study at any time as they wish without impacting their care. All information given was kept confidential by use of patient's initials and identification numbers and not names. Access to data or patient information was limited to those directly involved in the study and the attending clinician. Results of pleural fluid analysis, pleural tissue histopathology and MTB tissue cultures were given to the clinician in-charge of the patient on the unit. Results of TB culture were communicated to the patient through contact number provided on

consent forms by each patient. Elispot assay results were not returned to patients or clinicians because its validity for diagnosis of pleural TB was not known, but was being assessed in this study.

4.5 Follow up

Study subjects were requested to return for follow up at two months when the culture results were obtained. Those who failed to turn up were contacted through the phone number they provided in the consent forms.

4.6 Benefits of the study

- Laboratory tests were done at no cost to the patient.
- Results of all laboratory tests were availed to the clinician in charge of the patient as soon as they were available.
- The findings from this study are expected to help improve the management and diagnosis of TB pleural effusion in Uganda and elsewhere in the world.

4.7 Risks from study

There were no added risks to the patients from this study. The procedures like thoracocentesis and pleural biopsy were part of the usual diagnostic protocol for patients with pleural effusion in Mulago hospital pulmonology unit.

4.8 Dissemination of results

The results of the study shall be made available to the Department of Internal Medicine, Makerere University; School of Postgraduate studies, Makerere University; Sir Albert Cook library, Makerere Medical University; Uganda National Council of Science and Technology; clinicians taking care of the study patients and Mulago hospital TB unit.

CHAPTER FIVE

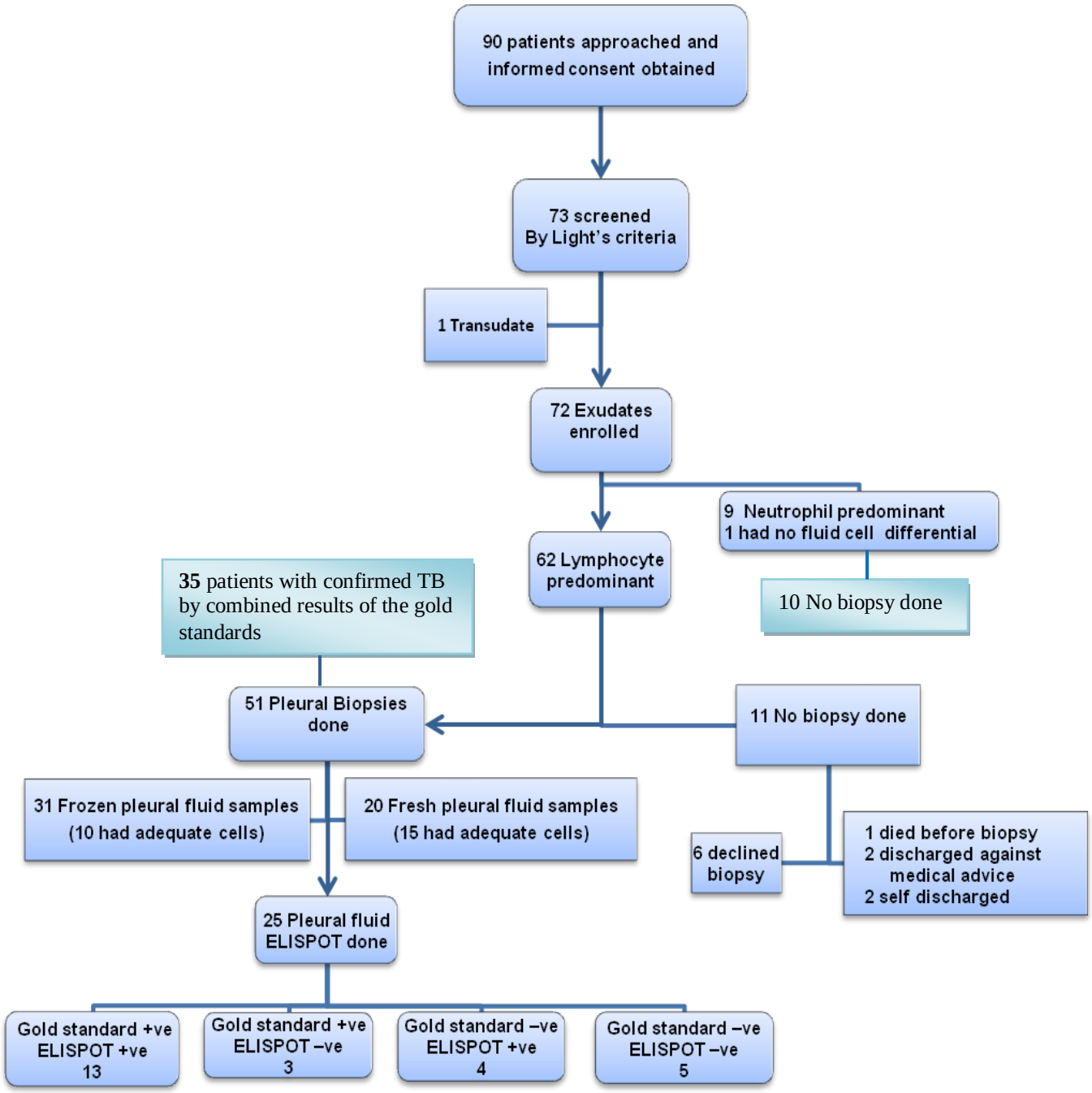
5.0 Results

5.1 Description of Study Process

From October 2008 to April 2009 and in June 2009, we approached 90 patients with pleural effusion on the basis of clinical features and chest X-ray. Informed consents were obtained and seventy three patients who fulfilled the criteria for suspected tuberculous pleural effusion were screened. The rest 17 were not screened as they had pyothorax at initial thoracocentesis done by clinician in-charge of the ward or were already on anti-TB medications or were mentally confused at initial evaluation. Seventy two patients had exudative pleural effusion by Light's criteria. One patient had transudative effusion. Among the seventy two exudates, sixty two (86%) had lymphocyte predominant effusion, 9 (12.5%) had neutrophil predominance and 1 had no differential cell counts done. Out of the 62 exudative lymphocyte predominant pleural effusions, 51 (82.3%) underwent pleural biopsy for histopathology with Haematoxylin and Eosin (H&E) staining and MTB tissue culture after obtaining consent for the procedure. In addition, pleural fluid samples were evaluated for MTB-specific Elispot assay using the commercial T-SPOT.TB. The pleural fluid samples had mononuclear cell extraction, viability assessment and cell counts.

This study was done in two phases. Thirty one pleural biopsies were done in the initial phase from October 2008 to April 2009 and pleural fluid Elispot done on stored frozen and thawed samples. Out of the 31 samples, 10 had good cell viability of above 67% and cell counts above 1,000,000. Due to the low cell yield in this phase, the study was halted and arrangements to do Elispot assay on fresh pleural fluid samples were made. In the second phase in June 2009, we subjected an additional 20 pleural fluid samples to Elispot assay on fresh pleural fluid mononuclear cells. Of the 20, 15 had adequate cell counts and viability. Thus, a total of 51 pleural biopsies were done, 51 pleural fluid samples were processed for Elispot assay and 25 pleural fluid Elispot assays were done (Figure 1).

Figure 1: Study Flow Diagram



5.2 Diagnosis of TB in the 51 patients who had pleural biopsies done

Table 1: Cross tabulation of pleural biopsy histopathology against pleural biopsy tissue MTB culture by LJ media.

	Pleural biopsy tissue culture for MTB			Total
	Positive	**Negative**	**Contaminated**	
Pleural biopsy Histopathology Positive	17 (73.9%)	11 (40.7%)	1 (100%)	**29 (56.8%)**
Negative	6 (26.1%)	16 (31.4%)	0 (0%)	**22 (43.1%)**
Total	**23 (45%)**	**27 (52.9%)**	**1 (0.2%)**	**51**

We used pleural tissue histopathology and pleural tissue cultures for TB as gold standards for the diagnosis of tuberculous pleural effusion. A patient had confirmed pleural tuberculosis if any or both the gold standards were positive. Of the 73 patients screened, 51 had pleural biopsies done. Thirty-five (68.6%) had confirmed diagnosis of tuberculous pleural effusion by the combined gold standards. Twenty nine (56.8%) patients had positive histopathology and 23 (45%) had positive culture.

Sixteen (31.4%) out of the 51 patients who had pleural biopsy had no TB by the combined results of the two gold standards. These patients had other diagnoses including bronchogenic carcinoma (n=5), lymphoma (n=3), cryptococcosis (n=1), Kaposi's sarcoma (n=2), and other (n=5). These patients were referred to the relevant units for further managaement. The patients with diagnosis "other" had chronic inflammation from pleural tissue histopathology; sputum smears were negative for AFBs and pleural tissue cultures were negative for TB.

The prevalence of confirmed TB pleural effusion among the patients who had exudative and lymphocyte predominant pleural effusion was 35 (68.6%) i.e. 29 positive histopathology plus 6 positive culture with negative histopathology.

5.3 Characteristics of patients with and without TB pleural effusion

5.3.1 Demographic, clinical and laboratory characteristics of 51 TB pleural effusion suspects by pleural biopsy results.

Overall, the two groups of patients with and without TB pleural effusion were similar in most of the characteristics. However, the patients with TB pleural effusion had higher axillary temperature, and tended to be more likely to have a cough than the non-TB patients (table 2). Twenty one (60%) patients with TB had a temperature greater than 37.4◦C compared to 5 (31.2%) without TB, p = 0.05.

Table 2: Demographic and clinical characteristics by TB diagnosis

Characteristics		Pleural TB n (%) (N=35)	No pleural TB n (%) (N=16)	P value
Age (years)	Median IQR	34 14 (28-42)	41.5 28 (28-56)	0.1
Females		13 (37.1%)	8 (50%)	0.39
Cough		33 (94.3%)	12 (75%)	0.07
Duration of cough >2 weeks		32 (97%)	11 (91.7%)	0.2
Weight loss		29 (82.9%)	14 (87.5%)	1.0
Fever		29 (82.9%)	10 (62.5%)	0.11
Excessive sweats		24 (68.6%)	8 (50%)	0.2
Chest pain		32 (91.4%)	16 (100%)	0.54
Dyspnoea		26 (74.3%)	14 (87.5)	0.47
Smoking		8 (22.9%)	2 (12.5%)	0.47
Alcohol		10 (28.6%)	5 (31.3%)	0.8
Karnofsky score	Median IQR	70 20	70 20	0.8
Left sided effusion		20 (57.1%)	7 (43.7)	0.37
Moderate effusion		25 (71.4%)	9 (56.3%)	0.3
Temperature >37.4		21 (60%)	5 (31.2%)	0.05
Respiratory rate >20		28 (80%)	10 (62.5%)	0.18

Patients with TB were younger with median age of 34 years (range of 28-42 years) compared to non-TB patients with a median age of 41 years (range of 28-56 years). Also more males had TB than females, while effusion tended more towards the left side. However, these were not statistically significant.

Table 3: Laboratory characteristics by TB diagnosis

Overall, the patients were similar in laboratory characteristics. In particular, patients with TB pleurisy had no difference with the non-TB group with regards to HIV positivity and CD4 cell counts (table 3).

Characteristics		Pleural TB n (%) (N=35)	No TB, n (%) (N=16)	P value
Sputum positive		5 (14.3%)	0 (0%)	0.1
HIV positive		20 (57.1%)	9 (56.2%)	0.9
Positive Pleural fluid ZN		5 (14.3%)	0 (0%)	0.1
Pleural biopsy tissue ZN		9 (25.7%)	0 (0%)	0.02
Pleural fluid lymphocyte %	Median	88	91	0.17
	IQR	10	5	
CD4 counts	n	19	9	
<101		8 (42.1%)	3 (33.3%)	1.0
101-200		4 (21.05%)	3 (33.3%)	0.6
201-350		5 (26.3%)	2 (22.2%)	1.0
>350		2 (10.5%)	1 (11.1%)	1.0

Fourteen percent of patients who had TB had pulmonary TB and 14 % had AFBs in pleural fluid. Many patients with TB pleurisy had CD4 counts of 100 cells/µL and below.

Five (n=51) patients had positive pleural fluid ZN stain, all of them had positive histopathology for TB and positive pleural tissue ZN stain. Four among the 5 had positive tissue culture for TB and the one with a negative tissue culture for TB was sputum smear negative. Two of the five patients were sputum smear positive TB.

Among the five sputum smear positive TB cases, two had positive pleural fluid ZN stain.

5.3.2 Characteristics of patients who had Elispot assay done

Table 4 portrays the clinical and laboratory characteristics of patients who were subjected to Elispot assay (n=25). Pleural fluid mononuclear cells were prepared by Ficoll–Hypaque gradient centrifugation from pleural fluid. In total, 16 (64%) had TB pleural effusion by the gold standards. Ten (40%) were females. Six (60%) of the females and 10 (66.7%) of the males had TB.

Fourteen (56%) were HIV positive. Among the HIV negative patients (n=11), 7 (63.6%) had TB and all the rest had adenocarcinoma. Out of the fourteen HIV positive patients, 9 (64%) had TB. Other diagnoses among the HIV positive patients included Burkitt's lymphoma, Kaposi's sarcoma, Cryptococcosis, acute pyogenic infection, Hodgkin's lymphoma and chronic inflammation (numbers for each other diagnoses were 1). However the patient with a diagnosis of acute pyogenic infection on histopathology had positive tissue culture for TB and Elispot was positive. There was no association between CD4 counts, percentage of lymphocyte in pleural fluid or the number of spots in either ESAT or CFP antigen wells.

Three (12%) who had positive pleural fluid ZN stain also had positive pleural tissue ZN stain and TB by histopathology; two had positive TB cultures; all of them were sputum smear negative; two had no response to both ESAT and CFP antigens. In total, six (24%) had positive pleural tissue ZN stain. There was no association between pleural fluid ZN stain and HIV serostatus.

Eight (32%) had TB by tissue culture; seven (87%) of eight patients also had TB by histopathology.

Table 4: Description of 25 patients who had MTB-specific Elispot assay done

Patient	Age (yrs)	Sex	HIV	CD4 count	% Lymph in PF	Sputum smear ZN	PF ZN	Histopathology	Tissue TB culture	TB Pleural effusion by gold standards	Tissue ZN	ESAT-6 PEMCs	CFP-10 PEMCs	PF ELISPOT result
1	46	Male	Positive	17	70	Negative	Negative	TB	Negative	Positive	Negative	3000	1353	Positive
2	21	Male	Negative		95	Negative	Negative	TB	Negative	Positive	Negative	3000	1511	Positive
3	61	Female	Negative		98	Negative	Positive	TB	Negative	Positive	Positive	3000	586	Positive
4	32	Male	Negative		88	Negative	Negative	TB	Negative	Positive	Negative	1777	1462	Positive
5	44	Female	Positive	329	96	Negative	Negative	TB	Negative	Positive	Positive	1279	1298	Positive
6	22	Female	Positive	550	85	Negative	Negative	TB	Negative	Positive	Negative	1178	752	Positive
7	49	Male	Negative		60	Negative	Negative	TB	Negative	Positive	Negative	1137	1172	Positive
8	36	Male	Positive	19	85	Negative	Negative	TB	Positive	Positive	Negative	791	312	Positive
9	34	Male	Positive	567	96	Negative	Negative	TB	Positive	Positive	Negative	760	710	Positive
10	21	Female	Negative		70	Positive	Negative	TB	Positive	Positive	Positive	637	331	Positive
11	28	Female	Negative		95	Negative	Negative	TB	Positive	Positive	Negative	541	282	Positive
12	42	Male	Positive	157	95	Negative	Negative	TB	Positive	Positive	Positive	147	761	Positive
13	42	Female	Positive	17	90	Negative	Negative	TB	Negative	Positive	Negative	37	29	Negative
14	30	Male	Positive	42	80	Negative	Positive	TB	Positive	Positive	Positive	0	0	Negative
15	30	Male	Negative		60	Negative	Positive	TB	Positive	Positive	Positive	0	0	Negative
16	35	Male	Positive	311	90	Negative	Negative	Acute pyogenic infection	Positive	Positive	Negative	789	1048	Positive
17	62	Male	Negative		92	Negative	Negative	Adenocarcinoma	Negative	Negative	Negative	586	392	Negative
18	34	Female	Positive	640	90	Negative	Negative	Burkitt's Lymphoma	Negative	Negative	Negative	436	3000	Positive
19	35	Female	Positive	60	90	Negative	Negative	Kaposi's Sarcoma	Negative	Negative	Negative	407	881	Positive
20	50	Male	Negative		96	Negative	Negative	Adenocarcinoma	Negative	Negative	Negative	342	27	Positive
21	27	Male	Positive	237	98	Negative	Negative	Hodgkin's Lymphoma	Negative	Negative	Negative	142	16	Positive
22	38	Female	Positive	60	90	Negative	Negative	Cryptococcosis	Negative	Negative	Negative	34	21	Negative
23	29	Male	Positive	165	98	Negative	Negative	Chronic inflammation	Negative	Negative	Negative	28	28	Negative
24	45	Male	Negative		60	Negative	Negative	Adenocarcinoma	Negative	Negative	Negative	8	4	Negative
25	60	Female	Negative		80	Negative	Negative	Adenocarcinoma	Negative	Negative	Negative	3	1	Negative

Elispot data are presented as the absolute numbers of spot-forming cells/250,000 pleural effusion mononuclear cells (PEMCs) on interferon gamma Elispot.
PF = pleural fluid
Patient 17 had a negative Elispot despite many spots in ESAT and CFP wells as the negative well had more spots than ESAT or CFP wells.
Patients were classified as having TB pleural effusion if either the pleural tissue histopathology or tissue culture revealed features of TB. So patient 16 was classified as TB.

5.4 Elispot wells of 25 patients from the study

Of the 51 patients with pleural biopsies, 25 had viable and adequate cells for Elispot. The remaining 26 had non-viable or inadequate cell counts. So Elispot was not done.

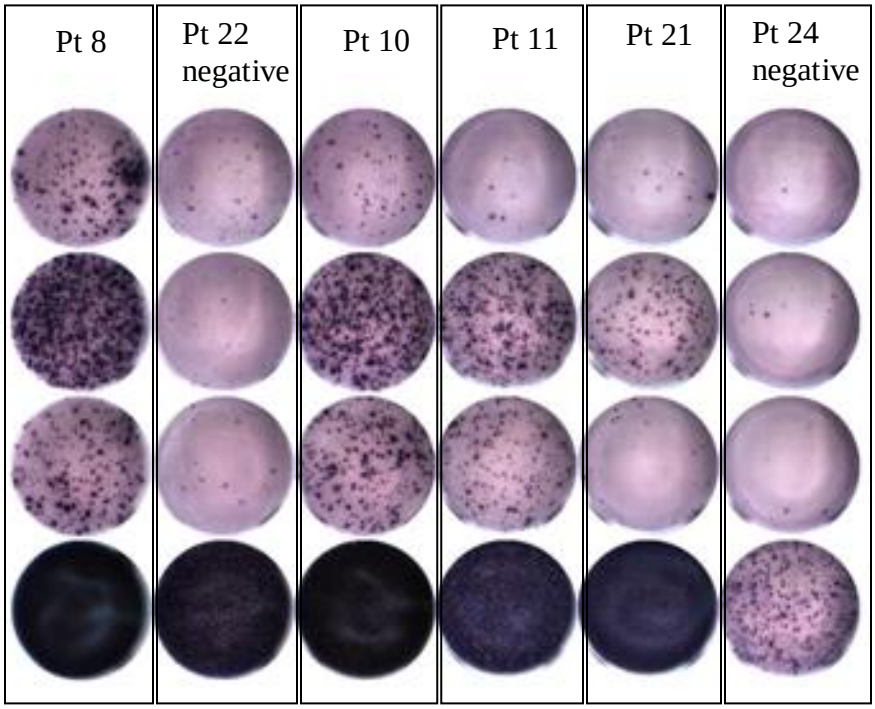
Figure 2 shows the Elispot results of the first 10 patients who had Elispot assay done on frozen and thawed pleural fluid mononuclear cells. These numbers correspond to those in table 4.

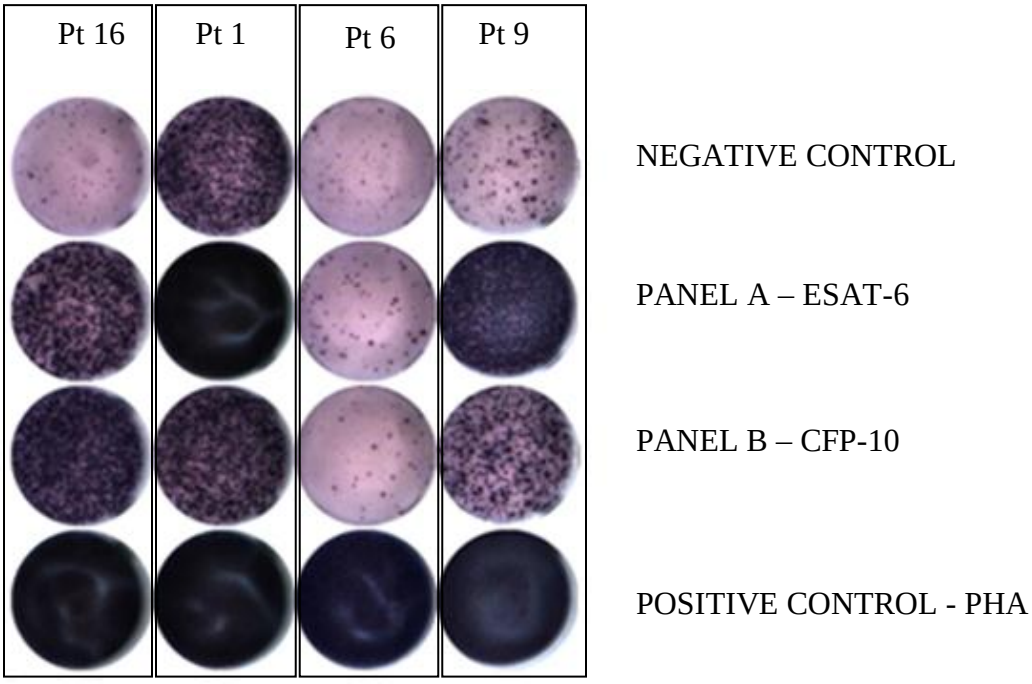
Figure 3 shows the Elispot results of the last 15 patients who had Elispot assay done on fresh pleural fluid mononuclear cells. These numbers correspond to those in table 4.

Of note, many patients had RBC contamination of the pleural fluid, which may have given a high background in the negative control well. Only 5 samples had spots less than 10 in the negative control well and 4 of them were Elispot negative. Patient 15 seems to have no spots in the positive control well as per the picture below due to low clarity; however this patient had 22 spots in the positive well as read by the immunospot reader after eliminating artefacts.

Figure 2: Photograph of Elispot wells from the first 10 patients, done on frozen and thawed pleural fluid samples.

Patients 22 and 24 were negative for T-SPOT.TB. The rest were positive.

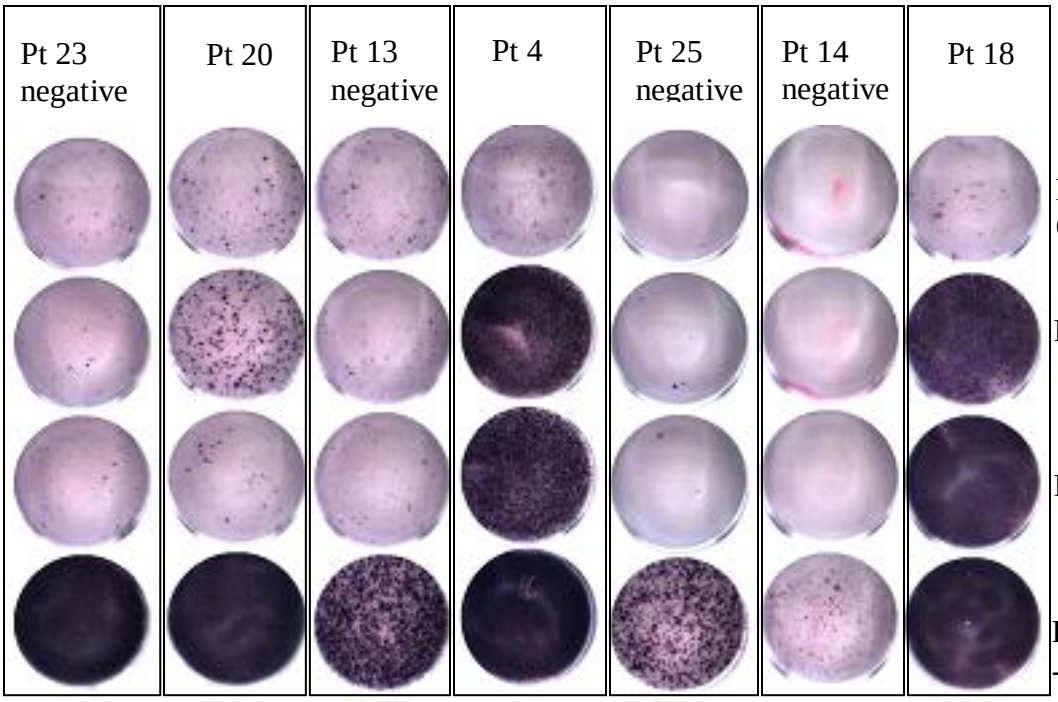


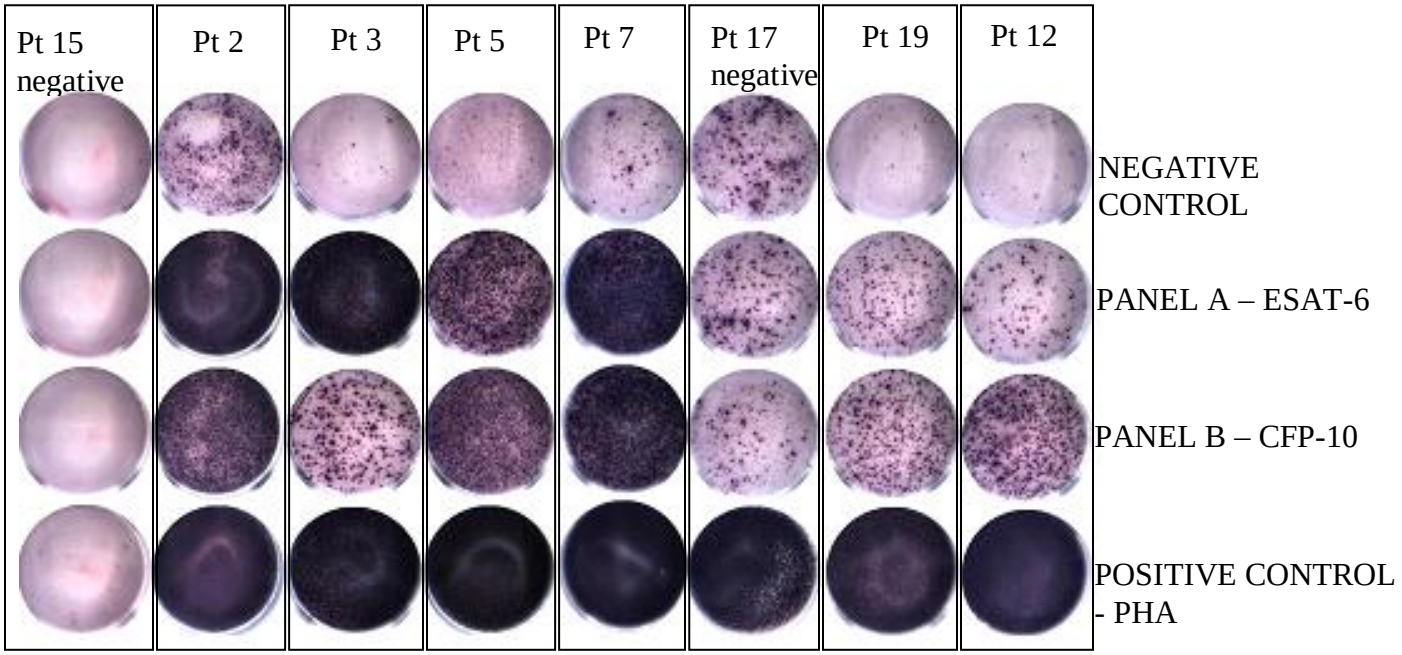


PHA - Phytohaemagglutinin

Figure 3: Photograph of Elispot wells from the last 15 patients, done on fresh pleural fluid samples.

Patients 23, 13, 25, 14, 15 and 17 had negative Elispot results. The rest were positive.

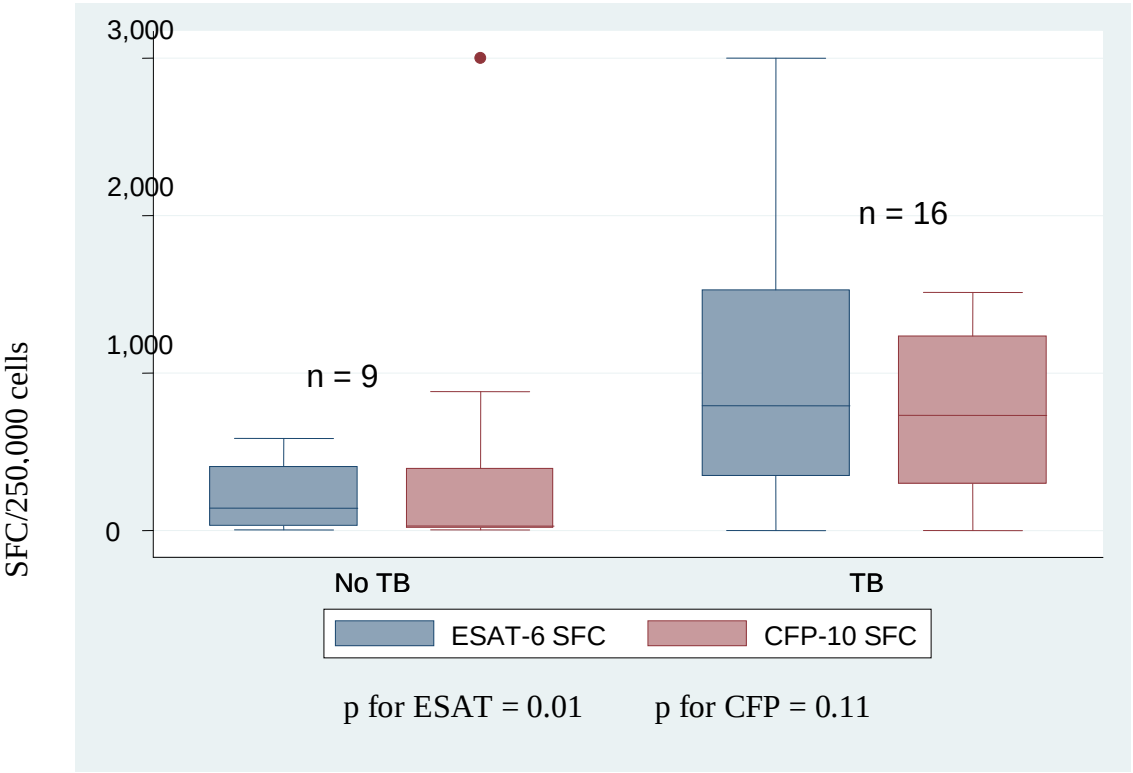




5.5 Comparison of Spot Forming Cells for ESAT-6 and CFP-10 antigens

Comparison of Spot Forming Cells (SFC) for ESAT-6 and CFP-10 antigens in pleural fluid mononuclear cells for patients with and without tuberculous pleural effusion (n=25) is shown in figure 4.

Figure 4: Box plot of Spot Forming Cells for ESAT-6 and CFP-10 antigens by Tuberculous pleural effusion status defined by gold standard (n=25).



The median SFC for ESAT-6 among those with TB pleurisy (n=16) was 790 (IQR 1184) compared to 142 (IQR 379) among those without TB pleurisy (n=9) and p value of 0.01. The median SFC for CFP-10 among those with TB and those without TB was not statistically different although there was a trend towards TB patients having more CFP spots (731, IQR 938) compared to non-TB patients (27, IQR 938) with a p value of 0.11.

5.6 Diagnostic value of pleural fluid MTB-specific Elispot assay

Using the combined results of pleural biopsy histopathology (H & E) and pleural biopsy tissue culture for TB as the gold standards, the accuracy of pleural fluid Elispot was determined by calculating the sensitivity (TP/TP+FN), specificity (TN/TN+FP), positive predictive value (TP/TP+FP), negative predictive value (TN/TN+FN) and likelihood ratios (table 5 and 6) where, TP = true positive, TN = true negative, FP = false positive, FN = false negative.

Table 5: Two by two table for gold standard (either histopathology or pleural biopsy tissue culture) and Elispot assay.

		Gold Standard		**Total**
		Positive	**Negative**	
Pleural fluid Elispot	**Positive**	13 (52%)	4 (16%)	**17**
	Negative	3 (12%)	5 (20%)	**8**
Total		**16**	**9**	**25**

From table 5, the diagnostic value of MTB-specific Elispot assay can be derived as follows:

Sensitivity = 13/16 = 81.3%

Specificity = 5/9 = 55.6%

Positive predictive value = 13/17 = 76.5%

Negative predictive value = 5/8 = 62.5%

Positive Likelihood ratio = Sensitivity/ (1-Specificity) = 0.813/0.444 = 1.83

Negative Likelihood ratio = (1-Sensitivity)/ Specificity = 0.187/0.556 = 0.34

ROC (Receiver Operator Characteristic) area = (Sensitivity+Specificity)/ 2 = (0.813+0.556)/2

= 0.684

Table 6: Table showing the prevalence and 95% Confidence Intervals of the various performance indices for Elispot assay compared to the gold standards.

		95% Confidence Interval	
Prevalence	64%	43%	82%
Sensitivity	81.3%	54.4%	96%
Specificity	55.6%	21.2%	86.3%
ROC area	0.684	0.486	0.883
Likelihood ratio (+)	1.83	0.849	3.94
Likelihood ratio (-)	0.338	0.104	1.09
Odds Ratio	5.42	0.947	31.2
PPV	76.5%	50.1%	93.2%
NPV	62.5%	24.5%	91.5%

The paired results of the 25 patients who had Elispot done, were subjected to statistical evaluation using a two by two table (table 6) to answer objectives 1 and 2 of the study. The prevalence of TB among the 25 patients was 64% with sensitivity of 81% and specificity of 55.6%. The 95% confidence intervals of the indices are as shown in table 6.

CHAPTER SIX

6.0 Discussion

6.1 Summary of study findings

We enrolled 72 patients with exudative pleural effusion and 51 patients with exudative lymphocyte predominant effusion had pleural biopsies done. Thirty five had confirmed TB while twenty five had Elispot done.

1. The sensitivity and specificity of MTB-specific Elispot assay were 81.3% and 55.6% respectively.
2. The positive predictive value, negative predictive value, positive and negative likelihood ratios of MTB-specific Elispot assay were 76.5%, 62.5%, 1.83 and 0.338 respectively.

6.2 Sensitivity and Specificity

In this study, the sensitivity of MTB-specific pleural fluid Elispot assay using T-SPOT.TB kit was low. Sensitivity is the proportion of patients with confirmed tuberculous pleural effusion who are positive by Elispot. Though data regarding the diagnostic accuracy of IGRAs for active extrapulmonary TB in general is limited, subgroup analysis of studies has demonstrated a sensitivity of 96-100% for Elispot-based tests [62, 75, 76]. In a recent study done in a low TB endemic area in Europe, the sensitivity of T-SPOT.TB on pleural fluid for diagnosis of tuberculous pleural effusion was 95 % [54]. Most recent study done in Taiwan, which has a high TB burden like Africa, showed a sensitivity of 94.7% when Elispot was performed on pleural fluid [77]. When compared to these previous studies, the sensitivity of Elispot was low in this study.

The diagnostic specificity of T-SPOT.TB in this study was low. Specificity is the proportion of individuals without tuberculous pleural effusion who are negative by Elispot assay. Thus, one out of every 2 patients had a false-positive result. The 95% Confidence Interval for specificity for TB Elispot was also very wide. There are no published data available from Uganda to validate the utility

of MTB specific Elispot assay where TB is endemic. A meta-analysis done by Jiang et al in 2007 showed a sensitivity ranging from 64 to 100% and specificity ranging from 86 to 100% [55, 56]. Most studies have shown low specificity for TB Elispot though these studies were done in low TB endemic areas outside Africa. Data from a study conducted in Europe showed a specificity of 76% [54]. Most recent published data from Taiwan demonstrated a specificity of 85.7% [77]. The specificity for elispot in this study was much lower than these previous studies.

The low sensitivity and specificity of the commercial T-SPOT.TB assay for the diagnosis of tuberculous pleural effusion could be explained due to the fact that probably Elispot is positive in those with a positive Tuberculin Skin Test (TST) i.e. it is unable to distinguish between infection and disease. In this study, four patients had a positive Elispot when the gold standards confirmed no TB. In Uganda, TB prevalence is high and many people are exposed. These people may have a positive Elispot without having active TB. For diagnosis of active TB pleurisy, a positive Elispot and TST will be most helpful where the pre-test probability of active TB is high and the pre-test probability of latent infection is low. This applies best to persons with clinical manifestations of active TB who lack risk factors of exposure to M. tuberculosis [79]. But this is not a situation that applies to individuals from countries with a high incidence of TB. It is also possible that these four patients who had a positive Elispot but no TB by reference standards could have had latent TB infection as they all had malignancies which are risk factors for latent TB. Also not all pleural biopsy specimens contain the TB granulomas, thus giving a negative histopathology and culture, resulting into many false positive Elispot results (thus low specificity). So there is a possibility that the four patients may have had malignancy and active TB at the same time. However, this could not be established as these patients were not followed up. This study was limited by sample size. Therefore, in future larger studies may provide a more accurate data about the diagnostic accuracy of pleural fluid Elispot for TB pleurisy.

6.3 Predictive values and Diagnostic Likelihood Ratios (DLRs)

The PPV and NPV in this study were 76.5% and 62.5%. Positive predictive value of MTB-specific Elispot assay is the proportion of patients with positive Elispot who are correctly diagnosed. It reflects the probability that a positive test reflects the underlying condition being tested for. Its value depends on the prevalence of the disease. Negative predictive value is the proportion of patients with negative elispot who are correctly diagnosed. Therefore, if a patient has a positive Elispot, there is 76.5% chance of having TB or a quarter does not have TB. But a negative predictive value of 62.5% means that 37.5% of patients with a negative Elispot actually have TB i.e. more than a third with negative test will actually have TB. Three patients, in the study, with TB had a false negative Elispot possibly because two of the patients were HIV positive and had CD4 counts below 50 cells/µL, although in a study by Lawn et al on peripheral blood, Elispot response was found to be independent of CD4 counts [(57)]. The third patient was HIV negative however had a low pleural fluid lymphocyte count.

Most studies done in low TB endemic areas do not report on the predictive values and DLRs due to the limited number of cases. According to the recent study in Taiwan, the PPV and NPV were 85.7% and 94.7% [(77)] for pleural fluid Elispot.

Our data demonstrate that a negative Elispot does not rule out TB but a positive result may be suggestive of TB. This is not in concordance with the findings from study in Taiwan.

The likelihood ratio indicates the value of the test for increasing certainty about a positive diagnosis. The “positive likelihood ratio” (LR+) tells us how much to increase the probability of disease if the test is positive, while the “negative likelihood ratio” (LR-) tells us how much to decrease it if the test is negative. A value more than 10 helps rule in disease and a value less than 0.1 helps rule out disease [(78)]. In this study, the positive and negative likelihood ratios were 1.83 and 0.34 respectively. Therefore, there is only a minimal increase in the likelihood of having TB with a positive Elispot and

a small decrease in the likelihood of having TB with a negative Elispot. So a positive Elispot may just mean past TB exposure and not disease. Hence the need to incorporate TSTs in future studies to differentiate infection from disease among those with a positive Elispot.

6.4 Area Under ROC Curve (AUC)

ROC curve is a graphical plot of the sensitivity vs. (1- specificity). When the "diagt" command was run in STATA® the ROC area was 0.68. Although the AUC is above 0.50, it is not high enough to give the Elispot assay a good predictive power for diagnosing TB. A figure of 0.8 and above would have been better in predicting TB.

6.5 Feasibility of pleural fluid Elispot in patients with suspected tuberculous pleural effusion in our setting

The Elispot assay may be useful among patients with clinically suspected tuberculous pleural effusion who cannot undergo pleural biopsy (eg bleeding tendencies, patients on anticoagulant therapy, patients with dementia who cannot cooperate, those on mechanical ventilation, and those with small pleural effusions where biopsy is difficult or unsafe [77].

From clinicians' perspective, Elispot assay results are available overnight [77] and hence a short turn over time unlike histopathology.

6.6 Other findings from the study

Clinical and laboratory characteristics were similar in those with TB compared to those without TB.

TB predominantly caused left sided pleural effusion, unlike other studies which showed right sided effusion [8].

Sputum smear for AFBs by ZN staining was positive in 14.5% with TB. It is reported that sputum smears are rarely positive in primary cases of TB. In contrast, sputum smear is positive in 50% of reactivation cases of TB [73].

The prevalence of TB among those with exudative lymphocyte predominant effusion was 68.6%. This was slighltly higher than Isabirye's study (66.7%) [51].

6.7 Conclusions

1. The diagnostic accuracy of commercial (T-SPOT.TB) Elispot assay is low when used alone. However, it may be a clinically useful adjunct test for diagnosing extrapulmonary TB.
2. The prevalence of TB pleural effusion among exudative lymphocyte predominant effusions was 68.6%, which was similar to Isabirye's study [51].
3. The median spot forming cell counts were significantly higher in patients with TB compared to those without for ESAT-6 antigen. The difference was not significant for CFP-10 antigen.

6.8 Recommendations

Based on the data from this study, Elispot assay may have a low diagnostic utility for TB pleurisy. Larger studies are needed in our setting to get more accurate data regarding the sensitivity and specificity of Elispot assay on pleural fluid. Future studies should also evaluate the cost-benefit of the test. Further studies should carefully consider procedures for collecting, processing and storing pleural fluid mononuclear cells for Elispot assay. This may mean doing assays fresh in the future or require further modifications in specimen processing to increase cell yield. Doing assays on fresh samples may not be plausible due to the pre-designed kits with 8 wells per plate.

6.9 Study Limitations

1. Although 51 pleural biopsies were done in this study, Elispot assay numbers were only 25 due to the low cell yield in the initial phase of the study due to unclear reason. So this study was limited by sample size.
2. Many samples had RBCs, though we do not have record of the red blood cell content in each sample of pleural fluid where Elispot was done. This could have led to false positive Elispot for pleural fluid due to the mixing of peripheral blood mononuclear cells because most Africans are exposed to TB.
3. TB sometimes presents with a small pleural effusion. In this study we excluded small pleural effusions as it was difficult to perform a blind pleural biopsy on such patients after removing a significant amount of the fluid for Elispot assay
4. The pleural fluid mononuclear cell counts and viability were significantly reduced after freezing and thawing the samples. The exact reason for this remains unclear.

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APPENDICES

APPENDIX 1: Performance status (Karnofsky scale)

Functional status	%	Characteristics
Able to carry on normal activity, no special care is needed.	100	Normal; no complaints; no evidence of disease.
	90	Able to carry on normal activity; minor signs or symptoms of disease.
	80	Normal activity with effort; some signs or symptoms of disease
Unable to work; able to live at home and care for most personal needs; a varying amount of assistance is needed	70	Cares for self; unable to carry on normal activity or to do active work.
	60	Requires occasional assistance but is able to care for most needs.
	50	Requires considerable assistance and frequent medical care.
Unable to care for self; requires equivalent of institutional or hospital care; disease may be progressing rapidly	40	Disabled; requires special care and assistance
	30	Severely disabled; hospitalization is indicated though death not imminent
	20	Very sick; hospitalization necessary; active supportive treatment necessary
	10	Moribund; fatal processes progressing rapidly
	0	Dead.

APPENDIX 2: Lights criteria

Exudates are defined by the presence of at least one of the following criteria:
(1) Pleural fluid–serum protein ratio greater than 0.5
(2) Pleural fluid–serum LDH ratio greater than 0.6, and
(3) Pleural fluid LDH concentration greater than 200 IU/L.

APPENDIX 3: Classes of Parapneumonic effusion

Class	Description	Features	Treatment
1	Insignificant parapneumonic effusion	Small <10mm thick on decubitus CXR	Antibiotics (no thoracocentesis)
2	Typical parapneumonic effusion	≥10mm thick Glucose >40 mg/dl pH > 7.20 Gram stain and culture negative	Antibiotics alone
3	Boderline complicated parapneumonic effusion	pH 7.0-7.2 and/or LDH> 1000IU/L Glucose > 40 mg/dl Gram stain and culture negative	Antibiotics plus serial thoracocentesis If loculated, consider small chest tube +/- thrombolytics
4	Simple complicated parapneumonic effusion	pH <7.00 and/or glucose < 40 mg/dl and/or Gram stain or culture positive No loculations	Small chest tube plus antibiotics
5	Complex complicated parapneumonic effusion	Same as Class 4 but multi loculated	Antibiotics plus thrombolytics via chest tube
6	Simple empyema	Frank pus present Single locule or free flowing	Antibiotics and large chest tube +/- decortication
7	Complex empyema	Frank pus present Multiple locules	Antibiotics plus thrombolytics via large chest tube; often requires thoracoscopy or decortication.

Source: Light RW. A new classification of parapneumonic effusions and empyema. Chest 1995; 108:299-301.

APPENDIX 4: Thoracocentesis: Indications, Contraindications and Complications

Indications:

1. Diagnostic purposes
2. Instillation of sclerosing agent
3. To relieve dyspnoea

Contraindications: none absolute. Relative contra indications include:

1. Bleeding diathesis
2. Mechanical ventilation
3. Uncooperative patient

Complications:

1. Pneumothorax (10% of patients develop pneumothorax)
2. Hemothorax

APPENDIX 5: Elispot procedure (JCRC, Uganda)

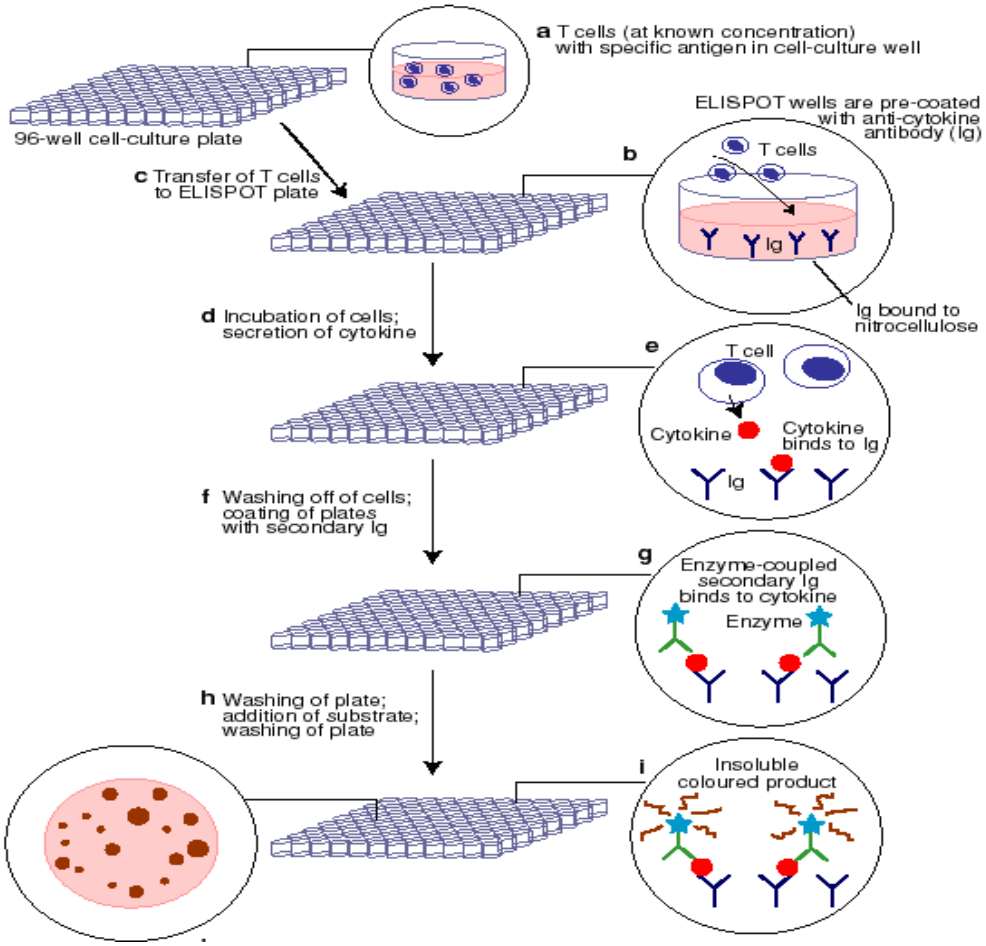
Procedure:

1. Prepare Pleural fluid mononuclear cells by Fycoll-Hypaque centrifugation gradient method.
2. Adjust pleural effusion mononuclear cells (PEMC) to 2.5 $X10^5$ in complete RPMI.
3. Four wells will be set for each patient.
4. Cover the rest of the plate with adhesive paper.
5. Add cell culture medium to each nil well (negative control).
6. Add 50 µl positive control solution to each cell viability control well.
7. Add 50 µl Panel A solution to each panel A well.
8. Add 50 µl Panel B solution to each panel B well.
9. Add 100 µl of PEMC to each of the duplicate wells (2.5 X 10^5 cells).
10. Incubate at 37°C 5% CO_2 for 16-20hrs. Avoid shaking the plate while in incubator.

Spot Developing:

11. Remove the plate from the incubator and discard the culture media (at this time remove substrate from the refrigerator and allow it to warm).
12. Wash four times with 200 µl of PBS.
13. Dilute stock conjugate 200 fold with PBS to make working solution (50 µl in 10 mL).
14. Add 50 µl of diluted conjugate to each well.
15. Incubate at 2-8°C for 1 hr.
16. Wash four times with PBS.
17. Flip out the remaining PBS and beat the plate on paper towels.
18. Add 50 µl of substrate working solutions.
19. Incubate at room temperature for 7 mins.
20. Thoroughly wash the plate with distilled or de-ionized water to stop the reaction.
21. Allow plate to dry overnight at room temperature or for 2-3 hrs at 37°C.
22. Count the number of spots.

APPENDIX 6: Diagram illustrating the steps in Elispot assay



Source: http://microvet.arizona.edu/Courses/MIC419/ToolBox/elispot.html

Either a monoclonal or polyclonal capture antibody is coated aseptically onto a polyvinylidene fluoride (PVDF)-backed microplate. These antibodies are chosen for their specificity for the analyte in question. The plate is blocked, usually with a serum protein that is non-reactive with any of the antibodies in the assay. After this, cells of interest are plated out at varying densities, along with antigen (e.g. MTB-specific antigens like ESAT-6 and CFP-10), and then placed in a humidified 37°C CO_2 incubator for a specified period of time. Cytokine like IFN gamma secreted by activated cells is captured locally by the coated antibody on the high surface area polyvinylidene fluoride membrane. Wells are washed to remove cells, debris, and media components. A biotinylated polyclonal antibody specific for the chosen analyte is added to the wells. This antibody is reactive with a distinct epitope of the target cytokine and thus is employed to detect the captured cytokine. Following a wash to remove any unbound biotinylated antibody, the detected cytokine is then visualized using an avidin-HRP (horseradish peroxidase), and a precipitating substrate (e.g., AEC - amino-9-ethylcarbazole, BCIP/NBT - an aqueous buffered solution containing NitroBlue Tetrazolium). The colored end product (a spot, usually a blackish blue) typically represents an individual cytokine-producing cell. The spots can be counted manually (e.g., with a dissecting microscope) or using an automated reader to capture the microwell images and to analyze spot number and size.

APPENDIX 7: Utility of IFN-gamma in the Diagnosis of Tuberculous Pleural Effusion

Study	Year	No. of patients	Assay method	Sensitivity, %	Specificity, %
Ribera et al	**1988**	**80**	**RIA**	**100**	**100**
Shimokata et al	**1991**	**40**	**RIA**	**100**	**95**
Aoki et al	**1994**	**39**	**ELISA**	**100**	**100**
Wongtim et al	**1999**	**66**	**ELISA**	**95**	**96**
Villegas et al	**2000**	**140**	**ELISA**	**78**	**97**
Villena et al	**2003**	**595**	**RIA**	**98**	**98**
Wong et al	**2003**	**66**	**ELISA**	**100**	**100**
Sharma and Banga	**2004**	**101**	**ELISA**	**90**	**97**
Poyraz et al	**2004**	**45**	**ELISA**	**87**	**95**
Gao and Tian	**2005**	**190**	**ELISA**	**84**	**96**

Source: *Chest* 2007;131;880-889

APPENDIX 8: Protocol for pleural tissue histopathology with H&E staining

Haematoxylin stains nuclei with the help of a mordant, such as aluminum, iron, or tungsten salts. Staining can be progressive, i.e. requiring no differentiation, or regressive- requiring differentiation. Eosin stains (counter stain) cytoplasm and other structures.

Reagents

- Haematoxylin (Harris, Mayers, Ehrlichs)
- Eosin (aqueous, 0.5-1%))
- Acid alcohol, 0.5-1%)

Procedure

- Sections (3-5µ) taken to water through xylene and alcohol
- Stain in haematoxylin for 5-15 minutes
- Wash in tap water
- Differentiate in acid alcohol (where applicable)
- Wash and let to “blue” in tap water
- Counter stain in eosin for 30 sec – 2 minutes
- Wash in tap water
- Dehydrate through alcohol and xylene
- Mount in Dextropropexyphene (DPX)

Results

- Nuclei- blue
- Muscle fibers- red/pink
- Cytoplasm- varying shades of pink
- RBCs- orange/red
- Fibrin- deep pink

APPENDIX 9: Pleural biopsy: Indications, Contraindications and Complications

Indications: in exudative pleural effusions with suspected

1. Malignancy (secondary, mesothelioma and lymphoma)
2. Granulomatous (TB, sarcoidosis, fungus)
3. Lupus (drug induced, denovo lupus)

Contraindications:

1. A dry tap during thoracocentesis
2. Empyema
3. Uncooperative patient
4. Uraemia
5. Coagulation defects like hemophilia and other factor deficiencies, thrombocytopenia and thrombasthenia, abnormal PT and PTT.
6. Severe anaemia in cardiac failure.

Complications:

1. Pneumothorax
2. Hemothorax
3. Empyema
4. Seeding of tumor
5. Extravasation of pleural fluid leading to massive swelling of back, abdominal wall, genitals.

APPENDIX 10: Procedure for Thoracocentesis

Equipments required: 21G sterile needle, sterile gloves, iodine tincture, 10mL sterile syringe, 1 red top container, 1 purple top container. Steps include:

1. Patients were positioned at the edge of the bed seated upright and slightly rotated to the opposite side to splay the ribs. Percussed for dullness to identify the superior and inferior margins of the effusion. Marked one-two rib spaces below the top of the effusion in the posterior axillary line using light pressure with a closed pen. Caution not to go below the 8^{th} intercostal space was taken.
2. The area was cleaned with iodine tincture and allowed to dry for one minute.
3. A 21G sterile needle with a 10mL syringe attached was inserted perpendicular to the rib above the superior margin and continued until there was a little resistance.
4. Pleural fluid was then aspirated to confirm position of needle in pleural space.
5. When in pleural space, 10mLs of pleural fluid was drawn in the same syringe.

The needle and syringe were removed and the site closed with a sterile dressing.

APPENDIX 11: Pleural biopsy procedure

Steps included

1. Patients were positioned at the edge of the bed seated upright and slightly rotated to the opposite side to splay the ribs. Percussed for dullness to identify the superior and inferior margins of the effusion. Marked one-two rib spaces below the top of the effusion in the posterior axillary line using light pressure with a closed pen. Caution not to go below the 8^{th} intercostal space was taken.
2. The area was cleaned with iodine tincture.
3. A 21G sterile needle with a 5mL syringe attached was inserted perpendicular to the rib above the superior margin and continues until there was a little resistance.
4. Pleural fluid was aspirated and the needle and syringe removed.

5. Infiltrated the area where aspirate was obtained from with 1% lignocaine. Infiltration was done to the skin around the area for biopsy, the underlying soft tissue, and the pleura.
6. A 1cm scalpel incision was made on the skin at the aspiration site.
7. Abrams needle was introduced into the pleural space with a firm constant pressure, reducing the pressure as the distinct 'give' was felt on entering the space.
8. The biopsy window was opened by turning the inner sleeve fully anti-clockwise.
9. The inner metallic rod was drawn out slowly until pleural fluid was able to flow freely. This confirmed that, if the biopsy window was in the pleural space.
10. The metallic rod was introduced back into the sleeve and making sure the biopsy window was closed the window of the outer trocar was pressed firmly against the inner chest wall, where it engaged the parietal pleura.
11. The window was opened by rotating it anticlockwise. While pressing firmly against the chest wall, the middle metallic rod was rotated clockwise to guillotine and retain the enclosed pleural tissue.
12. The biopsy needle was removed, tissue extracted and the procedure was repeated to obtain eight tissue samples, four of which was sent in 10% Formalin for histopathology and four of them in 0.9% saline solution for MTB-culture.
13. Pleurocentesis was completed through the Abrahms needle. Fifty millilitres of this sample was also sent in a conical tube to JCRC lab for Elispot assay. The rest of the sample was sent to pathology lab for cytology.
14. The biopsy needle was then removed, pressure applied to the area and the incision was sutured with Nylon 3.0
15. Sterile occlusive dressing was applied once hemostasis was achieved.

APPENDIX 12: Treatment regimen for TB in Uganda (NTLP)

Intensive phase - 2 months	Continuation phase - 6 months
Ethambutol (E) 5-25mg/kg Isoniazid (H) 5mg/kg Rifampicin (R) 10mg/kg Pyrazinamide (Z) 15-30mg/kg	Ethambutol (E) 5-25mg/kg Isoniazid (H) 5mg/kg

APPENDIX 13: Procedure for Zeihl-Neelsen (ZN) staining

Sputum smears of appropriate thickness were prepared using a wire loop, air dried and fixed by passing over a flame and left to cool. The slides were then covered with strong carbol fuchsin, heated to steaming and left to stand for 5 minutes. The cycle was repeated three times before the excess stain was washed off with running tap water. The slides were then decolorized by flooding with 3% acid-alcohol for 3 to 5 minutes and then washed with water before they were counter stained with 0.3% methylene blue for 2 minutes. The slides were again washed under the X100 oil immersion objective. And X10 eye piece. A minimum of 100 fields were examined per slide before declaring it positive or negative. A slide was considered positive if it had at least one bacillus, which appeared as red, beaded rods.

APPENDIX 14: QUESTIONNAIRE

The Accuracy Of Clinical Diagnosis Of Tuberculous Pleurisy Compared To MTB-Specific Pleural Fluid Elispot Assay Among Adults Admitted To Mulago Hospital.

Study number…… IPNO…… Date of recruitment…….

A. <u>Social Demographic characteristics</u>

Age (years)…….
Sex: Female □ Male □
Educational level of Participant: None □ Primary School (P1 to P7) □
Secondary School (S1 to S6) □ University □ Diploma □

B. <u>History</u>

1. Do you have any of the following symptoms? Tick the appropriate box.

	Symptom	Yes	No	Duration if present			
				<1 week	1 week	2 weeks	>2 weeks
a	Cough						
b	Unintentional weight loss						
c	Appetite loss						
d	Gland swelling						
e	Fevers						
f	Drenching night sweats						
g	Pleuritic chest pain						
h	Dyspnoea						

If the patient has at least 2 of (a), (b), (c), (d), (e) or (f) **and** (g) with or without (h) for 2 weeks, proceed to the next question. If not, do not proceed to the rest of the questionnaire.

2. Are you currently on anti-tuberculosis medication? 1. Yes □ 2. No □
If yes to question number 2, do not proceed to the rest of the questionnaire.
3. What is your HIV serostatus? 1. Positive □ 2. Negative □ 3. Do not know □
4. Are you a known diabetic? 1. Yes □ 2. No □
5. Do you have any well documented chronic illnesses? Tick the appropriate answer.
Heart failure 1. Yes □ 2. No □ 3. Do not know □
Nephrotic syndrome 1. Yes □ 2. No □ 3. Do not know □
Liver cirrhosis 1. Yes □ 2. No □ 3. Do not know □
6. Do you have any drug allergies? 1. Yes □ 2. No □ 3. Do not know □
7. In the last one year, have you been in close contact with a person who has been coughing for more than a month or on anti tuberculosis medication? 1. Yes □ 2. No □
8. Do you smoke cigarettes or pipes? 1. Yes □ 2. No □

9. Do you drink alcohol? 1. Yes □ 2. No □

C. Clinical Examination

Vital signs

- Axillary temperature …… (° C).
- Pulse rate (radial) …… beats per minute
- Blood Pressure Supine…….mmHg Standing ……..mmHg
  - Postural drop: Present □ Absent □
- Respiratory rate …… breaths per minute
- SpO2 measured at finger tip by a digital pulse oxymeter …… %
- Level of consciousness
  - Eye opening ……./4
  - Verbal response ……../5
  - Motor response ……../6

  } **Total GCS score = ……./15**

General examination

- Pallor of mucous membranes: Absent □ Mild □ Moderate □ Severe □
- Peripheral Lymphadenopathy: Present □ Absent □
- Pedal oedema: Present □ Absent □
- Weight in Kg ……

Respiratory system

- Location of trachea: Central □ Shifted to right □ Shifted to left □
- Percussion notes: Resonant □ Dull □ Stony dull □ Hyper-resonant □
  If abnormal, state the areas …….
- Breath sounds: Present □ Absent □ Reduced □
  If present or reduced, are they Bronchovesicular □ Bronchial □ Vesicular □ and state areas of abnormal findings …….

Cardiovascular system

Jugular venous pressure: Normal □ Raised □
Heart sounds: Normal□ Abnormal □ Specify ……..Gallop rhythm □
Haemic murmur (if patient is anemic): Present □ Absent □

Performance status (Karnofsky Score) …….. %

100 - Normal; no complaints; no evidence of disease.
90 - Able to carry on normal activity; minor signs or symptoms of disease.
80 - Normal activity with effort; some signs or symptoms of disease
70 - Cares for self; unable to carry on normal activity or to do active work.
60- Requires occasional assistance but is able to care for most needs.
50 - Requires considerable assistance and frequent medical care.
40 - Disabled; requires special care and assistance
30 - Severely disabled; hospitalization is indicated though death not imminent
20 - Very sick; hospitalization necessary; active supportive treatment necessary
10 - Moribund; fatal processes progressing rapidly
0 - Dead.

D. Laboratory results

CXR-PA:

- Pleural effusion: Present □ Absent □
- Size of effusion: Mild (<25% hemithorax) □ Moderate (25 – 75%) □ Large (>75%) □

Light's criteria:

1. Pleural fluid protein ….....
   Serum protein ..…….. } Pleural fluid-serum protein ratio = …….
2. Pleural fluid LDH …….
   Serum LDH …….. } Pleural fluid-serum LDH ratio = ……
3. Pleural fluid LDH concentration ……

Type of pleural effusion by Light's criteria: Exudate □ Transudate □

Pleural fluid cell counts:

WBC (total) ……. /mm^3

White cell differential counts ……

Sputum results of ZN stain for AFBs:

- Sample 1 Positive □ Negative □
- Sample 2 Positive □ Negative □
- Sample 3 Positive □ Negative □

HIV Serology: Positive □ Negative □

CD4 count if HIV positive …… cells/µl

Pleural fluid MTB-specific ELISPOT assay result: Positive □ Negative □

Pleural biopsy tissue histopathologic features of MTB: Positive □ Negative □

Pleural fluid cytology report:

……
……

Pleural biopsy tissue culture for MTB: Positive □ Negative □

Pleural tissue ZN stain for AFBs: Positive □ Negative □

APPENDIX 15a: CONSENT FORM I – SCREENING AND THORACOCENTESIS

CONSENT FORM

THE ACCURACY OF CLINICAL DIAGNOSIS OF TUBERCULOUS PLEURAL EFFUSION COMPARED TO MTB-SPECIFIC PLEURAL FLUID ELISPOT ASSAY AMONG ADULTS ADMITTED TO MULAGO HOSPITAL.

STUDY NUMBER: …… **IP NUMBER: ……**

You are requested to participate in a research conducted at the Mulago Hospital Ward 4C Pulmonology Unit by Dr. Rejani Lalitha. Your participation in this study is voluntary. You should read the information below, and ask questions about anything you do not understand, before deciding whether or not to participate.

PURPOSE OF THE STUDY

Tuberculosis is one of the commonest causes of disease and death in Uganda. Tuberculosis can cause disease in the lungs and other parts of the body. It can cause collection of "water" around the lungs. Confirmation of this disease due to Tuberculosis is very difficult and recently a new test was developed.

This study is going to use this new tuberculosis test called Elispot which will be done on the water obtained from your chest.

You have been identified as an eligible participant in this study because you have been suspected to have water collection around the chest due to tuberculosis.

PROCEDURES

If you volunteer to participate in this study, we would ask you to give or allow us to do the following things:

1. You will be expected to answer questions regarding your current illness, past medical history and drug history.
2. You will be examined by a member of the study team.
3. If after examination, you have water collection on one side of your chest, you will be asked to do an x-ray of your chest if you do not have a film already taken.

The above procedures are part of the standard care and are not experimental.

4. If on chest x-ray you are eligible for further tests, we will ask you to give or allow us to do the following things:
    a. You will be asked to allow us to remove 10 mLs (i.e. two teaspoonfuls) of "water" from the chest using a syringe and a needle.
    b. A test to determine the sugar level will be done at the bedside on the water taken from the chest.
    c. If found to be lower than 40 mg/dl, you will be excluded from the study but it will not affect your further care. But if above this level, 10 mLs of blood sample will be taken for measuring blood glucose, proteins and LDH and the rest of the water removed from the chest will be sent to lab for measuring proteins and LDH.

POTENTIAL RISKS/DISCOMFORTS
There are no tests that will be done outside the usual diagnostic protocol and so no potential risk is expected. The radiation dose when taking a chest x-ray is negligible. The procedure of removing "water" from the chest will cause some pain and may cause complications like entry of air into chest and leakage of blood into and from the chest.

ANTICIPATED BENEFITS TO THE SUBJECT
There will be no monetary benefits from participating in this study. The tests on blood and water from the chest will be done at no cost to you. The results will be forwarded to your doctors as soon as they are ready for better care. If you are discharged by the time the results are ready, you will be contacted by the PI or research assistant to inform you about the test results which were carried out on your samples and appropriate advice given.

CONFIDENTIALITY
The questionnaires will be given a study number and your name will not appear on it. The specimens will also be given the study number and your names will not appear on these
documents. Documents with your name and any other information and results will be kept confidential and locked up in a secure filing cabinet.

PARTICIPATION AND WITHDRAWAL
Your participation in this research is voluntary. If you choose not to participate, that will not affect your relationship with Mulago hospital ward 4C Pulmonology unit or your right to health care or other services to which you are otherwise entitled. If you decide to participate, you are free to withdraw your consent and discontinue participation at any time without prejudice.

WITHDRAWAL OF PARTICIPATION BY THE INVESTIGATOR
The investigator may withdraw you from participating in the research if circumstances arise which warrant doing so. The investigator will make the decision and let you know if it is not possible for you to continue. The decision may be made either to protect your health and safety, or because it is part of the research plan that people who develop certain conditions may not continue to participate.

IDENTIFICATION OF PRINCIPAL INVESTIGATOR
In the event of a research related injury or you experience an adverse reaction, please immediately contact me. If you have any questions about the research, please feel free to contact me. My contacts are as below:

Name: Dr. Rejani Lalitha,
Department of Internal Medicine
Makerere University
P.O Box: 7062, Kampala.
Mobile: 0773-142727

RIGHTS OF RESEARCH SUBJECTS
You may withdraw your consent at any time and discontinue participation without penalty. You are not waiving any legal claims, rights or remedies because of your participation in this research study. If you have any questions regarding your rights as a research subject, you may contact Dr. Harriet Mayanja-Kizza, the Head of Department of Medicine, Mulago Hospital.

SIGNATURE OF RESEARCH SUBJECT

I have read the information provided above. I have been given an opportunity to ask questions and all of my questions have been answered to my satisfaction. I have been given a copy of this form.

_____ _____

Name of Subject Signature and Date

_____

Address of the Subject including telephone contact.

SIGNATURE OF WITNESS

My signature as witness certifies that the subject signed this consent form in my presence as his/her voluntary act and deed.

_____ _____

Name of Witness Signature and Date

APPENDIX 15a - CONSENT FORM I – SCREENING AND THORACOCENTESIS

CONSENT FORM (Luganda)

The accuracy of clinical diagnosis of tuberculous pleural effusion compared to MTB-specific pleural fluid Elispot assay among adults admitted to mulago hospital.

STUDY NUMBER: …… **IP NUMBER: ……**

Osabibwa okwetaba mukunonyereza okugenda okubera mu dwarilo ly'eMulago ku wada 4C okujanjabibwa abalwade bakafuba. Okwetaba mukunoonyereza kuno kwakyeyagalire. Soma ekiwandiiko kino era obuuze ebibuuzo ku'ebyo byoba totegedde ebiri mukiwandiiko kino. Okunoonyereza kuno kukulibwa era kukunyonyoddwaako wamu ne ebibuuzo byo bikudidwaamu Dr. Rejani Lalitha. Ku ssimu eno 0773-142727

Ekigendererwa kyo'kunonyereza kuno

Obulwadde bwa kafuba bwebumu ku ndwadde ezikosezza enyo wamu no'kutta bana'Uganda ensangi zino. Akafuba kakosa amawuggwe, saako nebitundu by'omubiri ebirala. Mu mawuggwe, akafuba era kayonona olububi olubikka kumawuggwe. Kino kiretera olububi luno okuzaala amazzi mangi, nga lugezaako okulwanyisa obulwadde. Okukasa nti amazzi gano gavudde ku kafuba kizibu. Wabula kati waliwo okunonyereza okupya okuyinza okutwanguyizako mukuzula obulwadde bwakafuba mu lububi olubika kumawuggwe. Okunonyereza okupya kubulwadde bwa kafuba kuyitibwa Elispot, okujja kukolebwa ku mazzi agagiddwa mu kifuba. Naye olwo kunonyereza okwo'mutinddo, ojjakujibwako akabubi akamu ku'obwo obubika kumawuggwe okuva mu kifuba kyo nga bakozesa syringe ne trocar babiwereze mu Lab okukakasa obulwadde bwakafuba.
Osaanidde okwetaba mukunonyereza kuno kubanga ozuliddwa okuba na'mazzi mukifuba ekiletebwa obulwadde bwa'kafuba.

Emitendera

Bwoba okirizza okwetaba mu kunonyereza kuno kyeyagalire, tuja kukusaba okukola bino wamanga:

1. Ojja ku tuukirirwa onnyonnyolebwe era obuzibwe ebibuuzo ebikwata ku bulwadde bwo, era n'embera yobulamu bwo mubisera ebyemabega, nedagala lyobadde okozesa.

2. Ojakukeberwa omu kubasawo abanonyereza kubulwadde buno obwa'akafuba.

3. Ngomaze okukeberwa, siinga asangibwa nga olina amazzi mukifuba tujja kukusaba okubwe ekifananyi kyekifuba (x-ray). Tewetaaga kukuba kirala bwoba oliina kyewakubiddwa edda.

Emitendera egyo wagulu gyegimu ku egyo egiri wagulu mukunonyereza kubulwadde bono.

4. Ekifananyi kyekifuba bwekiraga nti osaniide okwetaba mukunonyereza kuno, tujja kukusaba okukolabino wamanga

    a. Ojjakusabibwa okukugyako amazzi mukifuba (10mLs) ne mpiso.
    b. Tujja kukebera obungi bwasukali alimumazzi getunaaba tugyeko.

c. Sukaali bwasangibwa ngali wansi wa 40mg/dl, togya kwetaba mukunonyereza kuno. Wabula kino tekijja kutawanya kujanjabibwa ko. Sukaali bwasangibwa ngali 40mg/dl oba okusingako awo, ojja kwetabamukunonyereza.
d. Buli mulwadde anetaba mukunonyereza ajjakujibwako omusaayi (10mLs) gukebeerebwe, era namazzi agagyidwa mukifuba gagyakwongerwa okunonyerezebwako.

Obuzubu bwoyinza okusanga.
Tewali kunonyereza kugenda kukukolebwako nga ogyeko ebyo byetumenye wagulu. Amasanyalaze (x-ray) aganakukubwa okufuna ekifananyi kye kifuba matono nyo era tegagya kukukosa. Oyinza okuwurila obulumi ngatukugyako amazzi mukifuba, olusi omusaayi oba empewo bijja kuyingira mukifuba, wabula tugya kukozesa obwegendereza obwawagulu okwewala bino era tewali kyabulabe kyona kyigenda kutusibwako ku bulamu bwo okuva ku ebyo wagulu. Bwo nooba osibudwa nga ebuvudde mukunonyereza tebinaba kufulumizibwa, akulira okunonyereza kuno oba abamuyanbako bajja kuyita bakuikubulire ebinaaba bivude mukunonyereza.

Ebirubgi byonafuna mukunonyereza.
Togendak kusasulwa osobole okwetaba mukunonyereza kuno, wabula omusaayi, amazzi na'kabubi kokumawugwe bigenda kugyibwako wamu n'okukebererwa bwerere. Ebinaava mukukebera kuno tugya kubiwa abasawo bo basobole okukulabirira obulungi

Okukuuma ebyama
Empapula zona zijja kuwebwa ennamba, erinnya ryo terijja kubeera ku mpapula zino. Ate empapula eziriko erinnya ryo zijja kukumibwa bulungi mu kiffo ekyekusifu.

Okwetaba mu kunonyereza kuno no'kukuvaamu
Okwetaba mukunonyereza kuno kwakyeyagalire. Oyinza okugaana okwetaba mu kunoonyereza kuno oba okukuvaamu ekisera kyona. Bino byona tebigenda kutabula bujjanjabi bwolina kufuna ku wada eno eyabalwadde ba kafuba mu dwaliro lino.

Okugaanibwa okwetaba mu kunonyereza kuno
Akulira okunonyereza kuno ayinza okuyimiriza okugenda mumaaso mu kunoonyereza kuno singa wabawo ensonga yona ngaraba tekusobozesa kugenda mumaaso mu kunonyereza kuno. Okusalawo kuno kuyinza okuba nga kuyanba bulamu bwo oba nga agoberera mitendera egifuga okunonyereza kuno. Nolwensonga ezo wagulu akulira okunonyereza kuno aba alina okubulira budde nensonga ekuyimizisiza.

Okumanya akulira okunonyereza
Bwewabawo obuzibu bwoba oba ekibuuzo kyona mukunonyereza kuno tukirira akukulira kundagiriro zino wamanga:
Dr. Rejani Lalitha
Department of Internal Medicine
Makerere university
P.o.Box 7072, Kampala
Mobile: 0773-142-727

Eddembe lyo'yo eyetabye mu kunonyereza
Olina eddenbe okugaana oba okusazaamu okwetaba mu kunoonyereza kuno ekisera kyona nga tosasudwa ngasi yona. Era tolina teeka lyona lyomenye mukusazaamu okwetaba mukunonyereza kuno.
Bwoba olina ekibuzo kyona kubyokumanya eddenbe lyo mu byokunonyereza, tukirira Dr. Harriet Mayanja-Kizza akulira Department of Medicine, Mulago Hospital.

Okukiriza kwoyo agenda okwetaba mukunoonyereza
Nsomye/bansomedde obubaka okuva mukiwandiiko ekimperedwa era nfunye no'mukisa okudibwaamu ebibuuzo byange byona byembuziiza bulungi. era nsazeewo okwetaba mukunoonyereza kuno kyeyagalire.

_____ _____
Erinnya ryomulwadde ekinkumu / omukono

Mbaddewo mukusa omukono/ekinkumu kulupapula luno era ntegedde ekigendererwa kyo kunonyereza kuno.

_____ _____
Erinnya ryabaddewo omukono gwadaddewo

Omulwadde munyonyodde ekigendererwa kyokunonyereza kuno.

_____ _____
Erinnya ry'o munonyerezi omukono

Enakuz'omwezi: _____

Essimu: _____

APPENDIX 15b: CONSENT FORM II – CONSENT FOR PLEURAL BIOPSY

THE ACCURACY OF CLINICAL DIAGNOSIS OF TUBERCULOUS PLEURAL EFFUSION COMPARED TO MTB-SPECIFIC PLEURAL FLUID ELISPOT ASSAY AMONG ADULTS ADMITTED TO MULAGO HOSPITAL.

STUDY NUMBER: ……. **IP NUMBER: …….**

You are requested to participate in a research conducted at the Mulago Hospital Ward 4C Pulmonology Unit by Dr. Rejani Lalitha. Your participation in this study is voluntary. You should read the information below, and ask questions about anything you do not understand, before deciding whether or not to participate.

PURPOSE OF THE STUDY

Tuberculosis is one of the commonest causes of disease and death in Uganda. Tuberculosis can cause disease in the lungs and other parts of the body. It can cause collection of "water" around the lungs. Confirmation of this disease due to Tuberculosis is very difficult and recently a new test was developed.

This study is going to use a new tuberculosis test called Elispot which will be done on the water obtained from the chest. But as part of the standard test, small parts of the tissue will be obtained from your chest just outside the lungs using a trocar and needle and sent to lab to look for evidence of tuberculosis.

You have been identified as an eligible participant in this study because you have been confirmed to have water collection around the chest probably due to tuberculosis.

PROCEDURES

If you volunteer to continue in participating in this study, we ask you to give or allow us to do the following things:

1. 5mLs of "water" will be removed from the chest using a syringe and needle. This will help us determine the appropriate site for the operation called 'pleural biopsy' where by small pieces of tissue will be removed from just outside the lungs for examination under microscope. Then you will be given an injection to reduce pain at the site of biopsy.
2. The biopsy will be done. At the end of the operation, any remaining 'water' will be removed and 100mLs sent to JCRC lab in Mengo and the rest sent to the Pathology lab in Makerere University Medical School.
3. You will be given Paracetamol tablets to control pain.
4. The small pieces of tissue will be sent to Makerere University Histology laboratory.

The results of the tissue taken from chest for culture of tuberculosis germs will take about two months to ready and you are requested to return for the results of the test after two months during the follow-up. But in the event that the pathologist finds evidence suggestive of tuberculosis (which usually takes about three days to be ready) you will be started on Tuberculosis medication immediately.

POTENTIAL RISKS/DISCOMFORTS

The procedure of removing "water" from the chest will cause some pain and may cause complications like entry of air into chest and leakage of blood into and from the chest. The procedure of pleural biopsy may cause the same problems as removing water from chest but also

there is a risk of introducing some germs into the water or water may leak out. These problems will be minimized as far as possible.

ANTICIPATED BENEFITS TO THE SUBJECT

There will be no monetary benefits from participating in this study. The tests on water and the tissue from the chest will be done at no cost to you. The results will be forwarded to your doctors as soon as they are ready for better care. If you are discharged by the time the results are ready, you will be contacted by the PI or research assistant to inform you about the test results which were carried out on your samples and appropriate advice given.

CONFIDENTIALITY

The specimens will be given the study number and your names will not appear on these documents. Documents with your name and any other information and results will be kept confidential and locked up in a secure filing cabinet.

PARTICIPATION AND WITHDRAWAL

Your participation in this research is voluntary. If you choose not to participate, that will not affect your relationship with Mulago hospital ward 4C Pulmonology unit or your right to health care or other services to which you are otherwise entitled. If you decide to participate, you are free to withdraw your consent and discontinue participation at any time without prejudice.

WITHDRAWAL OF PARTICIPATION BY THE INVESTIGATOR

The investigator may withdraw you from participating in the research if circumstances arise which warrant doing so. The investigator will make the decision and let you know if it is not possible for you to continue. The decision may be mad either to protect your health and safety, or because it is part of the research plan that people who develop certain conditions may not continue to participate.

IDENTIFICATION OF PRINCIPAL INVESTIGATOR

In the event of a research related injury or you experience an adverse reaction, please immediately contact me. If you have any questions about the research, please feel free to contact me. My contacts are as below:

Name: Dr. Rejani Lalitha
Department of Internal Medicine
Makerere University
P.O Box: 7062, Kampala.
Mobile: 0773-142727

RIGHTS OF RESEARCH SUBJECTS

You may withdraw your consent at any time and discontinue participation without penalty. You are not waiving any legal claims, rights or remedies because of your participation in this research study. If you have any questions regarding your rights as a research subject, you may contact
Dr. Harriet Mayanja-Kizza, the Head of Department of Medicine, Mulago Hospital.

SIGNATURE OF RESEARCH SUBJECT

I have read the information provided above. I have been given an opportunity to ask questions and all of my questions have been answered to my satisfaction. My participation is voluntary. I have been given a copy of this form.

_____ _____

Name of Subject Signature and Date

_____

Address of the Subject including telephone contact.

SIGNATURE OF WITNESS

My signature as witness certifies that the subject signed this consent form in my presence as his/her voluntary act and deed.

_____ _____

Name of Witness Signature and Date

APPENDIX 15b: CONSENT FORM II – CONSENT FOR PLEURAL BIOPSY (Luganda)

The accuracy of clinical diagnosis of tuberculous pleural effusion compared to MTB-specific pleural fluid Elispot assay among adults admitted to mulago hospital.

STUDY NUMBER: ……. **IP NUMBER: …….**

Osabibwa okwetaba mukunonyereza okugenda okubera mu dwarilo ly'eMulago ku wada 4C okujanjabibwa abalwade bakafuba. Okwetaba mukunoonyereza kuno kwakyeyagalire. Soma ekiwandiiko kino era obuuze ebibuuzo ku'ebyo byoba totegedde ebiri mukiwandiiko kino. Okunoonyereza kuno kukulibwa era kukunyonyoddwaako wamu ne ebibuuzo byo bikudidwaamu Dr. Rejani Lalitha. Ku ssimu eno 0773-142727

Ekigendererwa kyo'kunonyereza kuno
Obulwadde bwa kafuba bwebumu ku ndwadde ezikosezza enyo wamu no'kutta bana'Uganda ensangi zino. Akafuba kakosa amawuggwe, saako nebitundu by'omubiri ebirala. Mu mawuggwe, akafuba era kayonona olususbi olubikka kumawuggwe. Kino kiretera olususbi luno okuzaala amazzi mangi, nga lugezaako okulwanyisa obulwadde. Okukasa nti amazzi gano gavudde ku kafuba kizibu. Wabula kati waliwo okunonyereza okupya okuyinza okutwanguyizako mukuzula obulwadde bwakafuba mu lususbi olubika kumawuggwe. Okunonyereza okupya kubulwadde bwa kafuba kuyitibwa Elispot, okujja kukolebwa ku mazzi agagiddwa mu kifuba. Naye olwo kunonyereza okwo'mutinddo, ojjakujibwako akabubi akamu ku'obwo obubika kumawuggwe okuva mu kifuba kyo nga bakozesa syringe ne trocar babiwereze mu Lab okukakasa obulwadde bwakafuba.
Osaanidde okwetaba mukunonyereza kuno kubanga ozuliddwa okuba na'mazzi mukifuba ekiletebwa obulwadde bwa'kafuba.

Emitendera
Bwoba okirizza okwetaba mu kunonyereza kuno kyeyagalire, tuja kukusaba okukola bino wamanga:

1. Ojjakujibwako mills 5 eza' mazzi ku akwago agali mu kifuba nga bakozesa empiso. Kino kijja kutuyamba okumanya ekitudu we bagenda okukujjako akabubi akamu ku'obwo obubika amawugwe kakeberwe ne microscope tumanye oba kalimu akafuba. Kino kijakukolebwa nga tusoose okukuba akayiso akakendeeza obulumi. Akababi nga kamaze okujibwako ojakuwebwa amakerenda okundeza obulumi.

2. Akababi oba akanyama nga bamaze okukujibwaako. Mills 100 eza'mazzi ku ago aganaaba gasigadde mukifuba gajja kutwalibwa ku JCRC Lab e Mengo namalara agaanaba gasigadewo bagawereze mu Pathology Lab eye Makerere University Medical School gakeberebwe okunoonya akafuba.

3. Ojja kuwebwa eddagala elikendeza obulumi.

4. Obumu kususbi obutono bujja kuwerezebwa e Makerere University Histology Laboratory bukebererwe. Ebinazulwa mu kukebera obuwuka bwakafuba, bijja kukomezebwaawo oluvanyuma lwezzi ebiri. Osabibwa okudda okukima ebinazulibwamu kukebera kwobuwuka bwakafuba mu banga lya myezzi ebiri nga bwojjanjabibwa oba bwetukwekebejja. Mukiseera

ekyo ebinaava mu kukeberwa okusoka bijja kufuluma oluvanyuma lwenaku satu. Ssinga osangibwa nga olina akafuba ojakutandika eddagala lyakafuba amangu dala.

Obuzibu bwoyinza okusanga.
Amasanyalaze (x-ray) aganakukubwa okufuna ekifananyi kye kifuba matono nyo era tegagya kukukosa. Oyinza okuwurila okusiyiibwa n'okulumizibwa ngatugyako amazzi mukifuba, olusi omusaayi oba empewo bijja kuyingira okuyingira mukifuba, wabula tugya kukozesa obwegendereza obwawagulu okwewala bino era tewali kyabulabe kyona kyigenda kutusibwako ku bulamu bwo okuva ku ebyo wagulu. Bwo nooba osibudwa nga ebuvudde mukunonyereza tebinaba kufulumizibwa, akulira okunonyereza kuno oba abamuyanbako bajja kuyita bakuikubulire ebinaaba bivude mukunonyereza

Ebirubgi byonafuna mukunonyereza kuno.
Togendak kusasulwa osobole okwetaba mukunonyereza kuno, wabula omusaayi, amazzi na'kabubi kokumawugwe bigenda kugyibwako wamu n'okukebererwa bwerere. Ebinaava mukukebera kuno tugya kubiwa abasawo bo basobole okukulabirira obulungi.

Okukuuma ebyama
Empapula zona zijja kuwebwa ennamba, erinnya ryo terijja kubeera ku mpapula zino. Ate empapula eziriko erinnya ryo zijja kukumibwa bulungi mu kiffo ekyekusifu.

Okwetaba mu kunonyereza kuno no'kukuvaamu
Okwetaba mukunonyereza kuno kwakyeyagalire. Oyinza okugaana okwetaba mu kunoonyereza kuno oba okukuvaamu ekisera kyona. Bino byona tebigenda kutabula bujjanjabi bwolina kufuna ku wada eno eyabalwadde ba kafuba mu dwaliro lino.

Okugaanibwa okwetaba mu kunonyereza kuno
Akulira okunonyereza kuno ayinza okuyimiriza okugenda mumaaso mu kunoonyereza kuno singa wabawo ensonga yona ngaraba tekusobozesa kugenda mumaaso mu kunonyereza kuno. Okusalawo kuno kuyinza okuba nga kuyanba bulamu bwo oba nga agoberera mitendera egifuga okunonyereza kuno. Nolwensonga ezo wagulu akulira okunonyereza kuno aba alina okubulira budde nensonga ekuyimizisiza.

Okumanya akulira okunonyereza
Bwewabawo obuzibu bwoba oba ekibuuzo kyona mukunonyereza kuno tukirira akukulira kundagiriro zino wamanga:
Dr. Rejani Lalitha
Department of Internal Medicine
Makerere university
P.o.Box 7072, Kampala
Mobile: 0773-142-727

Eddembe lyo'yo eyetabye mu kunonyereza
Olina eddenbe okugaana oba okusazaamu okwetaba mu kunoonyereza kuno ekisera kyona nga tosasudwa ngasi yona. Era tolina teeka lyona lyomenye mukusazaamu okwetaba mukunonyereza kuno.
Bwoba olina ekibuzo kyona kubyokumanya eddenbe lyo mu byokunonyereza, tukirira Dr. Harriet Mayanja-Kizza akulira Department of Medicine, Mulago Hospital.

Okukiriza kwoyo agenda okwetaba mukunoonyereza
Nsomye/bansomedde obubaka okuva mukiwandiiko ekimperedwa era nfunye no'mukisa okudibwaamu ebibuuzo byange byona byembuziiza bulungi. era nsazeewo okwetaba mukunoonyereza kuno kyeyagalire.

_____ _____
Erinnya ryomulwadde ekinkumu / omukono

Mbaddewo mukusa omukono/ekinkumu kulupapula luno era ntegedde ekigendererwa kyo kunonyereza kuno.

_____ _____
Erinnya ryabaddewo omukono gwadaddewo

Omulwadde munyonyodde ekigendererwa kyokunonyereza kuno.

_____ _____
Erinnya ry'o munonyerezi omukono

Enakuz'omwezi: _____

Essimu: _____