Histopathological and mpt64 Immunohistochemical diagnosis of extra pulmonary tuberculosis at Muhimbili National Hospital

Caroline Ngimba, MD

MMed (Anatomical Pathology) Dissertation
Muhimbili University of Health and Allied Sciences
October, 2018

Muhimbili University of Health and Allied Sciences

Department of Pathology

HISTOPATHOLOGICAL AND MPT64 IMMUNOHISTOCHEMICAL DIAGNOSIS OF EXTRAPULMONARY TUBERCULOSIS AT MUHIMBILI NATIONAL HOSPITAL

By

Caroline Ngimba

A Dissertation Submitted in Partial Fulfilment of the Requirements for the Degree of Masters of Medicine (Anatomical Pathology) of the

Muhimbili University of Health and Allied Sciences
October, 2018

CERTIFICATION

The undersigned certify that she has read and hereby recommends for acceptance by Muhimbili University of Health and Allied Sciences a dissertation entitled **"Histopathological and MPT64 immunohistochemical Diagnosis of EPTB at Muhimbili National Hospital'',** in partial fulfilment of the requirements for the degree of Master of Medicine (Anatomical Pathology) of Muhimbili University of Health and Allied Sciences.

_____

Dr. Amos Mwakigonja

(Supervisor)

Date: _____

_____

Dr. Henry Mwakyoma

(Supervisor)

Date: _____

DECLARATION

AND

COPYRIGHT

I, **Caroline Ngimba**, declare that this dissertation is my own original work and that it has not been presented and will not be presented to any other university for a similar or any other degree award.

Signature…… Date……....……

This dissertation is copyright material protected under the Berne Convention, the Copyright Act 1999 and other international and national enactments, in that behalf, on intellectual property. It may not be reproduced by any means, in full or in part, except for short extracts in fair dealing, for research or private study, critical scholarly review or discourse with an acknowledgement, without the written permission of the Directorate of Postgraduate Studies, on behalf of both the author and the Muhimbili University of Health and Allied Sciences.

ACKNOWLEDGEMENTS

To all that I have achieved in my life may the glory, honour and praise be to the Almighty God.

I wish to express my sincere gratitude and appreciation to my supervisors Dr. Amos Mwakigonja and Dr. Henry Mwakyoma for their good supervision and encouragement during the development and writing of this dissertation.

I am also grateful to all staff members of Pathology department at MUHAS and MNH for their support during the course of my studies. I would like to mention Dr. Edda Vuhahula (Head of Department of Pathology at MUHAS) for her encouragement and support.

My sincere appreciation goes to my outstanding classmates; Doctors: Ndayisaba Marie Claire,Advera Ngaiza, Angela Pallangyo, Daudi Mshana and Moses Rugemarila for making my three years of MMed program enjoyable.

I am grateful and indebted to my mother for her unconditional love and support in all aspects of my life.

I also thank all my relatives who in different ways supported me morally and physically.

Last but of foremost importance I am thankful and indebted to my husband Melchior Kisahali, my sons Deogracias and Philip for their encouragement, motivation, support and patience throughout my training.

DEDICATION

To Almighty God

To my beloved parents, my mother Aneth Ngimba and my father the late Philip Ngimba

To my husband Melchior Kisahali

To my two sons, Deogracias Kisahali and Philip Kisahali

To all my teachers

To all my friends and colleagues

I dedicate this work.

ABSTRACT

Background: Extra pulmonary tuberculosis (EPTB) is reported to be on the rise worldwide particularly in developing countries. In the year 2014, the EPTB represented about twenty one per cent of the new cases of tuberculosis (TB) reported in Tanzania(1). The diagnosis of EPTB is more challenging than pulmonary tuberculosis (PTB) since it cannot be diagnosed by either sputum examination or chest imaging. The diagnosis of EPTB is made by identification of characteristic histomorphologic features on tissue biopsies from the affected organ and confirmed by Ziehl Nielsen stain (ZNS) that shows *Acid-fast bacilli (AFB*). However, ZNS has a low sensitivity thus it can be negative despite the presence of TB infection. New cost effective methods to identify AFB on tissues are being developed. One of them is immunohistochemistry (IHC) method using anti-MPT64 to detect mycobacterium TB complex proteins. Various studies were performed especially on cytological material and lymphoid tissues; scant information is available for other extrapulmonary sites particularly using formalin-fixed paraffin-embedded (FFPE) tissue sections.

Objectives: The study aimed at determining the EPTB frequency, demography, histomorphology, clinical presentation as well as anti-MPT64 immunoreactivity on FFPE archived surgical biopsies at Muhimbili National Hospital (MNH), Histopathology Unit.

Methods: FFPE tissue blocks and haematoxylin-eosin (H&E) stained slides of all biopsies which were signed out as EPTB from July 2015 to June 2017 were retrieved from the Histopathology Unit archive, at MNH. The slides of all included cases were retrieved and reviewed to confirm the diagnosis of EPTB by histomorphology prior to retrieval and resectioning for ZN staining and IHC. Demographic, clinical and histopathologic data were collected using excel data collection sheet exported to SPSS version 20 for analysis. Proportions of AFB positivity were calculated for ZNS and anti-MPT64 immunoreactivity. Continuous variables were summarized using mean, median, mode, standard deviation and range. Frequency tables, bar charts and pie charts were made using excel. The association between demographic, clinical data and positivity for AFB was analysed. The association was considered significant for a p-value less than 0.

Results: A total of 110 out of 165 tissue biopsies which were signed out as extrapulmonary TB based on histomorphology were further analysed. Biopsies were from patients aged between 2-76 years, with mean, median and mode of 36.3, 33 and 30 years respectively. Biopsies from female patients represented 60% (n=66). Necrotizing granuloma was the most (69%, n=76) frequent lesion amongst the biopsies collected. Furthermore, IHC and ZNS positivity were found in 71% and 8% of the biopsies respectively. All ZN positive biopsies were also positive to MPT64 by IHC. The lymph node presentation was more frequent (41.8%, n=46) compared to other sites.

Conclusions: EPTB is not infrequently encountered among biopsies submitted at MNH. Despite the challenges faced in establishing EPTB diagnosis, the histomorphology of necrotizing granulomas should be regarded as a strong indicator of EPTB, particularly in TB endemic areas. Furthermore, IHC by anti-MPT64 appears to be useful particularly in controversial cases where ZN staining is negative. TB lymphadenitis is the most common type of EPTB presentation at MNH. EPTB affects mostly young adults in reproductive age group and the female gender.

Recommendations: EPTB should be considered as a differential diagnosis in various clinical scenarios as it presents in a protean ways including mimicking malignancy, thus histological and/or cytological confirmation is important. All patients who present with enlarged lymphnodes should have TB infection ruled out. For granulomatous lesions it is important to do further work up including special stains for microorganisms in order exclude other causes. Furthermore, clinicopathological correlation is important in order to reach the diagnosis of EPTB and whenever there is a controversy; advanced tests including immunohistochemistry should be employed.

CONTENTS

CERTIFICATION i
DECLARATION AND COPYRIGHT ii
ACKNOWLEDGEMENTS iii
DEDICATION iv
ABSTRACT v
CONTENTS vii
LIST OF TABLES x
LIST OF FIGURES xi
LIST OF ABBREVIATIONS xii
DEFINITION OF TERMS xiii
CHAPTER ONE 1
1.0 INTRODUCTION AND LITERATURE REVIEW 1
1.1 Introduction 1
1.2. Overview of Extra pulmonary Tuberculosis 2
1.2.1 Transmission, Pathogenesis and Clinical presentation. 2
1.2.2 Laboratory diagnosis of EPTB 2
1.2.3 Treatment of EPTB 4
1.3 Magnitude of EPTB 4
1.4 Histopathology for extra pulmonary tuberculosis 5
1.5 AFB staining for Mycobacteria TB by Ziehl Nielsen Stain (ZNS) 6
1.6 Immunohistochemistry by anti- MPT64 for *Mycobacteria tuberculosis* 6
1.7 Anatomical sites for extra pulmonary tuberculosis 7
1.8 Extrapulmonary tuberculosis with age and sex 8
1.9 Conceptual framework 9
1.10 Problem statement 9
1.11 Study rationale 10
1.12 Research questions 11
1.13 OBJECTIVES 11
1.13.1 Broad objective: 11
1.13.2 Specific objectives: 11

CHAPTER TWO 12
2.0 MATERIALS AND METHODS 12
2.1 Study design 12
2.2 Study duration 12
2.3 Study Area 12
2.4 Study population 12
2.5 Inclusion Criteria 13
2.6 Exclusion Criteria 13
2.7 Sampling Method and Sample size 13
2.7.1. Sampling procedure 13
2.7.2 Sample size estimation 14
2.8 Laboratory Methods 14
2.8.1 Tissue block retrieval and Microtomy 14
2.8.2 H&E Staining 15
2.8.3 ZN staining 15
2.8.4 Immunohistochemical Staining 15
2.9 Data Collection 16
2.9.1 Demographic and Clinical characteristics 16
2.9.2 Microscopic interpretation 16
2.10 Quality control of the study 17
2.11 Statistical Analysis 17
2.12 Ethical Clearance 18
CHAPTER THREE 19
3.0 RESULTS 19
3.1 Frequency of EPTB diagnosis by histomorphology. 19
3.2 Demography and clinical characteristics of EPTB biopsies 19
3.3 Histormophological patterns of EPTB 21
3.4 Proportion of ZNS and anti-MPT64 IHC positive EPTB sections. 21
CHAPTER FOUR 30
4.0 DISCUSSION 30
CHAPTER FIVE 36

5.0 CONCLUSION AND RECOMMENDATIONS 36
5.1 Conclusion 36
5.2 Recommendations 36
REFERENCES 38
APPENDICES 43
Appendix I: Data collection sheet 43
Appendix II: Histopathology, ZN stain and MPT64 immunohistochemistry results 44
Appendix II: Ethical clearance approval 45
Appendix III: MNH research clearance 46
Appendix IV: Procedure for hematoxylin and eosin staining (MNH PROTOCAL) 47
Appendix V: Procedure for Ziehl Nielsen stain (MNH PROTOCAL) 48
Appendix VI: Procedure for immunohistochemistry (MNH PROTOCAL) 49

LIST OF TABLES

Table 1: Demographic and clinical characteristics of patients 20

Table 2: Association of anti-MPT64 immunoreactivity by demographic and clinical characteristics 25

Table 3: Association of gender and HIV serostatus 26

LIST OF FIGURES

Figure 1: Conceptual framework of this study 9

Figure 2: Algorithm showing biopsies selection on this study 13

Figure 3: Histomorphological pattern among the 110 EPTB biopsies 21

Figure 4: Proportion of ZNS results 22

Figure 5: Proportion of anti-MPT64 immunoreactivity 22

Figure 6: Histomorphology among MPT64 positive biopsies 23

Figure 7: Positive control photo images 27

Figure 8: Tuberculous Lymphadenitis photo images 28

Figure 9: Tuberculous Pleuritis photo images 29

LIST OF ABBREVIATIONS

AEC-Amino- ethlycarbazole

AFB- *Acid fast bacilli*

AIDS-Acquired immunodeficiency syndrome

BCG- Baccile Calmette Guerin

CD4-Cluster of differentiation 4

CNS-Central Nervous System

CPL-Central pathology Laboratory

DNA-Deoxyribonucleic acid

EPTB-Extra pulmonary Tuberculosis

FFPE-Formalin fixed paraffin embedded

FNAC-Fine needle Aspiration Cytology

GIT-Gastrointestinal

H/E-Haematoxylin and eosin

HIV-Human immunodeficiency syndrome

HRP- Horseradish peroxidase

IHC-Immunohistochemistry

MNH- Muhimbili National Hospital

MUHAS-Muhimbili University College of Health and Allied Sciences

MPT64-Mycobacterium Tuberculosis Protein 64

PCR-Polymerase chain reaction

SPSS-Statistical package for social sciences

TB-Tuberculosis

TBS- Tris buffer saline

WHO-World Health Organisation

ZNS- Ziehl Nielsen stain

DEFINITION OF TERMS

Immunohistochemistry (IHC) - is a method for identification and localisation of a specific antigen in tissues or cells based on antigen-antibody recognition; and visualized by microscopy.

Cell mediated immunity- is a type of immune response that involves the activation of phagocytes, antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen.

Granuloma- is a collection of activated macrophages (epithelioid cells) typically produced in response to infection, inflammation, or the presence of a foreign substance.

Histopathology- is the branch of pathology which deals with the study of disease in a tissue section.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Tuberculosis is a chronic infectious disease caused by *Mycobacterium tuberculosis*. It is one of the major public health concerns worldwide particularly, in developing countries. Tuberculosis is primarily a pulmonary disease but can also occur in extrapulmonary sites. Extrapulmonary tuberculosis (EPTB) can affect any organ in the body including the lymphatics, pleural, bone, joint, the gastrointestinal tract, genitourinary system, central nervous system, the head and neck, as well as breast and the skin (2,3).

Mycobacteria are generally grouped into Mycobacteria tuberculosis complex (MTC) and Non-Tuberculous Mycobacteria (NTM). *Mycobacteria Tuberculosis* are also known as *Acid Fast Bacilli (AFB)* due to high cell wall lipid content which readily binds carbol fuchsin (red) dye and subsequently resists decolourization during Ziehl-Nielsen staining (ZNS). *Mycobacteria* Tuberculosis complex is of clinical importance as it cause Tuberculosis in humans and comprises of *M. Tuberculosis, M. bovis, M. Africanum* and others (4). The non- tuberculous mycobacteria species include *M.avium, M. intracellulare, M. kansasii, M. fortuitum, M. chelonae, M. szulgai, M. paratuberculosis, M. scrofulaceum* and others, and these usually do not cause fatal disease in immunocompetent individuals, but in case of immunosuppression they may cause disseminated disease which is fatal and its treatment is different (3).

Currently the diagnosis of EPTB in surgical specimens depends on finding specific histopathological features in tissue biopsies from the affected organ or site and confirmation by ZNS. However ZNS has been reported to have low sensitivity (5) and thus can be negative despite the presence of TB infection. Mycobacteria tuberculosis protein 64 (MPT64) is an *M. tuberculosis complex* (MTC)-specific antigen secreted during mycobacterial growth (6) . Studies have shown secretory property of the mycobacteria specific antigen MPT-64 in tissue samples from various sites of the body which can be detected by IHC and therefore useful in the confirmation of mycobacteria infection.

1.2. Overview of Extra pulmonary Tuberculosis

1.2.1 Transmission, Pathogenesis and Clinical presentation.

After initial infection with *Mycobacteria tuberculosis (MTB)* bacilli the primary site of infection is the alveolar macrophages in the lungs. The infection may remain latent or progress to active TB at some point in lifetime(7). It is estimated that one third of world's population has latent TB and people infected with MTB have 10% risk of developing the disease (8). EPTB develops when mycobacteria reach the sites outside the lungs usually due to lymphohematogenous spread. EPTB patients may present with constitutional symptoms including fever, loss of weight, generalized body malaise and anorexia. Other signs and symptoms depend on the affected organ (9) such as enlarged lymph node(s) when lymphatics are involved, infertility when reproductive organs are involved, intestinal obstruction when the gastrointestinal tract (GIT) is involved and so on.

The risk factors for TB transmission include immunocompromised state including HIV infection and age extremes(10). Bacteriological status of the source of infection is also a risk factor for transmission of tuberculosis whereby smear positive cases are most infectious compared to smear negative cases(11). EPTB commonly affects children and people with immunosuppression including HIV infected individuals (12). A previous study done at the Vancouver General Hospital highlighted the possibility of TB transmission through EPTB cases. In that study there was a higher transmission rate (25% to 41%) when patients with EPTB were studied combined with Pulmonary TB cases compared to when there were only pulmonary TB cases [transmission was 17 to 21%] (13). HIV-infected individuals are associated with reduced CD4+ T helper cells particularly Th2 which is responsible for anti-mycobacteria immunity through production of interferon-γ and interleukin-2, this is associated with increased susceptibility to TB among them (5).

1.2.2 Laboratory diagnosis of EPTB

The diagnosis of EPTB is challenging. Clinical presentations of EPTB are diverse and thus require a high index of suspicion. Where laboratory tests can be performed, the diagnosis of EPTB can be made by various methods including tissue biopsy for histopathology, body aspirates for cytopathology, AFB staining, culture for mycobacteria, molecular methods

including Polymerase Chain Reaction(PCR) and Gene Xpert as well as mycobacteria antigen detection by Immunohistochemistry besides other methods.

Histopathological examination of tissue biopsies is one of the laboratory methods to diagnose EPTB. Typical morphological features are TB granulomas that consist of central caseous necrosis, infected macrophages surrounded by epithelioid cells, multinucleated giant cells of Langhan's type as well as a rim of mature small lymphocytes and fibrosis. However, in TB/HIV co-infection, the morphology of the granuloma lesion may not show typical architecture of caseating necrotizing granulomas. Furthermore, there are conditions which may present with granulomas including fungal infections, rheumatoid nodules, Wegener's granulomatosis, and necrobiotic postsurgical granulomas which may also complicate the diagnosis of TB. Thus, further workup is required in order to establish a definitive diagnosis of EPTB when suspected.

Staining for acid fast bacilli using Ziehl Nielsen stain is used to confirm the presence of *Mycobacteria tuberculosis*. ZN stain smear is viewed under conventional light microscopy and has limitation of detecting 1000bacilli/ml or more than 10000 bacilli per slide for acid fast bacilli to be detected (5). EPTB is usually associated with few bacilli thus ZN stain may sometimes be associated with false negatives results. The use fluorescence microscopy using a flourochrome and a light emitting diode (LED) has shown more sensitivity for microscopy when compared to ZN staining but they are more expensive and requires more expertise (5).

Culture for *Mycobacterium tuberculosis* is currently regarded as the gold standard method of diagnosis. It takes several weeks (2-6 weeks) to grow the organism and that this method can detect mycobacterium in amounts of 10-100bacilli/mil. The advantage of culture is that drug sensitivity can be performed simultaneously (14). Molecular studies such as PCR can be used to detect mycobacteria DNA in tissues. The use of PCR is associated with higher sensitivity (15) but requires high expertise and is too expensive, and also susceptible to false positives thus not recommended alone as a diagnostic test for EPTB.

The Xpert MTB/RIF assay has been used for rapid molecular diagnosis of TB. Currently, the World Health Organization has recommended the use of Xpert over conventional tests for diagnosis of TB in lymph nodes and other tissues (16).

Furthermore, various antibodies have been developed for the detection of mycobacterium antigens in tissue specimens. These antibodies range from polyclonal to monoclonal. Studies have shown that immunohistochemistry/immunocytochemistry is efficient and rapid method in the detection of mycobacteria from extrapulmonary sites (17–20). Immunohistochemistry can be used in addition to ZN stain due to ability to localize mycobacteria antigen in situation of paucibacillary which is the situation in EPTB (21).

1.2.3 Treatment of EPTB

The treatment of EPTB is by anti-tuberculosis drugs which are Isoniazid, Rifampicin, Pyrazinamide and Ethambutol, and usually using the same regimen of 4 drugs as for PTB. Special considerations however, are taken for treatment of TB meningitis, TB of bone and joints. TB meningitis is treated for 9-12 months due to serious risk of disability and mortality and also ethambutol should be replaced by streptomycin. If there is no suspicion for drug resistance adjuvant corticosteroids are recommended for TB meningitis and pericarditis (22).With early diagnosis and proper management EPTB has good prognosis.

1.3 Magnitude of EPTB

Tuberculosis is among the leading causes of death affecting 10.4 milion people worldwide (23). According to WHO Global Tuberculosis report during the year 2015 it was estimated that TB death occurred in 1.4 million cases and 0.4 million deaths among HIV positive people(23). In Tanzania, TB is in the list of top causes of morbidity and mortality along with Malaria and HIV and AIDS(24). Globally, EPTB occurs in between 10-25% cases infected by *Mycobacteria tuberculosis* (2). Studies have shown that EPTB accounts between 10-25% of all cases with TB in immunocompetent individuals and more than 50% in HIV positive patients (2). A study done in USA revealed that the prevalence of EPTB among cases diagnosed with TB was 22.9% and among them 9.2% had HIV co-infection (25).

The burden of TB is high in South East Asia and African countries. In the year 2015 the estimated number of new cases of TB was highest in Asia with 61% of new cases followed by Africa with 26% of new cases (23). Annual report by national Tuberculosis and leprosy control programme in Tanzania showed that In the year 2014 there were 60,575 new cases of all forms of Tuberculosis notified and among them and 21% were EPTB (1)

EPTB is high among people with HIV infection because these individuals are associated with reduced CD4+ T helper cell especially Th2 which is responsible for antimycobacteria immunity through production of interferon-γ and interleukin-2. This is associated with increased susceptibility to TB among HIV positive individuals (26). Individuals with advanced immunosuppression have increased risk of Extrapulmonary Tuberculosis (26–28). Africa has a highest burden of Tuberculosis/HIV in the world and more than 50% of TB cases are co-infected with HIV in Sub Saharan Africa (29). Tanzania is among the countries with high burden of Tuberculosis as well as TB/HIV coinfection in the world (30).

Various studies have shown that up to more than 50% of individuals with HIV are co-infected with Tuberculosis. A study done in Thailand showed that the prevalence of EPTB among HIV individuals was 40% (28). In Tanzania Meremo et al.,(2012) and P. Kamenju et al.,(2011) showed that HIV individuals were 48.8% and 15% respectively, more likely to have extrapulmonary Tuberculosis (31,32). In contrast to above findings, a study carried out in a high resource country with a low burden of Tuberculosis revealed that only 4% of patients with extrapulmonary TB were infected by HIV (33).

1.4 Histopathology for extra pulmonary tuberculosis

Histopathological diagnosis of tissue biopsies is done by looking for presence of granulomatous inflammation and caseous necrosis. Typical morphological features of TB granulomas include central infected macrophages which are surrounded with epithelioid cells, multinucleated giant cells of Langhan's type and caseous necrosis on histological specimens (34). A study which looked on the histological patterns of TB lymphadenitis revealed that most frequent of major histological features were the giant cells (88%)followed by caseation (84%) then epithelioid cells (80%), granuloma and caseation (68%), lymphocytes (31%), and histiocytes (4%) (21).

However, in situations where there is co-infection of Tuberculosis and HIV, the morphology of the granuloma lesion may not show typical patterns of necrotizing granulomas but also there are other conditions which may present with granulomatous inflammation which may raise suspicion of EPTB.

Most studies have shown necrotizing granuloma as the most frequent histomorphology associated with EPTB. A study which was done previously in a setting of low TB prevalence showed caseating granuloma were found in 62% of positive histology for EPTB and the rest was histology of non-caseating granulomas (33).Furthermore, other studies which dealt with biopsies from extrapulmonary TB showed more than half had histomorphology of caseating granulomas (35) while Eshete et al., (2011) showed among lymph node with histology diagnosis of EPTB more than three quarter had histology of necrotizing granulomas (36). Kitinya et al.,(1992) examined pathological changes of TB pleuritis among patients with HIV and found that reactive changes(granuloma with caseous necrosis) was found in 61% of biopsies (37).

1.5 AFB staining for Mycobacteria TB by Ziehl Nielsen Stain (ZNS)

Staining for acid fast bacilli can be done by using Ziehl Nielsen stain and viewed with standard light microscopy or rhodamine-auramine stain and viewed under fluorescence microscopy. However, ZN stain has limitation of detecting 1000 bacilli/ml or 10000 Bacilli per slide (5). Studies have revealed low detection rate of AFB staining on formalin fixed paraffin embedded tissue biopsies of extrapulmonary Tuberculosis (38,39). A previous report showed the ZNS positivity among paraffin embedded nodal tissue biopsies was 61.7% (36). Another study which was done in Yemen where by only 3% of lymph node biopsies with histological evidences of TB were picked by ZN stain (35). Another study which was done in Khartoum, Sudan on utility of ZN stain on diagnosis of TB lymphadenopathy found ZN stain was positive on only 4 (2.5%) biopsies out 161 biopsies which were previously reported as TB lymphadenitis by histomorphology (40). Purohit et al., (2007) on his study on lymph node and abdominal TB showed that ZN positivity was 11% and 0% respectively (20).

1.6 Immunohistochemistry by anti- MPT64 for *Mycobacteria tuberculosis*

Various antibodies have been developed for detection of mycobacterium antigens on tissue specimens. These antibodies range from polyclonal to monoclonal. Studies have shown that immunohistochemistry/immunocytochemistry is efficient and rapid method in the detection of mycobacteria from extrapulmonary sites (17).

Mycobacteria tuberculosis protein 64(MPT64) is one of the major antigens secreted bacilli. It is a 24KDa protein secreted during bacteria growth and can induce strong delayed type 4 hypersensitivity reaction (18). Studies have shown secretory property of the mycobacteria specific antigen MPT-64 in tissue samples from various sites of the body which can be detected by immunochemistry. This protein can distinguish mycobacteria TB complex species from other mycobacteria (6). So far most literature on MPT64 immunostaining of EPTB cases have been mainly lymphnodes and body effusions while TB in other anatomical sites is not well elucidated particularly in Tanzania.

These studies have shown that MPT64 assay can be applied to a wide range of clinical specimens with a significantly higher sensitivity and specificity as compared to the conventional methods of AFB microscopy and solid culture (19,20,41). MTP64 antibody has potential to reveal mycobacterial antigen in conditions of few and fragmented bacilli.
This immunohistochemical assay has shown to be efficient and simple diagnostic tool for EPTB to be employed in addition to ZN stain in the histopathology laboratory (41). Studies have shown that this immunochemical assay improved the detection rate in EPTB on FNA and surgical tissue biopsies (13,18,19). Purohit et al.,(2007) showed that MPT64 had sensitivity of 92% and specificity of 97% on lymphnodes TB (20). Thus it is important to evaluate the staining on ant-MPT64 on our surgical specimens from various extrapulmonary sites since data on this is either scanty or sketchy and as such not well documented.

1.7 Anatomical sites for extra pulmonary tuberculosis

EPTB can occur on any site in the body such as in the lymphatics, pleural and bone or joint as well as gastrointestinal tract, genitourinary system, Central nervous system, head and neck, breast and skin. Pericardial, meningeal and disseminated (military TB) forms of EPTB are associated with fatal outcomes (2).

Studies have suggested that the sites of EPTB may vary according to geographic location and population (42). A study done in Kabul, showed that Lymph node (37.3%) was the most frequent site for EPTB and Central Nervous system(20.3%) was the second in frequency, followed in descending order by skeletal, pleural, abdominal, cutaneous, genitourinary, pericardial, miliary, and breast tuberculosis (27). Lymph node are the most

frequent site for EPTB has this been shown by many other studies (27,36,43,44). Contrary to that a study done in Korea revealed that pleural (35.6%) was the most common site for EPTB followed by lymph node (18.3%), bone and joints (13.5%), upper airways (9%) and other locations such as pericardium, skin, middle ear, adrenal gland and thyroid gland constituted a total of 3.2% (42).Not only that but also a Turkish study showed most commonly seen two types of EPTB were genitourinary TB (27.2%) and meningeal TB (19.4%) (45). In the African perspective, the occurrence of extrapulmonary tuberculosis on different anatomical sites is also variable. A study done in Benin showed that pleural, spine and lymphnodes contributed about two third of cases of extrapulmonary Tuberculosis (46). Not only that but also another study done in Gondar Hospital in Ethiopia showed TB lymphadenitis occurred in 82.4% of all cases of Extrapulmonary TB. In Tanzania, a study which evaluated the clinical presentation of TB/HIV co-infection revealed that the pleural cavity was the most common site for extrapulmonary Tuberculosis (32). These findings showed that the variation in geographic location and population as well as host factors can influence the pattern of anatomical sites for EPTB though lymph node seems to be the most common site across studies.

The common site for EPTB among HIV individuals is lymph node but other sites such as central nervous system, pleural, pericardial, abdomen, disseminated TB and any part of the body can be involved (23). Leeds et al.(2012) showed that individuals with low CD4 count were more likely to present with CNS and disseminated TB form of EPTB (47).

1.8 Extrapulmonary tuberculosis with age and sex

Several studies have shown variable associations of extrapulmonary Tuberculosis with age and sex. A previous study conducted in a high burden country revealed younger age and female gender as independent risk factors for extrapulmonary Tuberculosis (44).

Another study showed that there was no significant gender differences in Extrapulmonary tuberculosis occurrence for patients less than 45 years but for more than 45 years cases women were more likely to develop extrapulmonary TB (48). A. Shanmuganathan et al., (2013) and Tiwari et al .,(2011) showed the distribution of extrapulmonary Tuberculosis was more common among males (50, 51).

Another study done in Sudan showed extrapulmonary Tuberculosis was more among females and young patients (51). Furthermore, a study done in Benin showed that extrapulmonary Tuberculosis distribution was mostly common among children less than 15 years and significantly high among females with regional variation (46). This finding has been noted in other studies (28,43).

1.9 Conceptual framework

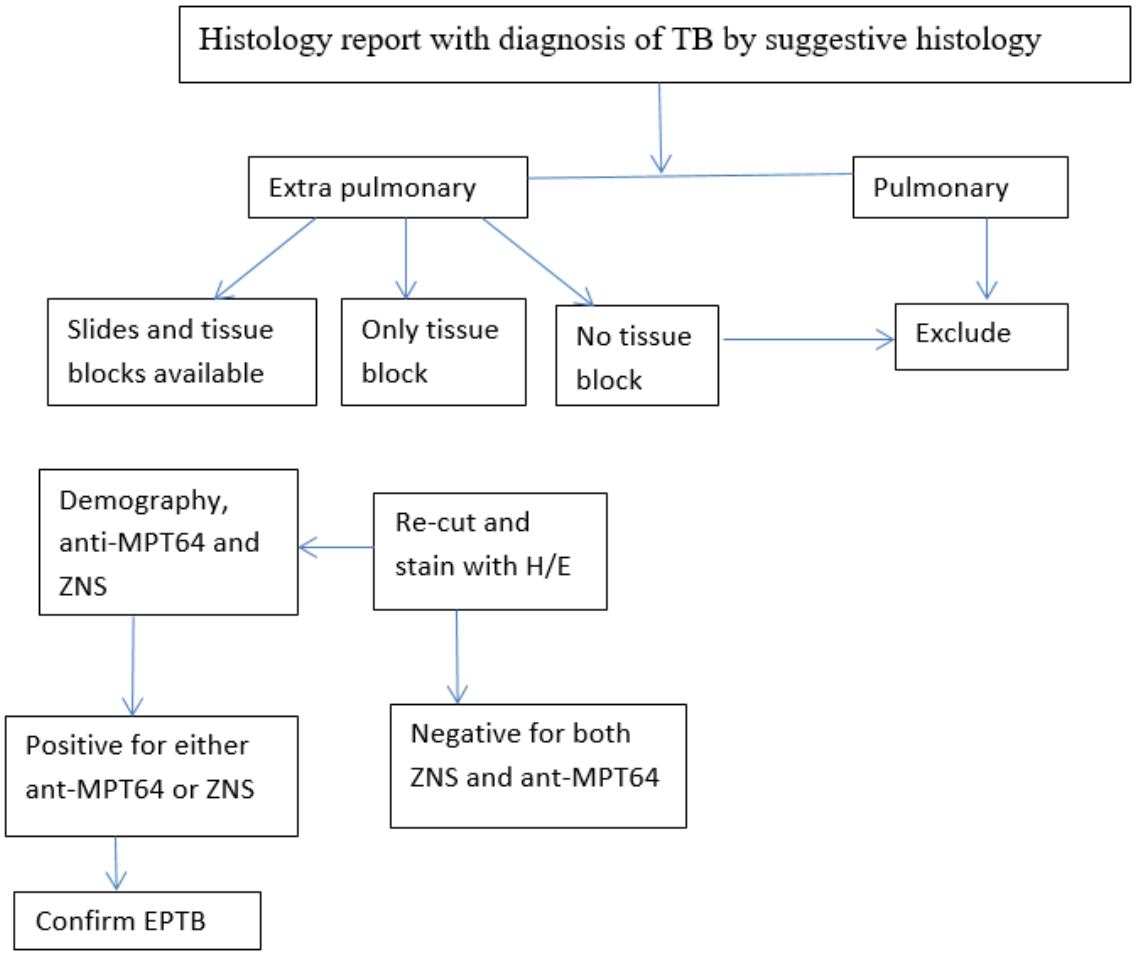


Figure 1: Conceptual framework of this study

1.10 Problem statement

In Tanzania data on EPTB is scanty. Most studies focused on Pulmonary TB. Few reports of EPTB were mostly on lymph nodes (20,24,31,41). Thus tuberculosis of extra nodal sites is hardly reported from Tanzania and those scanty reports did not include immunohistochemical evaluation. Furthermore, the diagnosis of EPTB is more challenging than PTB because it does not present with pathognomonic clinical signs and it cannot be

diagnosed by sputum examination or characteristic chest imaging features. The diagnosis of EPTB requires specimen from the affected organ most commonly tissue biopsy and cytological aspirates. On tissue biopsy, there are histomorphologic characteristics for TB infection but the confirmation of the presence of AFB is necessary. The ZNS is the current method used in our setting to confirm AFB on tissue sections however it has a low sensitivity thus it can be negative despite the presence of TB infection. A superior method by Immunochemistry using anti-MPT64 to detect mycobacterium TB complex proteins was developed. This method is more sensitive and is also useful in the absence of typical features of TB. Different studies in the literature on the use of anti-MPT64 IHC for TB diagnosis focused mostly on cytological and lymphoid tissue specimens and scant information is available for other extra pulmonary sites and further on the use of FFPE tissue sections. The aim of this study was to detect TB infection in FFPE archived tissue biopsies at MNH, as well as to determine the demographic and clinical profiles of patients with EPTB from 1st July 2015 up to 30th June 2017.

1.11 Study rationale

The results of this study will inform both clinicians and pathologists about the best approach to the diagnosis of EPTB when using FFPE biopsies. The study makes use of routine histomorphology, histochemistry (ZNS) as well as immunochemistry (MPT64) in the quest for effective diagnosis. The current study will detail the frequency of different anatomical sites of extra pulmonary tuberculosis in patients who submitted tissue biopsies to the Histopathology Unit at the Central Pathology Laboratory (CPL), MNH. Furthermore, the demographic findings/associations together with the histopathological presentation will help improve the index of suspicion and allow early detection of EPTB, promote diagnostic accuracy and allow prompt subsequent interventions. Furthermore, the findings of this study will provide important information to doctors, hospital management, policy makers as well as the community at large which will help promote awareness of EPTB and its diagnostic challenges and also help formulate interventions.

1.12 Research questions

- What is the frequency of EPTB diagnosis by histomorphology among surgical biopsies at MNH, Histopathology unit?
- What are the demographic and clinical characteristics of patients diagnosed as EPTB by histomorphology?
- What are the histomorphological patterns of EPTB among surgical biopsies?
- What is the proportion of ZNS and anti-MPT64 IHC positive EPTB biopsy sections?
- What is the association of demographic and clinical characteristics with anti-MPT64 immunoreactivity?

1.13 OBJECTIVES

1.13.1 Broad objective:

To determine the frequency, histomorphology, anti-MPT64 IHC as well as demographic and clinical presentation of EPTB among FFPE archived surgical biopsies at Muhimbili National Hospital (MNH), Histopathology Unit.

1.13.2 Specific objectives:

1. To determine the frequency of EPTB diagnosis by histomorphology among surgical biopsies.
2. To determine demographic and clinical characteristics of patients diagnosed as EPTB by histomorphology.
3. To determine the histomorphological patterns of EPTB among surgical biopsies.
4. To determine the proportion of ZNS and anti-MPT64 IHC positive EPTB biopsy sections.
5. To determine the association of demographic and clinical characteristics with anti-MPT64 immunoreactivity.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Study design

This was a retrospective cross-section laboratory-based study. All reported tissue biopsies diagnosed as EPTB by suggestive histological features and whose blocks, reports and files were available, were included in the study. Histopathological sections were reviewed by the primary investigator (pathology resident) and two supervising pathologists independently and only together in case of disagreement. Anti-MPT64 IHC and ZN stain were performed after histomorphological confirmation.

2.2 Study duration

This study was conducted for the duration of 2 years, from 1st July 2015 up to 30th June 2017.

2.3 Study Area

The study was conducted at the Histopathology unit in the Central Pathology Laboratory (CPL) at MNH, Dar es salaam, Tanzania. MNH is the National referral hospital and the University Teaching Hospital for MUHAS and other Universities, receiving patients from different regions of Tanzania. It also serves as Zonal referral hospital for Coastal, Southern parts of Central Tanzania. It has bed capacity of 1500 and attends 1000-1200 outpatient and inpatient per week. About 10,000 surgical pathological specimens are received in the laboratory annually and 70-100 of biopsies are diagnosed as EPTB. The tissue specimens for patients suspected to have extrapulmonary Tuberculosis are received from different departments such as ENT departments, Dental, General surgery, Muhimbili Orthopaedic institute, Gastroenterology, Ophthalmology, Internal medicine department and also from other hospitals in the country.

2.4 Study population

Our study population was composed of all tissue biopsies which were submitted to the Histopathology Unit at MNH between 1st July 2015 and 30th June 2017 and diagnosed as EPTB by suggestive features on histology.

2.5 Inclusion Criteria

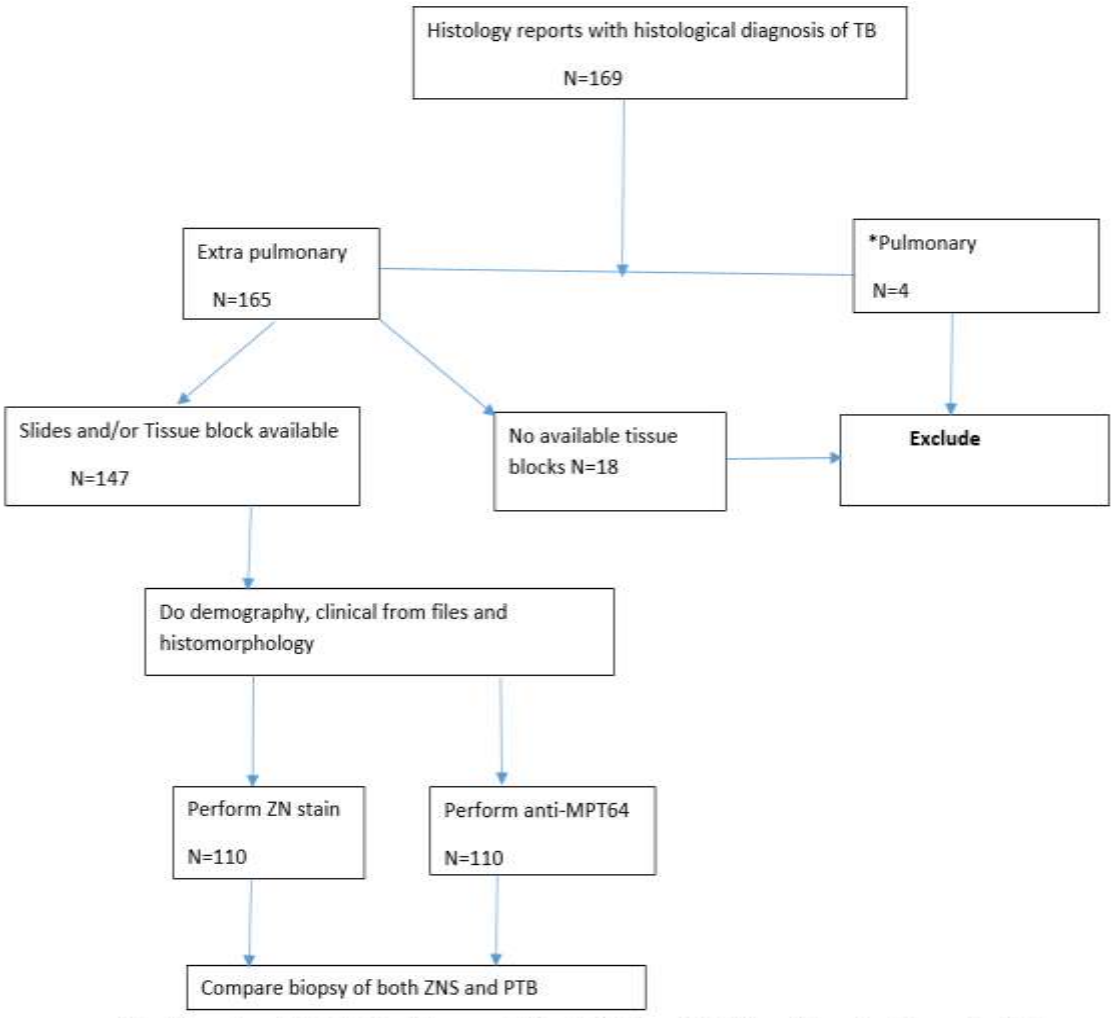
This study included all tissue biopsies diagnosed as EPTB by suggestive features on histomorphology between 1st July 2015 and 30th June 2017 and whose tissue blocks were available in the archives.

2.6 Exclusion Criteria

All tissue biopsies diagnosed of EPTB by suggestive histomorphology with missing tissue blocks were excluded.

2.7 Sampling Method and Sample size

2.7.1. Sampling procedure



One biopsy has both EPTB (on biopsy results) and PTB (from the file) and therefore it was classified as PTB as per WHO classification

Figure 2: Algorithm showing biopsies selection on this study

2.7.2 Sample size estimation

The sample size was calculated from open epi proportion formula:

$$n = \frac{DEFF*N*P*(1-p)}{(d^2/z^2_{1-\alpha/2})*(N-1)+p*(1-p)}$$

where:

n-sample size

Z = 1.96 at 95%

P = Proportion of those with the characteristic of interest of which for this study it was the proportion of extrapulmonary TB cases detected by anti-MPT64 immunohistochemistry in one of the previous study which was 64% (41)

d= Margin of error which was taken as 5% in this study

N=population size, this was obtained from the pilot study for one year, on which there were 70 biopsies diagnosed as EPTB by histomorphology. This study was for period of 2 years then the population size was 70 x 2=140

DEFF -Design effect (=1)

$$n = \frac{1 \times 140 \times 0.64 (1-0.64)}{0.05^2/1.96^2 \times (140-1) + 0.64(1-0.64)}$$

= 101 tissue biopsies.

2.8 Laboratory Methods

2.8.1 Tissue block retrieval and Microtomy

Tissue blocks and slides to be retrieved were identified by screening the histology reports kept in the archive of the histopathology unit where they are arranged by their laboratory accession number (HP number). Tissue blocks and slides which correspond with histology suggestive of EPTB were retrieved. For the purpose of this study, new sections for H&E staining were done if the quality of the previous was not optimal. Tissue blocks were put on ice blocks and cut using rotary microtome (Sakura model) SRM 200 CW. Sections for IHC had a thickness of 5 micrometre and 3micrometer for ZN and H/E, after sectioning were placed in floating water bath at 45^0C and then mounted on standard frosted glass slides each with ID number on the slides and allowed to drain and put in hot plate at 60^0c for minimum of 50 minutes.

2.8.2 H&E Staining

Sections were dewaxed in three changes of Xylene for three minutes each and brought down to water through descending grades of ethyl alcohol (absolute,95%,80% and 70%), After that they were stained in Harris haematoxylin for five minutes, washed in tap water, differentiated in 1% aqueous eosin for 5 minutes, washed in tap water, dehydrated in ascending grades of ethanol (70%,80%,95%) 10 dips each clear in changes of xylene and mounted in DPX and labelled accordingly (protocols for H &E at MNH).

2.8.3 ZN staining

Sections were dewaxed in three changes of xylene and brought down to distilled water, after that slides were put on staining rack with sections upwards. Slides were then flooded by Carbol fuchsin until steam rises, after 10 minutes the sections were washed in distilled water. This was followed by differentiation in 1% acid in 70% alcohol for 30min. After washing with distilled water, slides were bloat dried in whatma's filter paper. Then sections were put into two of each change of 95% and absolute a\\lcohol and cleared in two changes of xylene and mounted in DPX and labelled accordingly (protocols for H&E at MNH).

2.8.4 Immunohistochemical Staining

IHC by using MPT64 was performed by the use of Dakocytomation kit (Envi-sion +system-HPR, Dakocytomation Denmark A/S Glostrup, Denmark). Tissue sections were deparaffinized in xylene then hydrated in 100%, 95%, and 80%. A ring was done around the section using Pap pen, to limit the spread of antibody solutions, thereafter slides were put in antigen retrieval solution and heated on microwave at 350W. After that the endogenous peroxidise activity was inhibited by incubating the sections with hydrogen peroxidise for 15 minutes in humid chamber. The slides were treated with monoclonal anti-MPT64 antibody (in-house) at 1/250 dilution for one hour (41). Then the sections were incubated with anti-rabbit dextran polymer conjugated to horseradish peroxidise containing substrate and incubated for 45minutes, Amino ethylcarbazole-labelled chromogen was then applied and incubated for 15minutes before counterstaining with haematoxylin. All incubations were carried out at room temperature and sections were washed thoroughly in between incubations. Two known cases with TB which had numerous numbers of bacilli (multibacillary granulomas) were used as positive controls and two cases with diagnosis

other than TB (malignancy) were used as negative controls in every batch of staining. For negative control sample primary antibody was substituted by antibody diluent.

2.9 Data Collection

Data collection was carried out using data collection sheets, whereby the demographic, clinical and histopathologic data were recorded. The microscopic interpretation for histomorphology, ZNS and ant-MPT64 IHC was done by the principal investigator and reviewed by two supervising pathologists.

The histomorphology, mycobacteria staining by ZNS and anti-MPT64 immunoreactivity was evaluated by using light microscopy model Olympus CX 41 and few images from the result were taken by using Olympus soft imagine solutions by USB SC30 camera (100pixels).

2.9.1 Demographic and Clinical characteristics

The demographic characteristics recorded were age and sex of the patients. Clinical characteristics which were recorded included anatomical sites of presentation (site of biopsy), HIV status of the patients as well as a diagnosis which was suspected by the clinicians before biopsy results. HIV status was obtained from the clinical files if it was recorded as positive, negative or unknown.

2.9.2 Microscopic interpretation

2.9.2.1 Routine H/E Histormophology

On routine H/E histology, the histomorphological features/patterns were divided into two; 1) Caseating granulomas (presence of granuloma + Langhan’s giant cells + caseous necrosis)

2) Non-caseating granulomas (Granuloma + Langhan’s with/without foreign body giant cells+ absence of caseous necrosis).

The decision on histomorphology depended on presence of granuloma whether with caseous necrosis or without caseous necrosis. Patients/cases with low immunity may present with atypical caseating or non-caseating granuloma, therefore suggestive clinical presentation + caseous necrosis without typical granuloma was also considered as TB granuloma.

2.9.2.2 ZNS interpretation

ZN stained slides were evaluated first at 40x and then at 60x magnification (with oil emulsion) using light microscope and interpreted as positive if for red bacilli are seen and recorded as scant or numerous. The results were interpreted only if the positive control was also positive. If the test control (positive control) was negative, the staining was repeated.

2.9.2.3 Anti- MPT64 IHC interpretation

The MPT64 stained slides were evaluated at 10x magnification using light microscope and areas with positive signals were further evaluated at 40x magnification. Signals were interpreted as positive if detected as golden brown granular cytoplasmic staining in granuloma cells or extracellular granular deposits in necrotic areas and outside granuloma cells. The intensity was noted as either strong or weak. The results were interpreted only if the positive control was also positive .If the test control (positive control) was negative, the staining was repeated

2.10 Quality control of the study

The slides with histological diagnosis and immunohistochemistry results of extrapulmonary Tuberculosis were confirmed through review by principal investigator and confirmed by two pathologists who were the supervisors.

The tissue blocks for H/E, ZN stain and immunohistochemistry were prepared by following the standard operating procedures (Protocols for each procedure attached on annexes).

2.11 Statistical Analysis

Data was managed electronically by Statistical Package for Social Sciences (SPSS) version 20 computer software (SPSS Inc., Chicago Ill) after cross checking the filled data collection sheets. Proportions of AFB positivity were calculated for ZNS and anti-MPT64 immunoreactivity Continuous variables were summarized using mean, median, mode, standard deviation and range. Frequency tables, bar charts and pie charts were made using excel. The association between demographic, clinical data, histomorphology and positivity for AFB was analysed. The association was considered significant for a p-value less than 0.05. Where the sample size was very small the Fisher exact method was used to test the statistical significance.

2.12 Ethical Clearance

Ethical clearance was obtained from the MUHAS Ethical clearance committee (see attachment on appendices). Permission to access blocks, patient's files and use of machines and other materials was given by the Head of Histopathology unit at MNH. Strict confidentiality on Patient's information was maintained and only identification numbers were used.

CHAPTER THREE

3.0 RESULTS

3.1 Frequency of EPTB diagnosis by histomorphology.

During the two-year study conducted at Muhimbili National Hospital from 1st July 2015 to 30th June 2017, about 20276 biopsies were submitted to the department of pathology. About 0.83% (n= 169) tissue biopsies had histological diagnosis of Tuberculosis by suggestive histomorphological features. Ninety-eight per cent (n=165) of the 169 biopsies were EPTB.

Out of 165 biopsies with diagnosis of EPTB based on histomorphology by H/E, 110 biopsies were selected for detection of TB infection using ZNS and anti-MPT64 immunohistochemistry. Other biopsies were not stained due to missing tissue blocks in the archive and logistical constraints.

3.2 Demography and clinical characteristics of EPTB biopsies

Of the 110 biopsies with EPTB diagnosis based on histology, age was documented in 99% (N= 109) biopsies. The median age of our patient from which the biopsies were taken was 33 years with an age range from 2 to 76 years. The mean age was 36.03 years ± 16.77 SD and the mode was 30 years. Ninety per cent of our participants were adults, 18 years old and above. Females were the majority 60% (n=66). The lymph node was the leading anatomical site of presentation recorded at 42% (n=46). Clinically, tuberculosis was suspected by clinicians in 61.3% (n=65) of our patients and 4 patients had no clinical differential diagnosis. Sixteen out of 30 patients were HIV positive among those whose status was recorded. Details on demographic and clinical data are described in table 1.

Table 1: Demographic and clinical characteristics of patients

Characteristics	Frequency N (%)
Age group (years) (N=109)	
< 18	11 (10.1)
18-35	47 (43.1)
36-53	35 (32.1)
54 and above	16 (14.7)
Sex (N=110)	
Male	44 (40)
Female	66 (60)
Anatomical site (N=110)	
Lymph node	46 (41.8)
Abdomen	13 (11.8)
Bone	12 (10.9)
Pleura	10 (9.1)
Genito-urinary	8 (7.3)
Skin	6 (5.5)
Joints	3 (2.7)
Others*	12 (10.9)
Clinical diagnosis (N=106)	
EPTB	65 (61.3)
Others**	41 (38.7)
HIV status(N=30)	
Positive	16(53.3)
Negative	14(46.7)

**Others include breast, soft tissue, nasopharynx, brain.*

***Other diagnosis suspected by clinician such malignancy.*

3.3 Histormophological patterns of EPTB

The histomorphological features/patterns were subjectively divided into strong and weak evidence of TB. Strong evidences of TB on histomorphology included caseating granulomas (Granuloma+ Langhan's giant cells + caseous necrosis) while weak evidence of TB included non-caseating granulomas (Granuloma +Langhan's with/without foreign body giant cells+ absence of caseous necrosis).

In accordance with this classification, caseating granulomas were the most frequent histomorphological subtype 69% (n=76), while the non-caseating granulomas were 31% (n=34,) (Figure3).

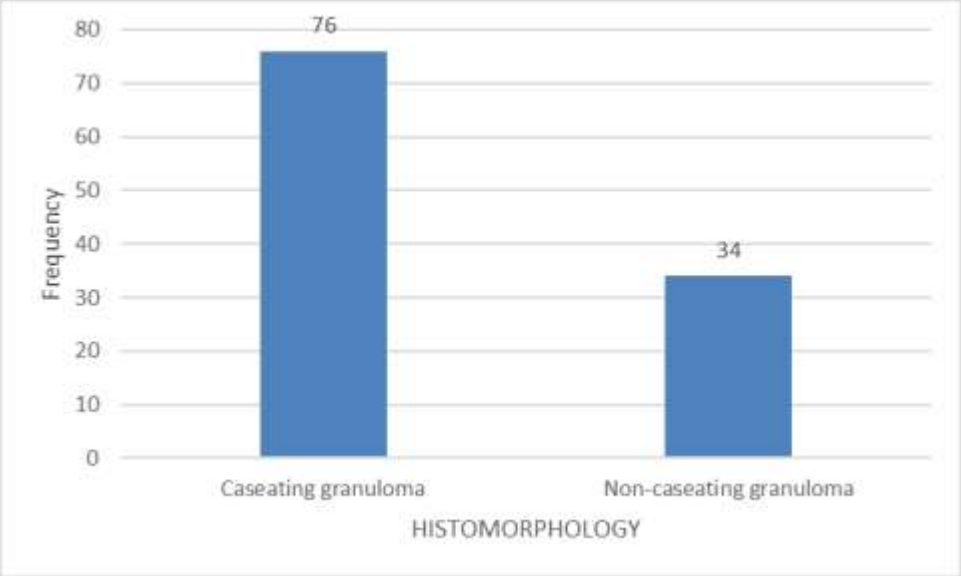


Figure 3: Histomorphological pattern among the 110 EPTB biopsies

3.4 Proportion of ZNS and anti-MPT64 IHC positive EPTB sections.

Out of 110 biopsies which were stained by ZN to demonstrate presence of *acid and alcohol fast* (AFB), only 9 biopsies (8.2%) were positive implying that despite suggestive features of EPTB on histology, majority of biopsies were negative on ZN stain (Table 2). 8(89%) out of 9 ZN positive biopsies had numerous numbers of bacilli implying that ZN positivity is seen mostly in multibacillary.



Figure 4: Proportion of ZNS results

Of the 110 EPTB biopsies the majority (71%) (n=78,) of biopsies appeared to show immunoreactivity to anti-MPT64 antibody by IHC (figure 4). The distribution of positivity was detected intracellularly in granuloma cells such as epithelioid cells, multinucleated giant cells and extracellularly implying that IHC detect mycobacteria protein (intact or fragmented bacilli) wherever it is, whether inside or outside the cells which is not for the case of ZN stain which require intact cell wall.

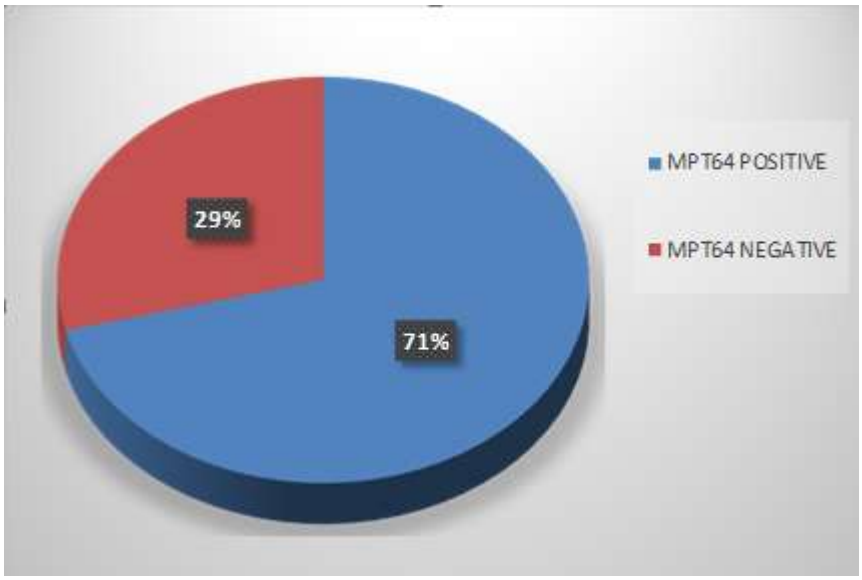


Figure 5: Proportion of anti-MPT64 immunoreactivity

The immunoreactivity by anti-MPT64 was mostly (80.2%) seen in caseating granulomas (n=61/76). In non-caseating granuloma anti-MPT64 IHC was positive in about a half of the biopsies (n=17/34). These findings suggests that caseating granulomas were more frequently associated with positive MPT64 results than the non-caseating granulomas (figure 5), and this difference was strongly statistically significant (P value=0.001).

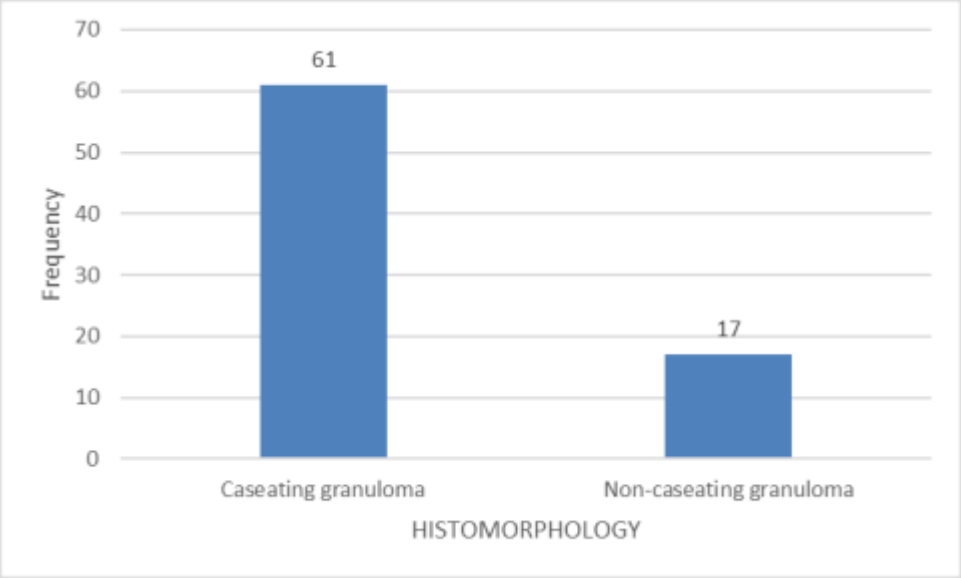


Figure 6: Histomorphology among MPT64 positive biopsies

All 9 biopsies which were positive by ZNS were also positive by MPT64, indicating that the anti-MPT64 was more likely to detect mycobacterial infection in tissue, as there were more biopsies of which the histology was highly suggestive but ZN was negative and ant-MPT64 was positive.

3.5 Association of demographic and clinical characteristics with anti-MPT64 immunoreactivity.

Of 78 biopsies positive by anti-MPT64 males constituted 29 (37.2%) and females were 49 (62.8%) although this difference was not statistically significant (p-value = 0.857). The peak age-group was between 18-35 years (42.9%, n=33) and the least (11. 7%) affected age group were children (<18 years) who were (n=9). The two most frequent sites of presentation were the lymph nodes and abdomen respectively and for both males and females, lymph node was the most common site of presentation. The second common site of presentation in males was the bones while in females it was the abdomen. Clinically, EPTB was suspected in 60.5% of 78 biopsies and the rest had other clinical suspected diagnoses including malignancy. Out of the 78 biopsies which were positive for MPT64 antibody, HIV status was known in only 24 (30.7%) patients while in the rest it was unknown. However, the differences in HIV serostatus was not statistically significant (p-value = 0.657, Fisher exact test). Details are found on table 3.

Table 2: Association of anti-MPT64 immunoreactivity by demographic and clinical characteristics

CHARACTERISTICS	ANTI-MPT64 IHC POSITIVE	ANTI-MPT64 IHC NEGATIVE	*P* value
Age group (years) (N=109)			
< 18	9(11.7%)	2(6.2%)	0.747
18-35	33(42.9%)	14(43.8%)	
36-53	23(29.8%)	12(37.5%)	
54 and above	12(15.6%)	4(12.5%)	
Sex (N=110)			0.857
Male	29(37.2%)	15(46.9%)	
Female	49(62.8%)	17(53.15)	
Anatomical site (N=110)			0.056
Lymphnode	38 (48.7%)	8 (25%)	
Abdomen	11 (14.1%)	2 (6.2%)	
Bone	9 (11.5%)	3 (9.4%)	
Pleura	4 (5.1%)	6 (18.8%)	
Genito-urinary	5 (6.4%)	3 (9.4%)	
Skin	2 (2.6%)	4 (12.5%)	
Joints	2 (2.6%)	1 (3.1%)	
Others*	7 (9.0%)	5 (15.6%)	
Clinical diagnosis (N=106)			0.485
EPTB	46 (60.5%)	19 (63.3%)	
Others**	30 (39.5%)	11(36.5%)	
HIV status (N=30)			0.657
Positive	12(50%)	4 (66.7)	
Negative	12(50%)	2 (33.3)	

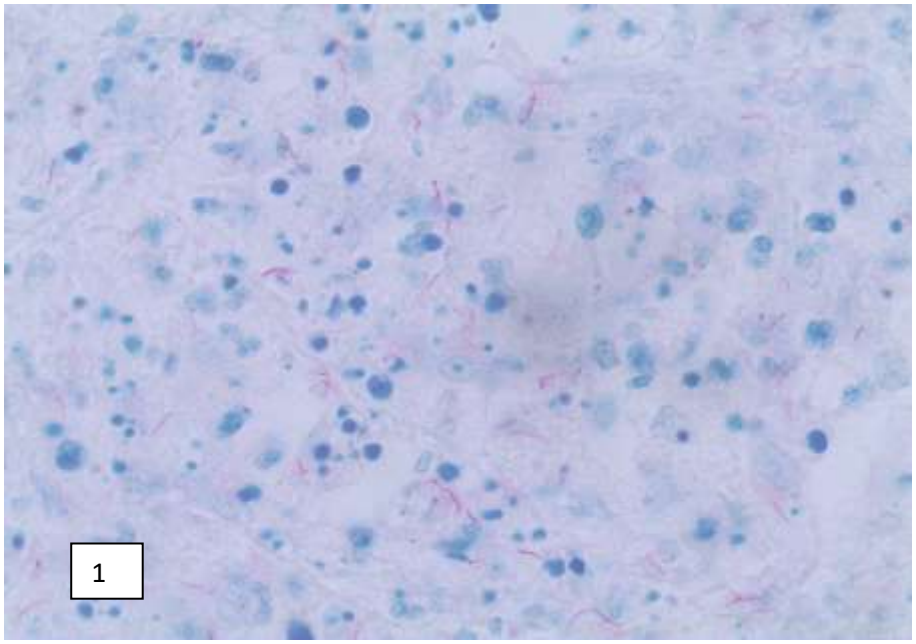
**Others include breast, soft tissue, nasopharynx, brain, **other diagnosis suspected by clinician such as malignancy*

Of the positive ant-MPT64 biopsies with HIV positive results (n=12), 2 biopsies (16.7%) were from males while 10 biopsies (83.3%) were from females. This suggests that females with HIV infection were more likely to have EPTB and this was statistically significant (P-value = 0.025).

Table 3: Association of gender and HIV serostatus

Gender	**HIV status**	
	Positive	Negative
Female	10(83.3%)	10(83.3%)
Male	2(16.7%)	2(16.7%)
Total	12(100%)	12(100%)

Figure 7: Positive control photo images



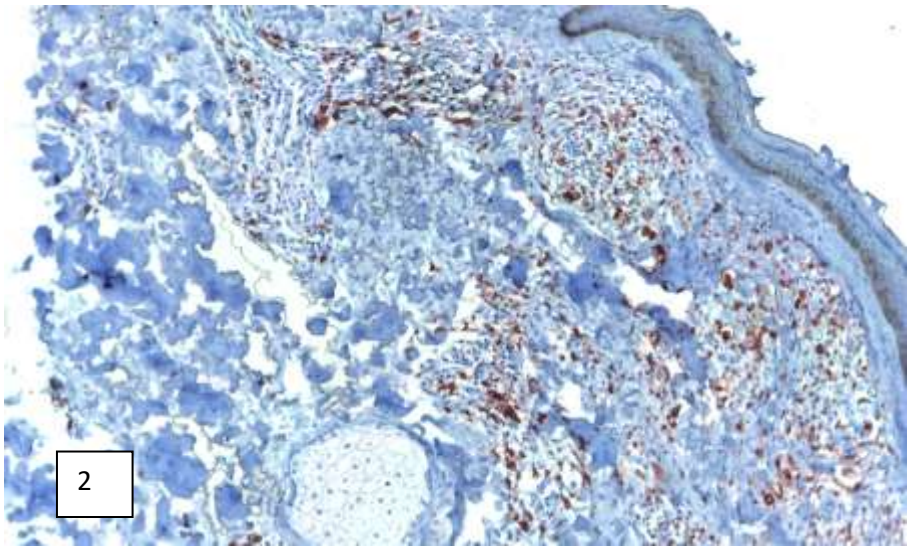
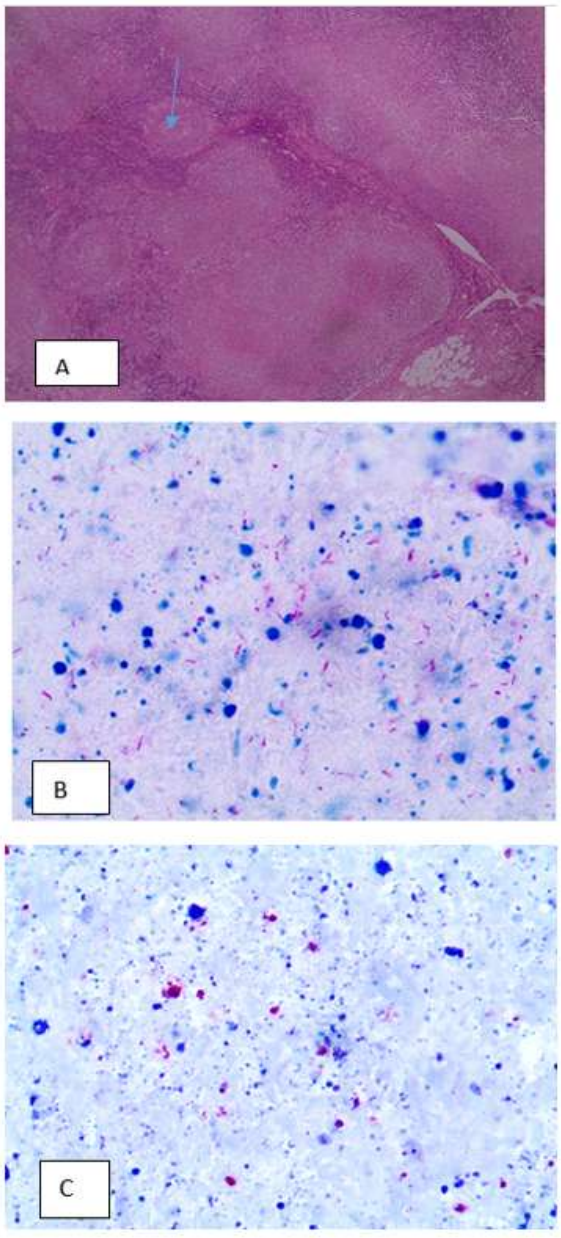


Image 1: ZN positive control showing red rods

Image 2: anti- MPT64 positive control

Figure 8: Tuberculous Lymphadenitis photo images

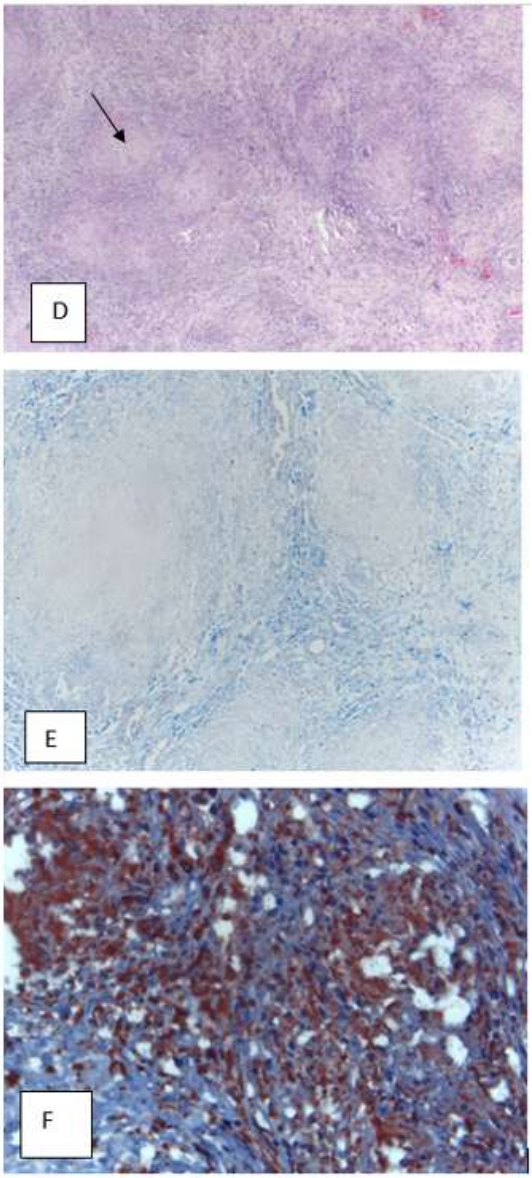


A-H/E section from the lymph node showing granulomas (arrow) 10x

B-ZN stained lymph node section positive for AFB (red rods) 40x

C- A section of Lymph node stained positive (brown) for anti-MPT64 antibody

Figure 9: Tuberculous Pleuritis photo images



D-Section from the pleura showing granulomas (arrow). 10x

E-ZN stained pleural section, negative for AFB (no red rods) 40x

F-A section of pleural TB stained positive (brown) for anti-MPT64 antibody 40x

CHAPTER FOUR

4.0 DISCUSSION

The present study aimed at determining the EPTB histomorphology from different anatomical sites of presentation and detect *Mycobacteria tuberculosis* by anti-MPT64 IHC on FFPE archived surgical biopsies. In this study, archival biopsies taken from 110 patients were recruited during the period from 1st July 2015 to 30th June 2017, at MNH. We found that extrapulmonary tuberculosis is not infrequent among surgical specimens in our settings and most of our biopsies showed caseous necrotizing granuloma histomorphology. The median age was 33 years; both children and adults were affected though the later were more frequent affected. Majority of our patients were female. Lymph node was the most common anatomical site of biopsy seen in about 42% of biopsies. Clinically, tuberculosis was suspected in more than half of our patients. Few cases were ZNS positive while IHC using anti-MPT64 positivity was recorded in more than half of the cases.

The frequency of diagnosis of EPTB was about 0.8 % of all surgical specimens which were submitted and reported during the study period. To the best of my knowledge, our study was the first to look on the frequency of EPTB among different surgical specimens with various diagnosis. Most of studies reported the frequency of EPTB among all TB cases, which was difficult in our index study as most of the pulmonary TB diagnosis are done by sputum or chest radiology rather than submission of lung biopsy.

The findings of our study are similar to other records from the literature which showed female predominance (52). However two other studies showed male predominance, one of them done by A. Shanmuganathan et al.,(2013) on which the male to female ratio was 58:42 (49) .The second study was conducted in a low TB prevalence setting and showed male predominance (33) which was similar the study done in USA by I. Leeds et al.,(2013) (47). The reason for this could be that the former studies were carried out in developed countries where HIV prevalence is much lower compared to our study setting.In developing countries, females are the most affected persons with HIV which increases their risk of developing extrapulmonary TB.

The median age was 33 years which appears to be slightly similar to a previous study done in Afghanistan whereby median age of patients with EPTB was 31.5(27). As expected both children and adults were more frequently affected though the later were more frequent which could partly be due to the fact that adults have had more exposure to the bacilli over time. Our cohort showed the people 18 years to 35years mostly harbour the infection of mycobacteria, followed by the age group 36 to 53years. This was in agreement with two previous studies that showed EPTB distribution was clustered in the reproductive age groups (27,49). The reason could be these age groups are also mostly affected by HIV infection, and as we already know HIV infection carries a risk of developing EPTB.

Clinically, tuberculosis was suspected a reasonable majority of our patients alluding to clinical-histopathological concordance, however, the findings that nearly 40% of histologically diagnosed EPTB had discordant clinical diagnosis indicates the sheer difficulty in the diagnosis of the same and as such the importance of developing assays and algorithms which can improve this.

Caseating granuloma is the strongest evidence of tuberculosis infection in tissue sections and this was the most frequent histomorphology finding encountered in our study. Our findings are similar to other previous studies: in Yemen where caseous necrotizing granuloma represented 68% of biopsies from TB lymphadenitis (35) and Eshete et al., (2011) also found caseous necrotizing granulomas in 85% of lymph node biopsies (36).

Another study which was done in a of low TB prevalence showed caseating granuloma are present in 62% of cases and the rest showed non-caseating granulomas (33). This is similar to what we noted in our index study in which one-third had non-caseating granulomas. Although EPTB may present with non-caseating granulomas, other diseases have to be ruled out including histoplasmosis, sarcoidosis, inflammatory bowel disease (IBD) and a confirmatory test is therefore necessary to reach on a definitive diagnosis. Chande et al., (2009) showed that about three quarters of biopsies had granulomatous inflammation consistent with TB lymphadenopathy (53). This is generally not inconsistent with our findings although we included both nodal and extra nodal sites in our index study in which almost three quarters had histomorphology consistent with TB.

In the current study, the positivity of ZN stain among biopsies with histology suggestive of EPTB was rather low (8.2%). One of the previous studies found the overall frequency ZNs positivity among biopsies with histology suggestive of EPTB was 26.1% (34).The higher positivity of this study compared to ours could be due to the reason that the previous study examined biopsies which had only strong features/evidences of EPTB on histology (caseating granulomas, Langhan's giant cells and epithelioid cells) while in our index study we included weak histopathological evidences as well. Another previous report showed the ZNs positivity among paraffin embedded nodal tissue biopsies was 61.7% (43). This was significantly higher compared to our findings and this difference could be due to the fact that the previous study examined ZNs positivity among lymphnode biopsies only of which the distribution of mycobacteria bacilli is higher compared to other sites. Our study included several other sites of biopsy as well. Our study showed a higher frequency of ZN positivity among histologically diagnosed biopsies when compared to another study which was done in Yemen where only 3% of lymph node biopsies with histological evidences of TB were detected on ZN (35).

A previous report from Khartoum, Sudan on utility of ZN stain on diagnosis of TB lymphadenopathy showed that ZN stain was positive in only 4 (2.5%) biopsies out 161 biopsies which were previously reported as TB lymphadenitis by histomorphology (40). The findings of this study were also slightly lower but rather consistent with our current findings and it included biopsies with caseating as well as non-caseating granulomas in histomorphology. Other studies which found comparable proportion of ZN stain positivity include; Tehmina et al., (2006) found the positivity of ZN stain in 10% (41) Purohit et al., (2007) on his study on lymp36h node and abdominal TB showed that ZN positivity was 11% and 0% respectively (20). In our current findings although overall ZN positivity was 8.2%, the site (lymph node) specific positivity for ZN was 11% (slightly higher) and for abdomen it was 4%. A former study (20) found 0% positivity on abdominal TB and several studies have shown the positivity of ZN stain on tissue biopsies ranging from 0%-40% thus our index findings are generally in agreement with these (5). The low positivity of ZN stain is presumably due to paucibacillary nature of extrapulmonary tuberculosis as it requires more than 10000bacilli per slide for acid fast bacilli to be detected(41). Therefore, the low positivity of ZN stain despite suggestive features of EPTB on histology in our

study could be attributed to the already reported low sensitivity of ZN stain on EPTB tissue biopsies.

The anti-MPT64 assay is specific to *Mycobacteria tuberculosis* complex species (MTBC) (18) and does not detect the non-tuberculous mycobacteria (NTM) species such as *mycobacteria avium* (3). In our study majority (71%) of biopsies (n=78) which had histological diagnosis of EPTB based on histomorphology were positive when stained by anti-MPT64 immunohistochemistry indicating that most of our EPTB patients were infected by *Mycobacteria complex species*.

Kamaldeen et al., (2008) showed the positivity of pleural TB by MPT64 immunohistochemistry to be 80%(20) which was slight higher compared to our current findings. The difference may be explained by the fact that the former study examined retrospectively tissue biopsies of patients who were diagnosed as Tuberculosis by several criteria including response to anti- tuberculous drugs as well as positive culture and/or positive acid-fast bacilli staining in addition to tuberculous granuloma on histology. Another study showed the positivity by MPT64 to be 64% among biopsies with granulomatous lymphadenitis(41) which is comparable to our results.

In a study done in India, 100% of anti- MPT64 positivity among biopsies of from various extrapulmonary sites was noted (54). This could be explained by the fact that the previous study examined biopsies which were already confirmed by either PCR or culture to be EPTB while our study we stained biopsies which had histology suggestive of EPTB. Not only that but also Purohit et al.,(2007) showed that MPT64 positivity on cervical lymph node was 80% and 73.7%,77.7%,80% for intestinal wall, peritoneum and mesenteric lymph node respectively(20) generally rather comparable to our current findings. Although we did not focus on anatomical site-specific positivity in our current study. This gives an opportunity for a further study to elucidate on the MPT64 positivity as well as sensitivity and specificity in each different site of biopsy.

As mentioned above, anti-MPT64 antibody characterizes only *Mycobacteria tuberculosis* complex species (MTBC), therefore non-tuberculous mycobacteria (NTM) which may give similar histomorphological pattern could have been missed in our study and this alludes to the MPT64 negative biopsies, some of which could still be cases of Mycobacterial

infection (false negatives). As the prevalence of non-tuberculous mycobacteria is not well known, in our settings, this could also contribute to difference observed in the findings of our study as compared to others.

As expected, all biopsies which were positive by ZN stain were also positive by MPT64 IHC. In our study we found that among biopsies which had positive ZN results, majority had numerous number of bacilli while only one had scant bacilli (< 2bacilli per high power field). Our findings differ from a previous study which found that although more than 50% of lymph node biopsies were positive by ZN stain the distribution of bacilli was scant in the majority(36).

Our current study found that MPT64 positivity was variable from markedly diffuse positivity to few positive signals. This could be explained by the fact that the distribution of mycobacteria bacilli on tissue is variable and the fact that the antibody only detects the protein and not necessarily the organism. This was similar to another study which showed that the intensity and extent of staining of anti-MPT64 immunohistochemistry varied from many cells of the granulomas to few cells of granulomas demonstrated staining(19).

The distribution of anti-MPT64 immunohistochemistry can be intracellular (cytoplasmic) and extracellular as it just detects the MPT64 protein wherever it is. In our study, we found MPT64 positivity was seen in various granuloma cells including epithelioid cells, lymphocytes as well as multinucleated giant cells. Implying that mycobacterial antigens were contained in these granuloma cells as anti-MPT64 can detect protein even if the mycobacterium was fragmented. For the case of ZN stained biopsies the distribution of the *bacilli* was seen both extracellular in necrotic areas and intracellular in epithelioid cells but we could not identify *mycobacteria bacilli* inside multinucleated giant cells. This can be explained by the fact that ZN stain positivity require intact cell wall and therefore fragmented *bacilli* inside multinucleated giant cells could have been fragmented were not detected by this stain.

Interestingly, we had one biopsy whose patient underwent craniotomy due to brain haemorrhage after head injury secondary to motor traffic accident and an incidental nodule was found on the dura and it was excised and submitted for histopathology which found a granulomatous inflammation consistent with EPTB. This biopsy was positive for anti-

MPT64 and negative for ZN stain. Prior to the accident the patient had no history of chronic illness. An implication for this patient is that the nodule could be a dormant infection by mycobacteria (EPTB) infection.

In our findings we noted a higher number of positivity by ant- MPT64 immunohistochemistry compared to ZN stain. This can be explained by the already established sensitivity of MPT64 which range from 80%-100% (19,20,41,55).

Our study findings showed that the lymph node was the most common anatomical site recorded and they are similar to other studies: in Kabul Afghanistan where TB lymphadenitis was found in 37.3% of all cases with EPTB and Gondar hospital in Ethiopia where TB of lymph node was found in 82.4% of cases with EPTB(26,56). A. Shanmugahan et al.,(2013) also reported lymph node was the most common site of presentation followed by pleural and abdomen (49) which did not differ much from our results, though we found the second common site of EPTB was abdomen and the third one was pleural. However, a previous study done in our settings (MNH) to evaluate TB/Co-HIV infection found that among the patients having TB/HIV co-infection; pleura was the most common TB involved site. In this study collected TB results and data were from different diagnostic modalities such as clinical criteria as well as laboratory confirmed tests (32). The difference in findings to our current study could be due to the fact that the present study dealt with only surgical biopsies, therefore most of the pleural TB were probably not submitted for histology and rather diagnosed by sending pleural fluid for smear, culture and gene Xpert. L. Chin et al., (2013) also found the pleura being the most common site of presentation for EPTB(48). In his study Chin et al., (2013) assessed patients having concomitant PTB and EPTB, therefore it was expected the pleural to be more affected due to close proximity between the two anatomical sites. There was another study which showed the genital urinary TB predominance in their cohort (52) different from lymph node and pleura. In general, the predominant EPTB involved site findings in various studies showed variations in geographic location, population, study design as well as host factors; however, lymph node seems to be the overall most common site.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

- EPTB is not uncommon at the Muhimbili National Hospital and effective diagnosis remains a challenge.
- TB lymphadenitis appears to be the most common site of presentation of EPTB in our setting.
- The most affected group is young adults in the reproductive age and females seem to be more affected by EPTB compared to males.
- The histomorphology of necrotizing granuloma appeared to be strongly associated with MPT64 positivity.
- As expected, anti-MPT64 positivity was more frequent compared to ZNS positivity which can be explained by the already documented sensitivity of this IHC stain.

5.2 Recommendations

To the Clinicians:

- EPTB should be considered as a differential diagnosis in different clinical scenarios as it presents in a protean ways which may mimic malignancy, therefore biopsy of the lesions for confirmation by histology is important.

- Patients who present with enlarged lympnodes should have TB infection investigated for.

To the Pathologist:

- For granulomatous lesions it is important to do further work up including special stains for microorganisms in order exclude other causes such as fungal infection.

- Clinical pathological correlation is important as well in order to make the diagnosis of EPTB and whenever there is a controversy; advanced tests including immunohistochemistry should be employed.

To MNH

- Immunohistochemistry for Mycobacteria TB should be available in the laboratory in addition to ZNS.

Study Limitations

Our study had some limitations. First, we could not compare the performance anti-MPT64 with other tests by using culture as a gold standard in diagnosis of TB infection, the reason being that our study was retrospective and dealt with archival parrafinized tissue biopsies which could not be cultured. Furthermore, budgetary constraints did not allow molecular tests such as PCR be performed to compare the anti-MPT64 performance.

Secondly, due to the retrospective nature of our study it was difficult to monitor and harmonize tissue processing factors including fixation which might affect the staining of the tissue. It is also important to note due to retrospective retrieval of data, in majority of biopsies HIV status was not documented, thus limiting the interpretation of the associations.

REFERENCES

1. Ministry of Health Community Development, Gender E and C. National Tuberculosis and leprosy programme,Annual report for 2014. 2014;(1).
2. Sharma SK, Mohan A. Extrapulmonary tuberculosis. Indian J Med Res. 2004;120:316–53.
3. Sinha P, Gupta A, Prakash P, Anupurba S, Tripathi R, Srivastava GN. Differentiation of Mycobacterium tuberculosis complex from non-tubercular mycobacteria by nested multiplex PCR targeting IS6110 , MTP40 and 32kD alpha antigen encoding gene fragments. BMC Infect Dis . 2016;16(123):1–10.
4. S.K.Sharma,A.Mohan T kadhirav. HIV-TB co-infection. Indian J Med Res. 2005;121:550–67.
5. Purohit M. Laboratory Diagnosis of Extra-pulmonary Tuberculosis (EPTB) in Resource- constrained Setting : State of the Art , Challenges and the Need. JCDR. 2015;4:1–6.
6. Jesuraj A, Kumar U, Srinivasa H. Fast and Accurate Identification of M . tuberculosis Complex Using an Immunochromatographic MPT64 Antigen Detection Test. J Tuberc Res. 2015;4(3):149–56.
7. Vijayan VK. Clinical aspects of tuberculosis. Indian J Clin Biochem. 2002;17(2):96–100.
8. The World Health Organization. WHO | Tuberculosis. WHO [Internet]. 2017 [cited 2017 Jan 27];
9. Pedersen JS, Clarke I, Mills J. Improved detection of mycobacteria species in formalin-fixed tissue sections. Histopathology. 2011;59(5):993–1005.
10. NTLP. National Guidelines for the Management of Tuberculosis . 2012.
11. Kumar V, Abbas AK, Fausto N, Aster JC. Robbins & Cotran Pathologic Basis of Disease - 8th Ed . 2010.
12. Elder NC. Extrapulmonary tuberculosis. A review. Arch Fam Med. 1992;1(1):91–8.
13. Thapa B. Smear negative pulmonary tuberculosis and infectivity. Int J Infect Microbiol [Internet]. 2013;2(3):68–9.
14. Davies PDO, Pai M. The diagnosis and misdiagnosis of tuberculosis. Int J Tuberc Lung Dis. 2008;12(11):1226–34.

15. Singh KK, Muralidhar M, Kumar A, Chattopadhyaya TK, Kapila K, Singh MK, et al. Comparison of in house polymerase chain reaction with conventional techniques for the detection of Mycobacterium tuberculosis DNA in granulomatous lymphadenopathy. J Clin Pathol . 2000;53(5):355–61.
16. Denkinger CM, Schumacher SG, Boehme CC, Dendukuri N, Pai M, Steingart KR. Xpert MTB/RIF assay for the diagnosis of extrapulmonary tuberculosis: A systematic review and meta-analysis. Eur Respir J. 2014;44(2):435–46.
17. Goel MM, Budhwar P. Immunohistochemical localization of Mycobacterium Tuberculosis complex antigen with antibody to 38 kDa antigen versus Ziehl Neelsen staining in tissue granulomas of extra-pulmonary tuberculosis. Indian J Tuberc. 2007;54(1):24–9.
18. Kanade S, Nataraj G, Suryawanshi R, Mehta P. Utility of MPT64 antigen detection aasy for rapid characterization of Mycobacteria in resourse constrained setting. Indian J Tuberc. 2012;(59):92–6.
19. Baba K, Dyrhol-Riise AM, Sviland L, Langeland N, Hoosen A a, Wiker HG, et al. Rapid and specific diagnosis of tuberculous pleuritis with immunohistochemistry by detecting Mycobacterium tuberculosis complex specific antigen MPT64 in patients from a HIV endemic area. Appl Immunohistochem Mol Morphol. 2008;16(6):554–61.
20. Purohit MR, Mustafa T, Wiker HG, Mørkve O, Sviland L. Immunohistochemical diagnosis of abdominal and lymph node tuberculosis by detecting Mycobacterium tuberculosis complex specific antigen MPT64. BioMed Cent. 2007;1(2):1–9.
21. Karimi S, Shamaei M, Pourabdollah M, Sadr M, Karbasi M, Kiani A, et al. Histopathological findings in immunohistological staining of the granulomatous tissue reaction associated with tuberculosis. Tuberc Res Treat . 2014;5.
22. The World Health Organization. Treatment of tuberculosis: guidelines. WHO 2010;4:95–6.
23. WHO. Global Tuberculosis Report. switzerland; 2016.
24. MoHSW. The First National Tuberculosis Prevalence Survey in the United Republic of Tanzania Final Report. 2013;(1):19.
25. Kipp AM, Stout JE, Hamilton CD, Van Rie A. Extrapulmonary tuberculosis, human immunodeficiency virus, and foreign birth in North Carolina, 1993 - 2006. BMC

Public Health . 2008;8(1):107.

26. Hernandez-Garduno E. Transmission of tuberculosis from smear negative patients: a molecular epidemiology study. 2004;59(4):286–90.
27. Fader T, Parks J, Khan NU, Manning R, Stokes S, Nasir NA. Extrapulmonary tuberculosis in Kabul, Afghanistan: A hospital-based retrospective review. Int J Infect Dis . 2010;14(2):102–10.
28. Kingkaew N, Sangtong B, Amnuaiphon W, Jongpaibulpatana J, Mankatittham W, Akksilp S, et al. HIV-associated extrapulmonary tuberculosis in Thailand: epidemiology and risk factors for death. Int J Infect Dis. 2009;13(6):722–9.
29. Zumla A, Petersen E, Nyirenda T, Chakaya J. Tackling the Tuberculosis Epidemic in sub-Saharan Africa - unique opportunities arising from the second European Developing Countries Clinical Trials Partnership (EDCTP) programme 2015-2024. Int J Infect Dis . 2015;32:46–9.
30. Falzon D, Getuhan H, Kanchar A, Mirzayev F, Raviglione M TH. Global Tuberculosis report 2015. WHO. 2015;20:1–19.
31. Meremo AJ, Kidenya BR, Mshana SE, Kabangila R, Kataraihya JB. High prevalence of tuberculosis among adults with fever admitted at a tertiary hospital in north-western Tanzania. Tanzan J Health Res. 2012;14(3):1–9.
32. Kamenju P, Aboud S. Tuberculosis-HIV co-infection among patients admitted at Muhimbili National Hospital in Dar es salaam, Tanzania. Tanzan J Health Res. 2011;13(1):21–6.
33. Pollett S, Banner P, O'Sullivan MVN, Ralph AP. Epidemiology, diagnosis and management of extra-pulmonary tuberculosis in a low-prevalence country: A four year retrospective study in an Australian Tertiary Infectious Diseases Unit. PLoS One. 2016;11(3):1–15.
34. Nassaji M, Azarhoush R, Ghorbani R, Kavian F. Acid fast staining in formalin-fixed tissue specimen of patients with extrapulmonary tuberculosis. Int J Sci Res Publ. 2014;4(10):4–8.
35. Ahmed HGE, Nassar AS, Ginawi I. Screening for tuberculosis and its histological pattern in patients with enlarged lymph node. Patholog Res Int. 2011;2011:1–4.
36. Eshete A, Zeyinudin A, Ali S, Abera S, Mohammed M. M . tuberculosis in Lymph Node Biopsy Paraffin-Embedded Sections. Tuberc Res Treat. 2011;13:1–5.

37. Kitinya JN, Richter C, Perenboom R, Chande H, Mtoni IM. Influence of HIV status on pathological changes in tuberculous pleuritis. 1992;(July):195–8.
38. Fukunaga H, Murakami T, Gondo T, Sugi K, Ishihara T. Sensitivity of acid-fast staining for Mycobacterium tuberculosis in formalin-fixed tissue. Am J Respir Crit Care Med. 2002;166(7):994–7.
39. Park DY, Kim JY, Choi KU, Lee JS, Lee CH, Sol MY, et al. Comparison of polymerase chain reaction with histopathologic features for diagnosis of tuberculosis in formalin-fixed, paraffin-embedded histologic specimens. Arch Pathol Lab Med . 2003;127(3):326–30.
40. Nourein IH, Ahmed HG, Abusharib AB, Badawi M. Diagnostic Utility of ZN , IH and PCR in Detection of Tuberculous Lymphadenopathy : A retrospective Study from Sudan. Intrernational J Sci Res. 2014;3(7):1402–6.
41. Mustafa T, Wiker HG, Mfinanga SGM, Mørkve O, Sviland L. Immunohistochemistry using a Mycobacterium tuberculosis complex specific antibody for improved diagnosis of tuberculous lymphadenitis. 2006;1606–14.
42. Yoon HJ, Song YG, Park W Il, Choi JP, Chang KH, Kim JM. Clinical manifestations and diagnosis of extrapulmonary tuberculosis. Yonsei Med J [Internet]. 2004;45(3):453–61.
43. Chandir S, Hussain H, Salahuddin N, Amir M, Ali F, Lotia I, et al. Extrapulmonary Tuberculosis : A retrospective review of 194 cases at a tertiary care hospital in Karachi , Pakistan S-103. J Pak Assoc. 2010;60(2):102–6.
44. Sreeramareddy CT, Panduru K V, Verma SC, Joshi HS, Bates MN. Comparison of pulmonary and extrapulmonary tuberculosis in Nepal-a hospital-based retrospective study. BMC Infect Dis. 2008;8(1):8.
45. Ganal S, Yang Z, Agarwa M, Koroglu M, Arici ZK DR. Demographic and microbial characteristics of extrapulmonary tuberculosis cases diagnosed in Matalya,Turkey 2001-2007. 1(11):154.
46. Ade S, Harries AD, Trébucq A, Ade G, Agodokpessi G, Adjonou C, et al. National profile and treatment outcomes of patients with extrapulmonary tuberculosis in Benin. PLoS One. 2014;9(4).

47. Leeds IL, Magee MJ, Kurbatova E V., Del Rio C, Blumberg HM, Leonard MK, et al. Site of extrapulmonary tuberculosis is associated with HIV infection. Clin Infect Dis. 2012;55(1):75–81.
48. Lin C, Chen T, Lu P, Lai C, Yang Y, Lin W, et al. Effects of Gender and Age on Development of Concurrent Extrapulmonary Tuberculosis in Patients with Pulmonary Tuberculosis : A Population Based Study. PLoS One. 2013;8(5).
49. Shanmuganathan A, Srinivasan R, Thilagavathy G, Satishkumar D, Sidduraj C, James B. Determination of Sites Involved , HIV Co – Infection & Utility of Diagnostic Modalities in EPTB. J Clin Diagnostic Res. 2013;7(8):1644–6.
50. Maynard-Smith L, Larke N, Peters JA, Lawn SD, Perkins M, Cunningham J, et al. Diagnostic accuracy of the Xpert MTB/RIF assay for extrapulmonary and pulmonary tuberculosis when testing non-respiratory samples: a systematic review. BMC Infect Dis. 2014;14(1).
51. Abdallah TEM, Toum FEM, Bashir OH, Mansoor TI, Yuosif MM, Elkhawad MAE, et al. Epidemiology of extra pulmonary tuberculosis in Eastern Sudan. Asian Pac J Trop Biomed . 2015;5(6):505–8.
52. Ganal S, Yang Z, Agarwa M, Koroglu M, Arici ZK DR. Demographic and microbial characteristics of extrapulmonary tuberculosis cases diagnosed in. BMC Public Health. 2011;11(154).
53. Sviland L, Chande H. East African Journal of Public Health Volume 4 Number 2, October 2007 47. 2007;4(2):47–50.
54. Purohit MR, Sviland L. Rapid and Specific Diagnosis of Extrapulmonary Tuberculosis by Immunostaining of Tissues and Aspirates With Anti-MPT64. 2017;25(4):282–8.
55. Davidsen M, Marijani M, Dyrhol-riise AM, Sviland L, Mustafa T. MPT64 antigen detection test improves routine diagnosis of extrapulmonary tuberculosis in a low-resource setting : A study from the tertiary care hospital in Zanzibar. PLoS One. 2018;5(13):1–15.
56. Zenebe Y, Anagaw B, et al. Smear positive extra pulmonary tuberculosis disease at University of Gondar Hospital, Northwest Ethiopia. BMC Res Notes . 2013;6:21.

APPENDICES

Appendix I: Data collection sheet

i) Patient's Particulars

1. ID NO. ☐

2. HP NO. ☐

3. Age at diagnosis (Years) ☐

4. Sex 1. Male ☐

2. Female

ii) Clinical data

I) Site of biopsy

1. Lymph node
2. Pleura
3. Bone
4. Abdomen
5. Genital urinary
6. Skin
7. Joints
8. Others (Specify)_____ ☐

II) Clinical diagnosis 1. EPTB 2.Others (specify)_____ ☐

III) HIV status 1) Positive 2)Negative 3) Unknown ☐

Appendix II: Histopathology, ZN stain and MPT64 immunohistochemistry results

HP No. ☐

Age ☐

Sex ☐

Histopathological findings

I) Caseating granuloma ☐

II) Non-caseating granuloma ☐

Immunohistochemistry by MPT64 antibody

1. Positive
2. Negative ☐

ZN stain results

1. Positive
2. Negative ☐

Appendix II: Ethical clearance approval

MUHIMBILI UNIVERSITY OF HEALTH AND ALLIED SCIENCES
OFFICE OF THE DIRECTOR OF POSTGRADUATE STUDIES

P.O. Box 65001
DAR ES SALAAM
TANZANIA
Web: www.muhas.ac.tz

Tel G/Line: +255-22-2150302/6 Ext. 1015
Direct Line: +255-22-2151378
Telefax: +255-22-2150465
E-mail: dpgs@muhas.ac.tz

Ref. No. MU/ PGS/SAEC/Vol. X/

21st August, 2017

Dr. Caroline Ngimba
MMed. Anatomical Pathology
MUHAS.

RE: APPROVAL OF ETHICAL CLEARANCE FOR A STUDY TITLED: "HISTOPATHOLOGICAL AND MPT64 IMMUNOHISTOCHEMISTRY CHARACTERISATION OF EXTRAPULMONARY TUBERCULOSIS AT THE MUHIMBILI NATIONAL HOSPITAL."

Reference is made to the above heading.

I am pleased to inform you that, the Chairman has, on behalf of the Senate, approved ethical clearance for the above-mentioned study. Hence you may proceed with the planned study.

The ethical clearance is valid for one year only, from 21st August, 2017 to 20th August, 2018. In case you do not complete data analysis and dissertation report writing by 20th August, 2018, you will have to apply for renewal of ethical clearance prior to the expiry date.

Prof. Andrea B. Pembe
DIRECTOR OF POSTGRADUATE STUDIES

cc: Director of Research and Publications
cc: Dean, School of Medicine

Appendix III: MNH research clearance

MUHIMBILI NATIONAL HOSPITAL

Cables: "MUHIMBILI"
Telephones: +255-22-2151367-9
FAX: +255-22-2150534
Web: www.mnh.or.tz

Postal Address:
P.O. Box 65000
DAR ES SALAAM
Tanzania

In reply please quote:

MNH/TRC/Research/2017/134

23rd August, 2017

Dr. Carolina Ngimba,
MUHAS

RE: PERMISSION TO COLLECT DATA AT MNH: 2017/18

Name of Student	Dr. Carolina Ngimba,
Title	**"HISTOPATHOLOGICAL AND MPT64 IMMUNOHISTOCHEMISTRY CHARACTERISTION OF EXTRAPULMONARY TUBERCULOSIS AT MUHIMBILI NATIONAL HOSPITAL".**
Institution	Muhimbili University of health and Allied Sciences
Supervisor	Dr. Amos R. Mwakigonja
Period	28/08/2017 to 30/02/2018 (6 months)

You have been permitted to collect data in respect to the undertaking of the above mentioned study.

Please ensure that you abide to the ethical principle and other conditions of yours approval.

Sincerely,

Dr. Faraja Chiwanga
Head of Teaching, Research and Consultancy Coordination Unit

HEAD, TEACHING RESEARCH & CONSULTANCY UNIT
MUHIMBILI NATIONAL HOSPITAL
P. O. BOX 65000
DAR ES SALAAM

Appendix IV: Procedure for hematoxylin and eosin staining (MNH PROTOCAL)

1. Dewax sections and bring down to water.
2. Stain in Harris haematoxylin for 10 minutes.
3. Rinse in running tape water.
4. Differentiate in 1% acid alcohol for 5-10 seconds.
5. Rinse in tap water
6. Blue the sections in tap water for 10 minutes.
7. Counter stain in 1% aqueous eosin for 3 minutes.
8. Rinse in tap water.
9. Dehydrate the sections through ascending grades of alcohols.
10. Clear in two changes of xylene (3 minutes in each)
11. Mount with DPX or mounting machine.

Appendix V: Procedure for Ziehl Nielsen stain (MNH PROTOCAL)

1. Deparaffinize the sections and bring down to distilled water.
2. Put slides, section upwards, on the staining rack.
3. Flood the slide section with Carbol- Fuchsin.
4. Warm until steam rises. (Do not boil) and leave for 10 minutes.
5. Wash well in distilled water.
6. Differentiate in 1% acid in 70% alcohol for 30 seconds.
7. Wash well in distilled water.
8. Counter stain with 0.2% Methylene Blue for 1 minute.
9. Bloat dry with whatma's filter paper.
10. Transfer to two of each change of 95% and absolute alcohol.
11. Clear in two changes of Xylene.

Add a drop of DPX on the stained slide and cover slip it or use mounting machine

Appendix VI: Procedure for immunohistochemistry (MNH PROTOCAL)

- Cut on Dako slides and bake for 30 minutes on hot plate.
- After rehydration place slides in antigen retrieval solution (citric acid at pH 6.0) in microwave and heat at 350W to the boiling point.
- Remove the slides and let it cool on the bench for 15-20 min, and wash in running (distilled water) for 3min
- Do not allow slides to dry from this step onwards.
- Add Tris Buffer Saline (TBS) for 3 minutes.
- Drain off TBS and cover sections with primary antibody at appropriate dilution and incubate for 60 minutes. For negative control substitute primary antibody with antibody diluent.
- Wash gently TBS on slide for 5 minutes
- Drain off TBS from slides and cover sections with 2-3 drops of Horse-radish Peroxidase (HRP) for 30 minutes, the reagent should be at room temperature before use.
- Wash gently for 5 minutes with TBS (make up AEC reagent by adding one drop of AEC to 1 ml of substrate buffer)
- Drain TBS from slides and apply AEC reagent to the sections and incubate for 5 minutes.
- Wash slides well in water
- Counterstain with haematoxylin for 30 seconds, dip in 1% acid alcohol 2 dips.
- Blue in tap water for 2 minutes.
- Tap of excess water and wipe the slides, put one or two drops of mounting medium on coverslip and gently place the cover slip over the slide.