Diagnosis and management of extrapulmonary tuberculosis in low-resource settings, a study from Zanzibar, Tanzania

Melissa Davidsen Jørstad

Thesis for the degree of Philosophiae Doctor (PhD)
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Contents

Scientific environment

The present research was conducted in collaboration between the following institutions:

1. University of Bergen (UoB): Centre for International Health at Department of Global Public Health and Primary Care, Department of Clinical Medicine, Department of Clinical Science.
2. Haukeland University Hospital (HUH): Department of Pathology, Department of Thoracic Medicine.
3. Mnazi Mmoja Hospital (MMH), Zanzibar, The United Republic of Tanzania.

Main supervisor was Professor Tehmina Mustafa (Centre for International Health, Department of Global Public Health and Primary Care, UoB) and co-supervisors were Professor Lisbet Sviland (Department of Clinical Medicine, UoB) and Professor Anne Ma Dyrhol-Riise (Department of Clinical Science, UoB).

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List of abbreviations

ADA	Adenosine deaminase
AEC	3-amino-9-ethylcarbazole
AFB	Acid fast bacilli
AIDS	Acquired immune deficiency syndrome
Anti-TB	Antituberculosis
ART	Antiretroviral therapy
BCG	Bacillus Calmette-Guérin
CFP-10	Culture filtrate protein 10
CNS	Central nervous system
CRS	Composite reference standard
CSF	Cerebrospinal fluid
CTRL	Central Tuberculosis Reference Laboratory
CXR	Chest x-ray
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DOT	Direct and supportive observation
DOTS	Directly Observed Treatment Short-course
DST	Drug susceptibility testing
E	Ethambutol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPTB	Extrapulmonary tuberculosis
EQ-5D-3L	EQ-5D 3 level version
EQ VAS	EQ visual analogue scale
ESAT-6	Early secreted antigenic target 6
FNAC	Fine needle aspiration cytology
FM	Fluorescence microscopy
H	Isoniazid

HCP	Health care provider
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
Hsp65	65-kilodalton heat shock protein
HUH	Haukeland University Hospital
ICC	Immunocytochemical
ICC/IHC staining	Immunostaining
IGRA	Interferon-γ release assay
IHC	Immunohistochemical
IL	Interleukin
INF-γ	Interferon gamma
IQR	Interquartile range
IRIS	Immune reconstitution inflammatory syndrome
kDa	Kilodalton
LAM	Lipoarabinomannan
LED-FM	Light emitting diode fluorescent microscopy
LF-LAM	Lateral flow urine lipoarabinomannan immunochromatographic assay
LJ	Lowenstein-Jensen
mAb	Monoclonal antibody
M.bovis	*Mycobacterium bovis*
MDR-TB	Multidrug-resistant tuberculosis
MGIT	Mycobacteria Growth Indicator Tube
MMH	Mnazi Mmoja Hospital
MoH	Ministry of Health
MPT64 test	The MPT64 antigen detection test
Mtb	*Mycobacterium Tuberculosis*
MtbC	Mtb complex
NAATs	Nucleic acid amplification tests

NDWG	New Diagnostics Working Group
NPV	Negative predictive value
NTM	Nontuberculous mycobacteria
PCR	Polymerase chain reaction
PHCC	Primary health care centre
PHCU	Primary health care unit
PHL-IdC	The Public Health Laboratory-Ivo de Carneri
PPD	Purified protein derivate
PPV	Positive predictive value
PTB	Pulmonary tuberculosis
QUADAS	Quality Assessment of Diagnostic Accuracy Studies
R	Rifampicin
RD	Region of difference
REK	The Regional Committee for Medical and Health Research Ethics
RNA	Ribonucleic acid
RR-TB	Rifampicin-resistant tuberculosis
S	Streptomycin
SOP	Standard operating procedure
STARD	Standards for Reporting of Diagnostic Accuracy
TB	Tuberculosis
TNF-α	Tumor necrosis factor-alpha
TST	Tuberculin skin test
UoB	University of Bergen
WHO	World Health Organization
Xpert	Xpert® MTB/RIF
Xpert Ultra	Xpert® MTB/RIF Ultra
Z	Pyrazinamide
ZAMREC	The Zanzibar Medical Research and Ethics Committee
ZIHTLP	The Zanzibar Integrated HIV, TB and Leprosy Programme

ZN	Ziehl-Neelsen
ZTLP	The Zanzibar Tuberculosis and Leprosy Programme

Abstract

Tuberculosis (TB) is still a major global public health concern. Of the 7 million incident TB cases recognized by the World Health Organization in 2018, 15% were extrapulmonary tuberculosis (EPTB) cases. Diagnosing EPTB remains a challenge despite continuing efforts and progress in the development of new TB diagnostic tools. Signs and symptoms of EPTB are often non-specific and similar in a range of other conditions. Laboratory confirmation of extrapulmonary disease is constrained by the difficulty of obtaining appropriate biological specimens, conventional diagnostic methods with low sensitivity and lack of an accurate, rapid, low-cost diagnostic test for EPTB. In addition, laboratory facilities are costly and often limited in low-resource settings. This may lead to diagnostic delay in initiating antituberculosis (anti-TB) treatment, on the other hand, starting empirical treatment, without laboratory confirmation, emphasises the need of close monitoring of treatment response.

The overall aim of this thesis was to improve the diagnosis and management of EPTB cases in a low-resource setting. Keeping the primary objective in mind, the secondary aims were to implement and assess the performance of a new diagnostic test based on immunochemical detection of the *Mycobacterium tuberculosis* complex specific antigen MTP64 (MPT64 test), for diagnosing EPTB in the routine diagnostics at Mnazi Mmoja Hospital (MMH) in Zanzibar. Further, to evaluate the health care seeking pathways and the diagnostic delays experienced by presumptive EPTB patients, identify factors associated with longer diagnostic delay, and to assess the impact of anti-TB treatment on self-rated health among EPTB cases. Next, to describe the clinical presentation of EPTB, and follow the study participants during anti-TB treatment to assess clinical parameters, independent of laboratory investigations, which could aid in the monitoring of treatment response among EPTB cases.

Presumptive EPTB patients of all ages were prospectively enrolled at MMH for thirteen months from august 2014. At inclusion, data were collected in a face-to face interview using the semi-structured study questionnaire, the results from a full physical examination were recorded and the adult patients initiating anti-TB treatment answered the EQ-5D-3L to evaluate the study participant`s self-rated health before

treatment. Further, a biological specimen was collected from the site of assumptive EPTB infection and subjected to the MPT64 test, GeneXpert® MTB/RIF assay and routine laboratory diagnostics. The included patients initiating anti-TB treatment were assessed after the intensive phase of treatment and at treatment completion. The adult study participants again reported their self-rated health using the EQ-5D-3L after completing anti-TB treatment. Utilizing a predefined composite reference standard, the patients were classified as TB (confirmed TB, probable TB or possible TB) or non-TB cases to assess the diagnostic tests performances and other variables.

We included 132 patients (median age 27 years, interquartile range 8-41 years), who were defined in accordance with the composite reference standard as TB cases (n=64 in paper I and III; n=69 in paper II) and non-TB cases (n=62 in paper I and III, n=63 in paper II). Six patients were classified as uncategorized cases in paper I and III. A higher proportion of positive test results was found for the MPT64 test in TB cases (45/69, 65%) as compared to ZN staining (8/69, 12%), culture (8/60, 13%) and the GeneXpert® MTB/RIF assay (6/38, 16%). The MPT64 test showed an overall sensitivity and specificity of 69% and 95%, respectively, with the best test performance demonstrated among TB lymphadenitis cases and in paediatric TB. Many EPTB cases experienced a delay exceeding two months from symptom onset until treatment was initiated, with health system delay as the main contributor to overall delay. The majority of adult TB cases described reduced work capacity with a median of 60 days due to the ongoing illness and using the EQ-5D instrument, a significantly improved self-perceived health status was noted after as compared to before anti-TB treatment. We further evaluated three clinical parameters, weight gain, clinical improvement and regression of objective findings during anti-TB treatment and found that most of the TB cases fulfilled ≥2 parameters after the intensive phase of anti-TB treatment.

The MPT64 test is implementable in the routine diagnostic laboratory in this low-resource setting and has the potential to improve the diagnosis of EPTB, especially for lymph node TB and paediatric TB in this and similar settings. With many EPTB patients experiencing long delays in the initiation of treatment together with the

reported reduced work capacity among the adult TB patients and improvement of self-reported health status after treatment, reducing the diagnostic delay and timely initiation of appropriate treatment can have crucial impact on the economic loss and morbidity of the affected patients. We propose that a combination of only clinical parameters can be incorporated in a simple assessment tool to aid health care workers in low-resource settings to monitor treatment response among EPTB patients. The findings from this study can be used to improve EPTB patient management in the current setting, but larger and more studies in various routine diagnostic settings are needed to expand the knowledge base regarding the MPT64 test and further evaluate and validate various simple clinical parameters to be incorporated as the suggested easy treatment response assessment tool.

List of publications

Paper I Jorstad MD, 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; 13(5):e0196723.

Paper II Jorstad MD, Aßmus J, Marijani M, Sviland L, Mustafa T.

Diagnostic delay in extrapulmonary tuberculosis and impact on patient morbidity: A study from Zanzibar.

PLoS One. 2018;13(9):e0203593.

Paper III Jorstad MD, Dyrhol-Riise AM, Aßmus J, Marijani M, Sviland L, Mustafa T.

Evaluation of treatment response in extrapulmonary tuberculosis in a low-resource setting.

BMC Infectious Diseases2019; 19:426

1. INTRODUCTION

1.1 The continuous fight against the tuberculosis epidemic

Tuberculosis (TB) infection continues to be a global public health concern and is among the top 10 leading causes of death worldwide and ranks above human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS) as a cause of death from a sole infectious agent, despite implementation of standard care and a global fall in TB incidence (2% per year) and mortality rates (3% per year) (1-3). With the announcement of the Directly Observed Treatment Short-course (DOTS) strategy by the World Health Organization (WHO) in 1994 the public sector TB programmes were strengthened to handle drug-susceptible TB disease, providing standardized short-course chemotherapy under direct and supportive observation to at least all sputum smear positive pulmonary TB (PTB) patients and monitoring programme performance (4). Building on the DOTS strategy, the WHO`s Stop TB Strategy launched in 2006 and the Stop TB Partnerships' Global Plan to Stop TB 2006-2015 addressed DOTS expansion, HIV/TB coinfection and drug-resistant TB, in addition to advocating the need for research (4). In the post-2015 global TB strategy, the global fight against TB was continued, with the United Nations Sustainable Development Goals and WHO`s End TB strategy sharing the common vision to end the worldwide TB epidemic (5).

"A world free of tuberculosis – zero deaths, disease and suffering due to tuberculosis"(5)

The targets are to reduce the number of TB deaths by 95% and 90% reduction of TB incidence rate by year 2035, compared with 2015 numbers. In addition, no families should face catastrophic costs because of TB disease. The strategy is built on several pillars and components, included in these are early diagnosis and appropriate treatment of all people with TB, and developing and swift uptake of novel tools, strategies and interventions (5).

In 2018, the worldwide estimated number of new TB cases was 10 million (range, 9-11.1 million), with 9% of the cases among persons living with HIV (2). The burden of HIV-associated active TB is highest in the WHO African region. Globally, 70% of the estimated new TB cases were notified (2). This points the fact, that even though millions of TB patients are diagnosed and receive successful treatment every year, still a substantial number of TB cases are either unnotified or undetected. Extrapulmonary TB (EPTB) patients have received less attention in international TB guidelines, global TB control strategies and public health research. In addition to PTB being the main form of the disease, the lower priority given to EPTB could be due to EPTB not being a significant contributor to the transmission of TB disease. Still, the contribution of EPTB to the morbidity (6) and TB-related mortality (7, 8) is significant and delay in the diagnosis could possibly cause more advanced disease, complications and sequelae and increased economic constrains for the affected patients and their families.

Increased priority should be given to the timely and effective diagnosis, rapid initiation of specific antituberculosis (anti-TB) treatment and monitoring of adequate response to treatment in EPTB patients, in line with the End TB strategy. But, the diagnosis of EPTB is challenging due to unspecific and various clinical presentations, the difficulty of obtaining adequate specimens for laboratory investigations, the paucibacillary nature of the disease leading to decreased sensitivity of routine laboratory diagnostic tests (9, 10). Further, an accurate, low-cost and rapid diagnostic method for the diagnosis of EPTB is missing. All could contribute to a delay in the diagnosis and initiation of treatment. Anti-TB treatment is often initiated based on patient medical history and signs and symptoms suggestive of EPTB, without bacteriological confirmation. The international standards for TB care state that a specimen from the site of infection should be collected in all patients with presumptive EPTB and bacteriologically confirmation of TB disease as a basic principle still holds also among EPTB cases (3). There is a need to develop new methods for diagnosing EPTB that are feasible in low-resource settings. Studies are required to assess factors leading to diagnostic delay among EPTB patients. This would lead to identification of areas of intervention to increase case detection and early diagnosis and treatment of EPTB.

1.2 Epidemiology of extrapulmonary tuberculosis

Even though primarily considered a pulmonary disease, TB can affect any organ in the body. The term EPTB describes occurrence of TB at other body sites than the lung (6). PTB is the major contributor to the transmission and burden of TB disease, but EPTB accounts for a considerable proportion of the total TB burden and was reported in 15% of the notified incident TB cases in 2018 (2). Involvement of extrapulmonary sites can appear in isolation or accompany PTB. There has been described a decreasing trend in the overall number of TB cases, but an increasing proportion of EPTB among the total notified TB patients (11, 12). In a low HIV prevalent population, at least 15-20% of the TB cases are extrapulmonary (3), but in settings with extensive diagnostic and reporting system the proportion of EPTB among the total TB cases is reported to be even higher (13, 14). In addition, in special subgroups, such as in immunocompromised patients and at younger ages there is a higher proportion of EPTB (15-18). Among TB/HIV coinfected patients the occurrence of extrapulmonary manifestations increases with declining CD4 cell counts (19), and has been reported to be present in 45-56% of patients with TB and HIV infection/AIDS (20, 21), and in 70% of TB/HIV cases with CD4 cell count ≤ 100 cells/μL (19). Similarly, compared to more immunocompetent individuals, extrapulmonary disease is seen in a high proportion of patients receiving tumor necrosis factor-alpha (TNF-α) antagonists treatment (22). Other factors described to be associated with increased risk of EPTB compared to PTB is female gender, end-stage renal disease and ethnicity/geographic origin (16, 17, 23, 24). Extrapulmonary site of TB disease has been found associated with Asian or African origin compared with Western European origin (14, 23), and a study from the United States reported non-Hispanic blacks to have higher risk of EPTB than non-Hispanic whites (17).

Despite availability of anti-TB treatment, unfavorable treatment outcome and mortality in EPTB is relatively high. Various studies have reported death rates between 4,5-29% among EPTB patients (7, 24-26), and treatment outcome described to be associated with HIV infection and site of EPTB disease (8, 26).

1.3 Pathophysiology of tuberculosis infection

1.3.1 Microbiology and transmission of disease

It is 138 years since Robert Koch presented his work on the successful isolation of the tubercle bacilli, the causative agent of TB (27). TB disease in humans is primarily caused by *Mycobacterium tuberculosis* (Mtb) (28). Mtb belongs to the Mtb complex (MtbC) of organisms, and other species in the MtbC able to cause human disease are *Mycobacterium africanum, Mycobacterium bovis (M.bovis), Mycobacterium microti, Mycobacterium canetti, Mycobacterium pinnipedii and Mycobacterium caprae* (28-31).

Transmission of TB is almost entirely from person-to-person by the airborne route, when an individual inhales droplet nuclei containing tubercle bacilli expelled from a person with active pulmonary or respiratory tract TB (32). The risk of infection after exposure is depended on several factors, such as length, frequency and proximity to the infectious individual, the infectiousness of the TB diseased person, environmental factors and susceptibility of the exposed individual. An individual`s infectiousness is reflected in the amount of tubercle bacilli seen in sputum specimens, with smear positive PTB cases as the most potent sources (33), though smear negative PTB cases also play a part in the transmission of TB disease (34). TB can also be transmitted through zoonotic transmission, from animals to humans. Zoonotic TB, largely as a result of *M.bovis* from cattle, occurring through the ingestion of contaminated animal products, like untreated milk or meat, or potentially airborne transmission if close contact with infected animals, can be important in some settings (30).

1.3.2 Outcomes of tuberculosis infection

In the vast majority, primary TB infection occurs after droplet nuclei containing Mtb bacilli, small enough in size (1-5 μm), pass through the respiratory tract and reach the pulmonary alveoli of the new host (31). However, exposure to Mtb does not necessarily lead to infection as the pathogen can be eliminated due to either innate or adaptive immune response (35). The original focus of infection in the lungs, the Ghon focus, and the accompanying hilar lymphadenopathy form the primary (Ghon) complex (36). In most cases the primary infection is asymptomatic (37). The

progression of primary infection to active disease (primary TB) is mostly seen in children or immunocompromised adults, such as those with HIV co-infection. Primary TB results from local spread in the lung or hematogenous or lymphatic dissemination of the bacilli to various organs and tissues (32), and may result in pulmonary TB, miliary TB and EPTB such as pleuritis, lymphadenitis (mostly cervical), meningitis and pericarditis (32). The other outcomes of the primary infection are elimination of the bacilli by immune responses leading to clearance of the infection or containment of the infection leaving the bacilli in a quiescent stage, i.e. latent TB infection. Post-primary TB occurring years after the initial infection is a result of either endogenous reactivation of persistent bacilli or due to exogenous reinfection. In post-primary TB, the disease process characteristically remains localized, and the lungs are usually affected with typical upper lobe involvement and comprehensive tissue destruction with cavitation (32). Still, any organ can be involved, with pleuritis and lymphadenitis as the most common extrapulmonary sites (32). Figure 1 gives a simple summary of the outcomes of Mtb infection.

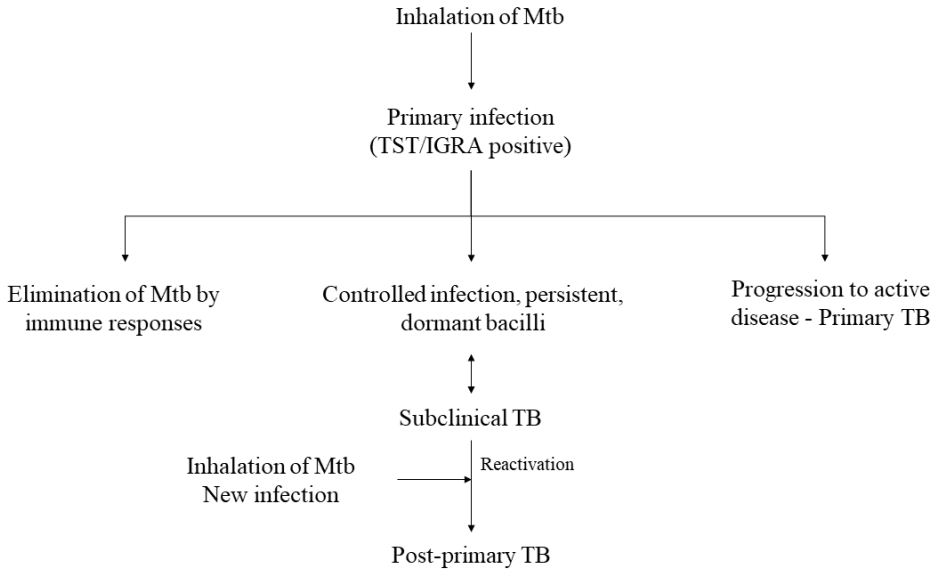

Figure 1. Simplified schematic overview of the outcomes of *Mycobacterium tuberculosis* infection.
Abbreviations: Mtb, *Mycobacterium tuberculosis*; TST, tuberculin skin test; IGRA, interferon-γ release assay; TB, tuberculosis.

Development of a cell-mediated, adaptive immune response to Mtb antigens in exposed individuals, detectable around 3-8 weeks after the initial infection (38), indicated by tuberculin skin test (TST) conversion or positive interferon-γ release assay (IGRA), is usually thought to represent Mtb infection (39, 40). Still, the relative number of individuals surely harbouring dormant Mtb after TST or IGRA conversion is unknown (41), since individuals with successful elimination of the pathogen might have positive TST or IGRA due to memory T cell responses (35). A widely quoted estimate that 1/3 of the world population is immune sensitized and thus potentially harbouring latent TB infection, could be an over-estimate of the infection pool (37, 42). Among immunocompetent individuals the estimated lifetime risk of developing active TB disease following a single infection is ~10% (43-45), with the majority of active TB cases occurring within some years after the initial infection (37, 46). Even though there is a persistent risk of reactivation throughout a person's lifetime, the risk of developing disease seems to decrease with time since infection (45, 47). The outcome of infection, the risk of progression to active disease or risk of reactivation of latent TB infection depends to a great extent on the host`s immune status. Several predisposing factors is associated with an increased risk of active TB disease such as TNF-α antagonists treatment (48, 49), end-stage renal failure (50, 51), organ transplant recipients (50), diabetes (52, 53), underweight, under- and malnutrition (50, 54), smoking (55), malignancies (54) and silicosis (56, 57). However, HIV infection is the most potent risk factor for developing TB, with the risk over 20-times greater among HIV infected compared to HIV uninfected individuals (29).

TB patients have traditionally, from a public health and clinical perspective, been categorized as having either latent TB infection (noninfectious, asymptomatic) or active TB disease (potentially infectious, symptomatic disease) (35, 58). Recent research demonstrates that the classical binary division of TB may be an oversimplification and that the transition from latent TB infection to active disease involves early, asymptomatic disease states, where microbiological evidence and/or radiological manifestations are the only signs of active TB disease (59, 60). It is thus proposed that TB infection can better be regarded as a continuous disease spectrum extending from elimination of infection by immune responses, to controlled infection

with persisting inactive bacilli, to subclinical disease with active bacterial replication, to fulminant and potentially life-threatening disease at the outermost extreme (35, 39, 61).

1.3.3 Immune response and granuloma formation

The immune response to Mtb and the host-pathogen interactions, involving an interplay between the bacteria and the innate and adaptive immune system, are complex, multifactorial and to some extent incompletely defined. Entering the alveolar space, the innate immune system provides the first line of defense against Mtb (29), and the first host-pathogen interaction appears to be mediated through pattern recognition receptors located on phagocytic cells, leading to receptor-mediated phagocytosis of Mtb (62-64). Generation of adaptive immunity is important for control and containment of the infection. With Mtb being an intracellular pathogen protective immunity relies to a great extent on cell-mediated immune responses, with the CD4+ T-helper 1 cell in a prominent role (65, 66). Macrophages and DCs are crucial in initiating, stimulating and directing adaptive antigen-specific T cell immunity, with presentation of Mtb antigens and production of cytokines and costimulatory signals (67). DCs and macrophages present mycobacterial antigens loaded on major histocompatibility complex to T cells (65). Further, they secrete proinflammatory cytokines such as interleukin (IL) 1, IL-6, IL-12, TNF-α, recruiting cells to the site of infection, stimulate interferon gamma (INF-γ) producing cells and promote formation and maintenance of the granuloma (64, 65, 67). Effector T cells respond by secreting cytokines like TNF-α, INF-γ and IL-2. TNF-α and INF-γ in turn activate macrophages and increase their microbicidal capacity, recruit additional cells to the site of infection and maintain the granulomas cellular integrity (64, 68-70). IL-2 is mainly involved in T cell differentiation into memory and effector cells (71). Thus, some weeks after the infection, cell-mediated immunity develops, and effectively controls the infection (72). Progressive assembly of cells at the site of infection, into an organized, compact aggregate of macrophages, interspersed with lymphocytes, DCs and various other inflammatory cells, surrounded by a rim of fibroblasts, leads to the formation of the TB granuloma (73).

The granuloma formation is the histopathological hallmark of TB infection. At the early, initial stage, the granuloma is a cluster of mature macrophages, distinguished by higher number of organelles and increased cytoplasmic size (74, 75). Granuloma macrophages can go through additional morphological changes and differentiate into specialized cells, such as foam cells (identified by lipid accumulation in the cytoplasm), some will fuse to produce multinucleated giant cells or transform to epithelioid macrophages (epitheloid histiocytes) (74-77). Epitheloid macrophages are elongated, slender, large cells, where interlocking pseudopods associate and closely pack adjacent cells (76). When adaptive immunity develops, the granuloma evolves into a more intact, organized structure, with a lymphocytic cuff surrounding the macrophage-rich center (78). The macrophage cell death by necrosis leads to the accumulation of necrotic material creating a morphological pattern, caseous necrosis, which is most commonly seen in TB granulomas (74, 79). HIV coinfection leads to numeric or functional depletion of Mtb-specific CD4+ T cells, resulting in diminished cell-mediated immune responses, impairment of the lymphocyte-macrophage immune axis and ability to form competent granulomas (29). With increasing immunodeficiency, the classic TB granuloma are increasingly disorganized (59, 80).

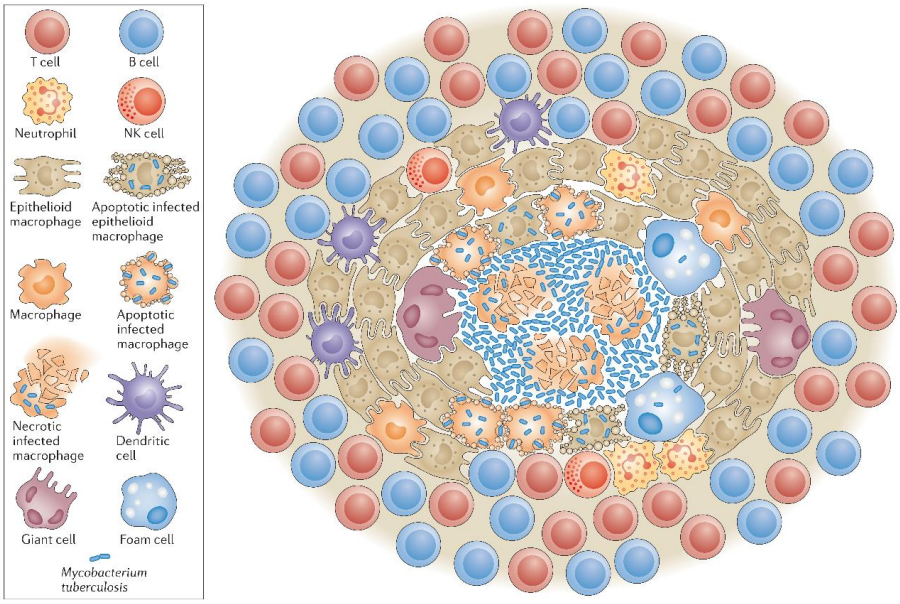

Figure 2. Structure and cellular constituents of the tuberculous granuloma. Ramakrishnan L. Revisiting the role of the granuloma in tuberculosis. Nat Rev Immunol. 2012;12(5):352-66 (74). Reprinted with permission from Macmillan Publishers Limited, Nature Reviews Immunology.

1.4 Clinical presentation of extrapulmonary tuberculosis

TB is one of the diseases referred to as "The Great Imitator" as it can resemble various other disease presentations (81). EPTB cases can present unspecific constitutional symptoms like weight loss, fever, night sweats, reduced appetite and general malaise, in addition to specific signs and symptoms according to the site of infection (6). Still, the signs and symptoms in EPTB are often insidious and nonspecific. TB lymphadenitis and TB pleuritis are predominantly reported as the most common sites of EPTB infection (12, 82, 83), both among HIV negative and HIV positive patients (6). However, in some settings bone and joint TB and genitourinary TB have been described as the most frequent sites (17, 84), thus suggesting that sites of EPTB may differ according to population and geographic location (85). Concomitant PTB is a common finding among EPTB patients (15, 84, 86).

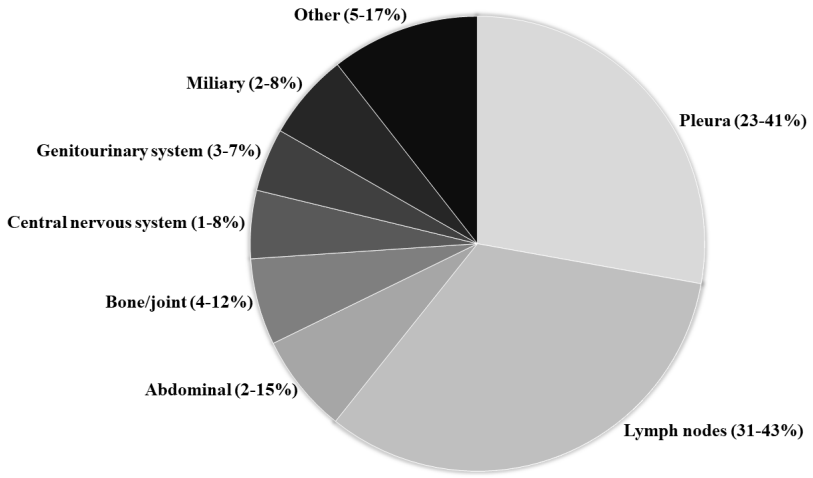

Figure 3. Distribution of sites of extrapulmonary tuberculosis disease. Data derived and adapted from references (16, 18, 82, 83, 85, 87-89).

In lymph node TB, peripheral lymph nodes are affected in the majority of cases, with cervical lymph node involvement as the most common site (90). The presentation is frequently a unilateral, discrete, firm, painless neck mass above 2 cm in diameter (91). With time, as a results of periadenitis, fixation and matting of the nodes can be found, and if left untreated abscess formation and spontaneous drainage of the node with sinus formation can develop (90). TB lymphadenitis commonly affects young adults and children (92, 93) and is reported to be more frequent among women than men (94). Constitutional symptoms can be absent but described more often in males compared to females (94) and in patients with HIV coinfection (95).

An acute presentation of symptoms is often seen among TB pleuritis cases (96). The most frequent presenting symptoms are pleuritic chest pain, non-productive cough and fever (97). The patients can have varying degrees of dyspnea depending on the magnitude of pleural effusion. The pleural effusion following TB pleuritis is generally unilateral (98), of any size, but reported in a study of 254 pleural TB patients to occupy less than 2/3 of the hemithorax in the majority of patients (99). The size of pleural effusion has been described to be similar in HIV negative and HIV positive patients, but a longer and more severe illness is often seen among HIV positive patients (100).

Spinal TB (Pott`s disease) is the most frequent form of bone and joint TB (6), and it is more common among younger individuals in TB-endemic countries, while in developed countries, the disease is generally seen among the adult population (101-103). The clinical presentation depends on the duration and severity of the disease. Characteristic features are local pain, stiffness and muscle spasms, cold abscess and prominent spinal deformity (102). Symptoms onset is generally slow and insidious and constitutional symptoms are reported in about 20-30 % of the patients (102, 103). Back pain is almost always present, usually localized to the involved site, and spinal deformities and neurological deficits are common (102, 103). The clinical presentation, the site of spinal TB and number of vertebral bodies involved have been reported to be similar in HIV negative and HIV positive patients (104, 105).

Abdominal TB is mostly seen in young adults and intestinal TB disease and peritoneal involvement are the most prevalent forms (106). Abdominal swelling and pain are typical presenting symptoms of peritoneal TB, in addition to constitutional symptoms such as weight loss, fever and night sweats, with ascites as the most frequent physical sign (107). Intestinal TB can involve all areas of the intestine, but commonly affects the ileocecal region (108). The presenting symptoms with chronic diarrhea, abdominal pain, intestinal obstruction, hematochezia and constitutional symptoms can resemble Crohn`s disease (108).

The most devastating form of EPTB is TB of the central nervous system (CNS), where the mortality rates are high and there is a risk of disabling neurological sequela (86, 109). Increased risk of CNS TB is found in young children and TB/HIV coinfected patients (86, 110). Clinically, the most common presentation, TB meningitis, often has an insidious onset with a prodromal phase including symptoms such as fatigue, fever, headache, vomiting, weight loss and behavioral changes, followed by seizures, neurological deficits and altered consciousness (90, 111). Some patients can present with a rapid progression and sudden onset of symptoms simulating other bacterial meningitides (111, 112). Studies comparing the clinical manifestations of TB meningitis in HIV positive and HIV negative cases report similar presenting symptoms in both groups (113). However, others have described altered level of consciousness or

cognitive dysfunction to be more prominent in HIV-infected cases (86, 114) and that this patient group more likely presents with additional sites of EPTB (112).

Disseminated/miliary TB has a wide range of clinical manifestations, from a prolonged vague illness to acute fulminant disease. Symptoms and signs depend on the organs involved (115). Still, the presenting symptoms are usually nonspecific, and dominated by constitutional symptoms such as fever, fatigue, reduced appetite, weight loss and night sweats (115-117). Among HIV negative patients with miliary TB, predisposing conditions such as malignancy, organ failure, diabetes mellitus or immunosuppressive therapy are present in about 40-45% of the cases (117-119). In HIV positive patients with early HIV infection (CD4+ count >200 cells/μL) the prevalence of miliary TB is similar to that reported among immunocompetent patients, while it is reported more often with advancing stages of HIV infection (115). In children, occult dissemination following primary infection is common, but seldom progresses to disseminated TB, with the exception in immunocompromised children and in young children (< 3 years) (110). The mortality rate of miliary TB remains high despite available effective treatment (115, 116).

1.5 The diagnosis of extrapulmonary tuberculosis in low-resource settings

EPTB poses more diagnostic challenges compared to PTB. First, the clinician must have a clinical suspicion of EPTB, and given that extrapulmonary disease may present with a wide range of signs and symptoms depending on the site affected, the patient's immune status and disease stage, the EPTB differential diagnosis may come late in a clinical evaluation. Next, laboratory facilities and diagnostic capacity are often limited in low-resource settings. The biological samples for laboratory investigations often require invasive sampling, sometimes from relatively inaccessible sites, which can pose a risk to harm the patient and raises the need of facilities and trained personnel performing these procedures. Further, the access to competent laboratory technicians performing the various sample processing methods of the heterogeneous specimen material must be in place. Adding to the challenge is that many forms of EPTB are

paucibacillary, i.e. disease due to a lesser number of bacteria, reducing the sensitivity of the most commonly used laboratory diagnostic methods (9, 120-123). There are also limited possibilities of adaption of newer and improved diagnostic techniques in low-resource settings.

The diagnosis of EPTB is thus often based on an integration of clinical suspicion of TB, and if laboratory and imaging facilities are available, results from different investigations, without a microbiologically confirmed diagnosis. The decision to start empirical anti-TB treatment could potentially lead to both over- and underdiagnoses of TB disease. This highlights the need to improve laboratory diagnostic methods for EPTB, with the objective of increased confirmation and case detection of active forms of EPTB disease, providing a rapid and reliable diagnosis regardless of the site of infection, age and HIV status. The aim should be to develop a diagnostic test which is highly specific and sensitive (improved accuracy compared to existing tests), quick to results, inexpensive, cost-effective, easy to use and interpret and doable in TB-endemic low-resource settings. In addition, it would be valuable if the diagnostic test is performed using technological platforms already established in low-resource, high TB prevalent settings.

1.5.1 Current laboratory methods for diagnosis of active extrapulmonary tuberculosis disease

Direct laboratory methods detect or demonstrate the Mtb organism itself or its product, indirect methods detects the Mtb organisms impact on the host, measuring the host`s cellular or humoral immune response against Mtb (124).

1.5.1.1 Direct methods

Microscopy

Demonstration of acid-fast bacilli (AFB) directly in clinical specimens using the light microscope and Ziehl-Neelsen (ZN) staining method still plays a fundamental role and is often the only TB diagnostic tool available in many settings. Under optimal conditions the detection threshold of AFB microscopy is between 10^4-10^5 bacilli/ml (125), and although usually identifying the most infectious PTB patients, the sensitivity is variable and relatively low (range 20-80%) in detecting active PTB cases

(126). Microscopy shows limited sensitivity in paucibacillary disease, such as pediatric TB, HIV-associated TB and extrapulmonary disease (110, 124, 127-129). The suboptimal sensitivity of AFB microscopy in identifying EPTB disease (sensitivity 0-40%) has been shown in several studies including various sites of infection and sample material (9, 122, 123, 130-133). Measures to improve the diagnostic performance of ZN microscopy such as examining larger CSF volume and using ≥ 30 minutes to examine the ZN stained slide (134, 135) and cytocentrifugation of pleural fluid and pleural biopsies (136), have been described to increase the yield of ZN microscopy, but it is still suboptimal.

Numerous studies have described improved diagnostic performance of sputum smear microscopy by the use of conventional fluorescence microscopy (FM) using fluorochrome dye (auramine-rhodamine or auramine O) compared with ZN staining and light microscopy (137, 138). In addition, the slides can be evaluated at lower power magnification which reduces the screening time (139). A systematic review reported higher sensitivity (average improvement 10%) and similar specificity of FM compared with conventional ZN sputum smear microscopy (140). Improved sensitivity of fluorescent methods in comparison to ZN microscopy has also been described in extrapulmonary specimens (141, 142). Development of the light emitting diode fluorescent microscopy (LED-FM), which is cheaper, require less power, has a light source with longer lifespan (>50 000 hours) and not requiring a dark room, have made the FM technology (either as LED microscopes or by the conversion of conventional FM to LED light sources) more feasible in resource-constrained settings (126, 139, 143). After reviewing the evidence, where the LED-FM was found at least as sensitive and specific as conventional FM, the WHO issued a policy statement recommending LED-FM replacing conventional FM in settings already using FM and a switch from ZN light microscopy to LED-FM following a careful implementation plan (126). However, the sensitivity of fluorescent microscopy in EPTB samples is low. Regarding the specificity of AFB microscopy, smears can have particles other than MtbC organisms that are acid-fast, such as precipitates, inorganic material, artefacts, fibers and pollen (144). In addition, acid-fast staining does not differentiate organisms in the MtbC, nor the MtbC organism from other acid-fast microorganisms like

Nocardia species (partially acid-fast) and nontuberculous mycobacteria (NTM) (144, 145). Identification of the mycobacterial species is vital as the management of MtbC infection and NTM disease differs (146).

Culture

Culture is still regarded as the gold standard for bacteriological confirmation of TB disease (147). Culture is a lengthy and relatively cumbersome process, starting with clinical specimens often needed to be transported to reference laboratories (148), decontamination of non-sterile specimens, inoculation and incubation of media, detection of growth and identification of mycobacteria (149). Traditionally, mycobacteria are cultured on solid media which are either agar-based, e.g. Middlebrook (7H10/7H11), or egg-based, e.g. Ogawa and Lowenstein-Jensen (LJ) media (150). LJ is the culture medium most commonly used worldwide, whereas Middlebrook media are seldom used in routine diagnostics in resource-limited settings due to higher costs (149). Observation of growth characteristics, colony morphology (distinctive appearance on Middlebrook agar) and microscopy of the cultured material can provide a presumptive TB diagnosis, but additional tests are needed for a confirmatory identification of isolates of the MtbC and its differentiation from NTM (151-153). In addition to species identification, culture is necessary for phenotypic drug susceptibility testing (DST) (154). Mycobacteria may often be detected on solid media in <4 weeks, but incubation for 8 weeks is required before solid culture is classified as negative (155). The slow growth of Mtb inevitably delays the results. Further, Mtb culturing is time-consuming, complex and thus requires skilled laboratory personnel and a well-functioning infrastructure and health care system and specialised laboratories with high biosafety level. In sub-Saharan Africa many clinics do not have access to a laboratory performing TB cultures (156). Culture shows a higher sensitivity than microscopy as only 10-100 bacilli/ml material are required to detect Mtb (157). Still, in EPTB disease the sensitivity of culture has been reported in several studies to display suboptimal sensitivity (9, 10, 158-160).

Figure 4. **Culture test tubes**. Test tubes showing growth on Lowenstein-Jensen media. Photo: Melissa Davidsen Jørstad.

Mycobacterial culture and growth detection through commercial broths systems, such as the radiometric BACTEC™ 460 TB system, the Mycobacteria Growth Indicator Tube (MGIT™) system and the nonradiometric BACTEC™ MGIT™ 960 system (BD Diagnostic Systems, Sparks, MD, USA) are seen as major improvements. DST can also be performed using liquid culture systems (161). Liquid culture systems are more sensitive and give faster results than culturing on solid media, but are also more expensive (162). Further, higher contamination rates are found among liquid culture systems and the possibility of increased frequency of isolating NTM emphasises the need of available rapid species identification tests and clear, feasible guidelines on how the clinical staff should manage NTM results (162-164). Optimal yield of culture is by a combination of solid media and automated liquid systems (165). In 2007, the WHO endorsed phased implementation and use of liquid culture and DST in low- and medium-income settings (166), and now recommends the use of both solid and liquid culture, regarding liquid culture as the gold standard (167). However, TB laboratories in resource-limited settings often use only solid media, largely due to cost of liquid culture (162).

Molecular methods

Nucleic acid amplification tests (NAATs) are molecular techniques allowing detection and amplification of minute amounts of target genetic (deoxyribonucleic acid (DNA) or ribonucleic acid (RNA)) material and have the capability to produce abundant

quantities of identical copies of the nucleic acid target sequence if the particular sequence is present in the sample (168). Several amplification methods are available, but the first and most commonly used method is the polymerase chain reaction (PCR) (169). Amplification of the nucleic acid is accomplished in a thermocycler. Advantages of PCR is the reduced time to detect Mtb compared to culture, it can be performed in fresh specimens and in formalin-fixed, paraffin-embedded tissue and alcohol-fixed aspirate smears and using the genetic material, species identification and sometimes detection of genes encoding antibiotic resistance can be done (160, 168, 170-172). Further, the benefit that the technique can detect minute amounts of DNA is also a limitation since cross-contamination might lead to false-positive results. Meta-analyses and systematic reviews, including both commercial and in-house NAATs for the diagnosis of various EPTB cases, report inconsistent and profoundly variable results (168, 173-176). Commercial NAAT, can play a role as a rule-in-test in EPTB disease due to often high specificity, but the moderate and highly variable sensitivity impedes the use as a rule-out-test (173-175, 177). The abundant cost, technical skills and laboratory equipment needed have precluded the scale-up and widespread use of conventional NAATs in routine diagnostics in low-resource settings.

Development of the Xpert® MTB/RIF (Xpert) assay (Cepheid, Sunnyvale, CA, USA), a cartridge-based, fully automated nucleic acid amplification assay, utilizing heminested real-time PCR for amplification of a Mtb-specific DNA sequence of the *rpoB* gene, concurrently detecting both Mtb and rifampicin resistance, is undoubtedly seen as a landmark event in TB diagnostics (178-181). The Xpert assay was in December 2010 endorsed by the WHO, and the initial policy statement published early in 2011, recommended using the Xpert assay as the first diagnostic test in sputum specimens among presumptive PTB patients suspected of having HIV-associated TB or multidrug-resistant tuberculosis (MDR-TB) or as an add-on test to microscopy in other patients (182). The assay was initially developed, assessed and optimized for the diagnosis of PTB cases using sputum, and was found sensitive and specific in the diagnosis of PTB among adults, showing increased TB case detection in culture-positive cases compared to microscopy, though exhibiting lower sensitivity in smear-negative compared to smear-positive cases (183, 184). Evaluation of the Xpert assay in

the diagnosis of EPTB have been more complex due to a variety of specimen types, diversity of sample processing before analysis and suboptimal reference standards (185). Studies including various extrapulmonary sample material report highly heterogeneous sensitivity, also varying depending on the sample material (10, 185-187). Based on the growing body of evidence, the WHO published a policy update in 2013, expanding the recommendations using the Xpert assay for the diagnosis of PTB among adults, and added guidance for the use in children and in subgroups of EPTB patients (188). The next generation of the assay, the Xpert® MTB/RIF Ultra (Xpert Ultra) was recently launched, and further endorsed by the WHO, offering improved performance compared to the Xpert assay (189, 190). In sputum samples the Xpert Ultra assay was reported to have decreased specificity compared to the Xpert assay, but higher sensitivity than the Xpert assay in paucibacillary disease (smear-negative, culture-positive) and among patients with HIV-associated PTB (191). Studies assessing the Xpert Ultra assay in different extrapulmonary clinical samples are continuously being published (159, 192-196). Although the results look encouraging, the sensitivity vary across the diverse sample types, thus more studies, where results are classified according to sample types, are needed (197). Updated guidelines was released by the WHO in 2020, strongly recommending the use of the Xpert/Xpert Ultra assay as the initial diagnostic test in CSF among presumptive TB meningitis cases (198). Further, conditionally recommending the use of the Xpert assay in other extrapulmonary sample materials and the Xpert Ultra assay in lymph node biopsies and aspirates. There are several advantages with the Xpert/Xpert Ultra assay such as rapidity of the test results compared to culture, detection of both Mtb and rifampicin resistance (rifampicin resistance being strongly indicative of MDR-TB (181)), relatively simple to perform and thus easy to train health care workers in its use, not prone to sample cross-contamination and requires minimal biological safety facilities (185, 199, 200). However, some aspects of the diagnostic test can generate operational problems such as the need of a stable electricity supply, annual recalibration of the instrument, possibility of swap or repair if module failure, the instrument has a critical temperature ceiling of 15-30°C and the need of regular supply of cartridges to be stored at a temperature range of 2-28°C (167, 201). Next, the test is expensive and thus

funding and an assurance of reliable procurement of consumables and equipment is of vital importance in low-resource settings (201). While more studies on diagnostic performance and operational feasibility are necessary to evaluate the Xpert/ Xpert Ultra assay in the routine diagnosis of EPTB in low-resource settings to further guide the tests potential use, cost- effectiveness analyses and health impact studies assessing the actual impact on patient management and outcome also among EPTB patients need to be performed.

Antigen detection tests

Mtb antigens can be present in Mtb infected tissue, in fluids surrounding the tissues and after entering the circulation the antigens could be eliminated in the urine (202). Detection of Mtb antigens directly in clinical samples, such as sputum, CSF, peritoneal or pleural effusion, urine, fine needle aspirates cytology (FNAC) and biopsies from various tissues could potentially present rapid and direct evidence of active TB disease. Various formats of antigen detection tests have been developed such as enzyme-linked immunosorbent assays (ELISA), lateral flow immunochromatographic assays sometimes called strip-tests or dipstick tests, agglutination tests and antigen detection using immunocytochemical (ICC) or immunohistochemical (IHC) staining techniques (ICC/IHC staining hereinafter referred to as immunostaining) (203-208). Diverse target antigens have been assessed for their diagnostic potential in TB disease like 65-kilodalton (kDa) heat shock protein (Hsp65), 38-kDa protein, 45/47-kDa proteins, Ag85 complex, early secreted antigenic target 6 (ESAT-6), *M.bovis* bacillus Calmette-Guérin (BCG) antigens and lipoarabinomannan (LAM) antigen using different test designs in a diversity of sample material (124, 209).

Of the different antigens, detection of LAM antigens in urine have been one of the most frequently targeted antigen in studies (209). The LAM antigen was in early studies shown to be detectable in urine from active TB cases (210). The first commercially diagnostic assays detecting LAM in urine was LAM ELISAs (211). After development of the LAM ELISAs several studies evaluating the diagnostic performance were conducted in diverse patients groups, mostly including presumptive PTB patients, showing variable diagnostic accuracy (212, 213). The conclusion from a meta-analysis in 2011 was that LAM urinary assays had “suboptimal sensitivity for

routine clinical use", but it was noted a higher sensitivity of the assay among HIV positive (pooled estimated sensitivity 51%) compared to HIV negative patients (pooled estimated sensitivity 14%), and highest sensitivity among HIV positive TB patients with advanced immunosuppression (214). Further studies among HIV positive, adult patients, confirmed the high specificity, and greatest sensitivity, although at best only moderate, among HIV positive cases with CD4 cell count < 50 cells/μL (215, 216). Development of the commercial lateral flow urine LAM immunochromatographic assay (LF-LAM), Alere Determine™ TB LAM Ag (Alere Inc, Waltham, MA, USA), which could be used as a point-of-care test, was seen as a potential breakthrough. Although the LF-LAM showed similar results in urine samples as LAM ELISA with highest sensitivity in patients with reduced CD4 cell counts, the sensitivity of LF-LAM remained suboptimal (205, 211, 217). The latest policy update for the use of LF-LAM was issued in 2019, recommending the LF-LAM in all HIV positive patients with presumptive TB, regardless of the CD4 cell count (218). The LF-LAM was further recommended irrespective of TB symptoms in seriously ill HIV positive patients and among hospitalized patients with CD4 cell count ≤ 200 cells/μL (≤ 100 cells/μL in out-patients) (218). LAM antigen detection tests have been investigated in other extrapulmonary specimens such as CSF and pleural fluid showing low sensitivity (219, 220), and is not recommended in other sample material than urine (221).

Detection of Mtb antigens by immunostaining utilizing monoclonal or polyclonal antibodies could be an alternative to conventional AFB staining in biopsy tissue sections and cytological smears. Immunostaining applying specific antibodies could potentially detect any mycobacterial antigen and it is possible to demonstrate Mtb antigens within phagocytic cells, extracellular organisms, fragmented bacilli and antigenic debris, i.e. intact Mtb cell wall is not needed (208). Other advantages are that alcohol-fixated smears could be stored and transported to facilities performing immunostaining, the staining techniques are relatively simple, not prone to contamination and with operational advantages over conventional PCR, and thus suitable in laboratories in low-resource settings. Demonstration of mycobacterial antigens in extrapulmonary specimens and the use of immunostaining in the diagnosis of TB have been investigated using in-house made or commercially available

monoclonal or polyclonal primary antibodies (222-227). Many of the published studies investigating the potential of immunostaining to improve the diagnosis of TB have used commercially available polyclonal anti-BCG antibodies (207, 224-226, 228, 229) or in-house polyclonal anti-Mtb antibodies (227, 230, 231) in various tissue. One study by Kohli et al. assessing the value of IHC staining using anti-BCG antibodies in detecting mycobacterial antigens in various tissue sections described a higher sensitivity (96% versus 31%), but a lower specificity of IHC staining than ZN staining (35% versus 96%) (226). Similarly, Mukherjee et al. reported IHC staining to identify 37/50 (74%) cases of TB lymphadenitis, whereas in only 22/50 (44%) cases ZN staining showed AFB (225). However, polyclonal anti-BCG and anti-Mtb antibodies are not specific for the MtbC complex organisms, and thus cannot differentiate between species of the MtbC complex and other mycobacteria, in addition anti-BCG antibodies are known to show cross-reactivity with additional bacteria and fungi (229, 232, 233). Goel et al. investigated the potential of ICC staining in FNAC from lymph nodes as an adjunct to cytological diagnosis of TB lymphadenitis using species-specific primary monoclonal antibody (mAb) to 38-kDa protein antigen, and reported a sensitivity of ICC staining > 95% in both archival and fresh FNAC smears (208). The 38-kDa protein antigen is reported to be a quantitatively Mtb-specific antigen compared with BCG (the protein is present in BCG culture fluid but in lower concentrations than in Mtb culture fluids) (234), and even though the gene encoding the antigen is described absent in several NTM, it is found in *M. intracellulare* (235) and suggested to be present in *M. malmoense* (236). Thus, detection of the 38-kDa protein antigen cannot indicate infection due to MtbC with certainty. MAbs (61.3, 60.15, 105.10 and 2.16) reacting with different mycobacterial proteins, 35-kDa protein, 28-kDa proteins and Hsp65, was assessed by Barbolini et al. for detection of mycobacterial antigens in lymph node, joint and lung tissue of TB patients (223). The mAbs reacting with 28-kDa protein and Hsp65 show broad cross-reactivity in mycobacteria, while the mAb 61.3 was described to recognize an epitope on the 35-kDa protein showing a more limited cross-reactivity (223, 237). Sumi et. al evaluated IHC in the diagnosis of TB lymphadenitis using polyclonal primary antibodies directed towards mycobacterial antigens, HspX, Tb8.4, PIcA and ESAT-6 (222). They

reported the anti-ESAT-6 antibodies to be highly sensitive and specific, advocating the use of IHC with primary anti-ESAT-6 antibodies among TB lymphadenitis patients where conventional laboratory diagnostic methods cannot confirm the TB disease (222). The use of primary antibodies targeting antigens restricted to MtbC organism, but absent in *M. bovis* BCG substrains and NTM, could increase the performance of immunostaining and discriminate TB infection from NTM. The MPT64 antigen is a 26-kDa protein produced and actively secreted by MtbC organisms (238-241). The protein was initially purified and isolated from *M. bovis* BCG Tokyo culture filtrates by Harboe et al. (242). Subsequent studies indicated that the gene encoding the antigen was absent in several *M. bovis* BCG substrains (239, 243), and further showed that the DNA segment containing the corresponding gene, region of difference (RD) 2, was deleted in some BCG substrains (244). In addition, the antigen MPT64 has not been demonstrated in NTM (242, 245). Studies assessing the diagnostic potential of immunostaining in tissue sections and cytological smears from different extrapulmonary sites using in-house polyclonal primary anti-MPT64 antibodies show promising results, with higher sensitivities than AFB staining and Mtb culture in paucibacillary extrapulmonary TB disease (122, 123, 246, 247).

Although various studies have suggested a potential role of immunostaining as a diagnostic adjunct to conventional laboratory methods for the confirmatory diagnosis of EPTB, it has not been extensively studied and embraced, and thus conceivably an underutilized diagnostic method for EPTB disease. Hence, the exact application and role of immunostaining in the routine laboratory diagnosis of EPTB should be assessed in further studies in endemic areas.

1.5.1.2 Indirect methods

Antibody-based serological tests

TB serological tests usually refer to tests detecting antibodies, i.e. humoral immune responses to Mtb antigens, as opposed to tests relying on Mtb antigens being recognized by cellular immune responses, such as in IGRA tests, or Mtb antigens being directly detected in specimens, e.g. LAM in urine (248, 249). Serological tests have the potential to be developed into point-of-care tests, as they are rapid and

usually have simple technological and training requirements (250). However, commercial serological antibody-based tests for the diagnosis of both PTB and EPTB show insufficient diagnostic accuracy and have restricted clinical value (251), and the use of these tests are discouraged in international TB care guidelines (3). After reviewing the available evidence, the WHO, in 2011, issued a negative policy statement (249). The statement announced a strong recommendation against the use of the present serological tests for the diagnosis of PTB and EPTB (249). Still, serological tests are marketed and extensively used in several high TB-burden countries (252).

Tuberculin skin test and Interferon-γ release assays

There are two established methods for the immunodiagnosis of latent TB infection, the TST and IGRAs (253), but the role of these tests in the diagnosis of active TB is still unclear (254, 255). The two tests are indirect markers of Mtb exposure and indicate adaptive immunity and immunological sensitization towards Mtb antigens, but not inevitably infection with Mtb (41). They do not differentiate between latent and active TB disease, nor accurately predict progression to active disease (40, 255), and show reduced sensitivity among immunosuppressed individuals (40).

The TST consists of intradermal injection of purified protein derivate (PPD) of tuberculin, where a delayed-type hypersensitivity inflammatory reaction occurs after 2-3 days in individuals with a cell-mediated immune response towards these antigens (40). In the interpretation of a positive test the three dimensions; size of skin induration, positive predictive value and risk of active disease should be considered (256). The PPD is a mixture of Mtb antigens, and contains antigens shared by NTM and MtbC organisms, including *M. bovis* BCG strains (257). Thus, prior BCG vaccination, especially repeated BCG booster vaccination and post-infancy vaccination, and exposure to NTM can give false positive results, and comprise the test`s specificity (258). The sensitivity of the TST is limited in certain subgroups, such as malnourished individuals and people living with HIV (40), and even among active TB patients, with no obvious immunosuppression, a considerable proportion has negative results on TST (259).

The IGRAs are *in-vitro* blood test measuring INF-γ release by T cells following stimulation by Mtb specific antigens (260). The antigens used are culture filtrate protein 10 (CFP-10) and ESAT-6 (40). The QuantiFERON®-TB Gold In-Tube test (Cellestis/Qiagen, Carnegie, Australia) includes one additional Mtb specific antigen, TB7.7 (Rv2654). ESAT-6 and CFP-10 are encoded by genes located in the RD1 segment of the MtbC genome, a genomic region missing in *M. bovis* BCG substrains (244, 261) and the antigens are absent in most NTM (262), and thus show increased specificity compared to PPD (257). However, like the TST, not being able to distinguish latent and active TB, the specificity for active TB will be low in settings with a high TB burden (263). It has also been reported suboptimal sensitivity in active TB disease and no consistent proof that IGRAs are more sensitive than TST for the diagnosis of active TB in low/middle-income countries (40, 254). Fan et al. concluded in a systematic review and meta-analysis that IGRAs, notably in low/middle-income countries, have limited value as screening tools and "rule-out-tests" for EPTB (264). The diagnostic performance of IGRAs for the diagnosis of EPTB have been assessed using other specimens than blood. Moderate diagnostic accuracy has been reported using pleural fluid and CSF IGRA among patients with presumptive TB pleuritis and TB meningitis, respectively (265, 266).

Adenosine deaminase activity, white blood cell differential count and other basic biochemistry analyses

Estimation of adenosine deaminase (ADA) activity is a widely studied marker in effusions and fluids for the EPTB diagnosis. ADA is an enzyme catalysing the conversion of 2'deoxyadenosine and adenosine into 2'deoxyinosine and inosine, respectively (267). It is released by activated immune cells and even though seen as a unspecific marker of inflammation (268), using various ADA activity cut-off values, it has been shown that measuring ADA activity may be helpful as an indirect and adjunctive diagnostic marker of EPTB in different effusions and fluids (269-271). However, the clinician must have knowledge of situations increasing the likelihood of false-positive and false-negative results. Low levels can be found in the early disease phase and in current smokers and the older patients (272, 273). Whereas, increased

ADA levels can be observed in other conditions, including empyema caused by other bacteria, cancer, parapneumonic effusion, rheumatoid effusions and haematological malignancies (272). As an example, ADA cannot differentiate between bacterial and TB meningitis (274). Further, the diagnostic utility of the test depends on the local TB prevalence. Among populations with presumptive TB effusions from high TB prevalence settings, an increased ADA level provide a high post-test probability of TB disease (268, 272). However, in low TB prevalence settings, the positive predictive value is low, whereas the negative predictive value is higher (272, 275). The combination of ADA ≥ 40-50 U/L and lymphocyte/neutrophil ratio ≥ 0.75 or lymphocyte/total white cell count ratio ≥ 0.50 in pleural effusions have been reported to increase the specificity for the TB pleuritis diagnosis (276, 277).

In fluids and effusions, estimation of the white blood cell count, white blood cell differential count, pH, protein and glucose concentration and lactate dehydrogenase level are generally part of the routine laboratory diagnostic work up. In the CSF, findings usually seen as supporting a TB diagnosis among patients with clinical presumptive TB meningitis are pleocytosis (> 20 cells/µl) with a predominance of lymphocytes, CSF/blood glucose ratio < 0,5-0,6 or absolute glucose concentration in CSF < 2,2 mmol/L and protein concentration > 1g/L (278). In pleural TB, the effusion is usually a straw-coloured, exudative (using lactate dehydrogenase or protein criteria (279)), lymphocyte predominant fluid, generally showing a protein concentration > 30 g/L, elevated lactate dehydrogenase (often exceeding 500 IU/L), glucose concentrations between 3,3-5,6 mmol/L and pH < 7,4 in the majority of cases (272). In 2006 the WHO published recommendations on how to improve the diagnosis of smear-negative PTB and EPTB in resource-constrained settings (91). As for pleural effusion, TB pleuritis should be suspected if clinical presumptive TB, a clear and straw-coloured unilateral effusion with protein level > 30g/L and ≥ 50% lymphocytes (91). In patients with clinical features of TB meningitis, a clear CSF with lymphocytes, low glucose and high protein, and negative Gram stain and cryptococcal antigen (or fungal culture and India ink) in the CSF, should give a high suspicion of TB meningitis (91).

Cytology/histopathology

Cytological examination of effusions, fluids and FNAC smears from various tissues is often performed as it is relatively easy and gives rapid results. In TB, cytology of fluids and effusions usually shows a lymphocytic predominance, however lymphocytic pleocytosis is not specific for TB infection as it can be found in other conditions such as viral and fungal infection and malignancies (280, 281). Further, TB cases may also present a polymorphonuclear cell predominant fluid or effusion (282, 283). FNAC of palpable mass lesions, the most frequent site being aspirates from peripheral lymph nodes, is a widely used diagnostic method in the diagnosis of EPTB. FNAC is a safe, simple, minimally invasive technique, providing material for various investigations including cytological examination, and practical in peripheral health care units and feasible in resource-limited settings (284). Excision biopsy with histopathological examination is advised in cases with negative or equivocal cytology results. The invasive nature and unavailability of personnel and facilities performing biopsy and histopathological procedures constrain the use in many resource-limited settings. Several studies have assessed the value of the cytomorphologic diagnosis of TB lymphadenitis using FNAC (285, 286). There are well defined cytological and histological criteria suggestive of TB infection with chronic granulomatous inflammation with or without caseous necrosis defined as the classic features seen in TB disease (285, 287). Immunocompetent patients often present with the classic morphological pattern of epitheloid granulomata with multinucleated giant cells and caseous necrosis, whereas immunocompromised patients often show “dirty” necrosis with cellular debris and abundant neutrophils, but there is a wide range of morphological pictures between these extremes (285, 287). The classical patterns of granulomatous inflammation are not specific for TB disease, and other conditions such as NTM, other bacterial, viral, parasitic or fungal infections, sarcoidosis and malignancies may present a similar cyto/histomorphological picture (288). Thus, differentiating Mtb infection from other etiologies of granulomatous inflammation is not possible based on cytology/histology alone. NTM are a common cause of mycobacterial lymphadenitis, especially among children and HIV-infected individuals

(289, 290). Still, these features are strongly suggestive of an inflammatory response to Mtb in TB endemic countries.

Considering the currently available diagnostic tests for EPTB, an algorithm including clinical signs and symptoms, imaging results and a combination of available laboratory diagnostic tools is the best diagnostic approach in presumptive EPTB patients at present.

1.6 Care pathway and diagnostic delay in extrapulmonary tuberculosis

In many TB endemic settings, TB case detection is still primarily dependent on passive case finding. As shown in prevalence surveys, relying on passive case finding in high-prevalence settings is not enough to detect all TB cases in the community (291, 292) and there is still a large gap in notified and estimated TB cases (293). Active case finding is acknowledged as a complementary strategy, and comprises any approach for TB case finding not reliant on patients reporting to the health care provider (HCP) of own accord (294). Systematic screening among possible risk groups (295) and investigation of contacts of TB patients (296) are examples of active case finding strategies covered in guidelines published by the WHO, and give some guidance to active case finding in low-resource settings (147, 297). Still, implementation and feasibility of active case finding can be challenging for a health care system already struggling to handle the current burden of known TB disease. Thus, in low-resource settings most patients are detected trough passive case finding. Passive case finding depends on affected individuals' awareness of symptoms, access to health care facilities and on the individual taking appropriate care seeking actions after symptom onset (147). In a study among 1220 EPTB cases in India a minority of patients (12%) sought medical advice as their first action after onset of symptoms, while the majority practiced self-medication, consulted traditional healers or drug stores (298). A review including studies from high TB and HIV burden sub-Saharan African countries reported that first visit to traditional healers was consistently associated with patients delay and travel time for return visits associated with health system delay (299). Thus,

assessing the level of EPTB knowledge, awareness of signs and symptoms of EPTB and the patients` health care seeking actions after onset of symptoms are valuable when trying to identify potential areas of intervention to reduce diagnostic delay and increase case detection among this patient group. Further, when the individual visits the HCP, the HCP needs to recognize the signs and symptoms as presumptive EPTB and take relevant actions to confirm the diagnosis and timely initiate effective treatment (147). Studies have shown that patients often have repeated visits to HCPs before being started on anti-TB treatment (298, 300). In low-resource settings the scarcity of laboratory and imaging facilities especially in peripheral health care units, can potentially delay the diagnosis. Factors with possible impact on delay are various sociodemographic characteristics such as gender, age, household size, residence, educational level and occupation, patients health care seeking behaviour, awareness and knowledge about EPTB among the patients and formal and non-formal HCPs, travel time or distance to health care facilities, type of the first HCP visited and the number of HCPs consulted.

Assessing the length of patient, health system and total delay, the number of patients experiencing unacceptable delays, the various care seeking pathways among EPTB patients and factors associated with the distinct delays is important. Delayed diagnosis and initiation of anti-TB treatment could increase morbidity, mortality and economic loss for the patients and affected families (301-303). Studies from various settings including both EPTB and PTB patients describe EPTB associated with longer delays (304, 305). Still, the majority of studies on diagnostic and treatment delay have been aimed toward the adult population and among PTB patients (299, 300). It is essential to increase the evidence base regarding the extent of the distinct delays experienced by EPTB patients, preferentially divided according to the various sites of EPTB infection, and evaluate potential factors contributing towards patient and health system delays in these specific patient groups. Achieving an increased understanding can aid actors working with EPTB patients to place the efforts and provide a basis for development of evidence-based strategies and interventions to accomplish the goal of improved and prompt detection and treatment of all TB patients. In this regard searching among studies performed in other settings can be helpful, but the factors affecting delay can

vary between settings and populations, and thus cautions should be taken when generalizing findings and conclusions from one setting to another. In some settings patient delay accounts for the major part of the total delay (306, 307), while in other settings, studies report health system delay as the main contributor (301, 308). It is thus meaningful to assess the various delays, variables associated with the delays, TB knowledge and patients care seeking pathways in each individual setting. Even though there have been some studies performed at the Tanzanian mainland (309-311), data exclusively and specifically for EPTB patients at Zanzibar are missing, and studies in this setting and among this patient group are needed.

1.7 Treatment of extrapulmonary tuberculosis

Standard TB treatment consist of four first-line antimicrobials; isoniazid (H), rifampicin (R), pyrazinamide (Z) and ethambutol (E) (35). National and international guidelines currently recommend 6 months of chemotherapy as the standard first-line treatment regimen for new TB cases with known or presumed drug-susceptible TB (312-314). The treatment is divided in two phases, the intensive or initial phase and the continuation or consolidation phase. The 2 months intensive phase comprises a four-drug treatment regimen with H, R, Z and E, which is followed by a two-drug regimen with H and R in the 4 months continuation phase (312). Children are generally recommended to receive the same 6 months regimen (2HRZE/4HR), but ethambutol could be omitted in peripheral TB lymphadenitis in HIV negative children and in children who live in settings with low prevalence of HIV and/or isoniazid resistance (315). Extended treatment regimens with first-line drugs, have been recommended by expert groups in drug-susceptible osteoarticular TB and TB meningitis (312, 313). Adjuvant corticosteroid treatment is advised in the initial weeks of treatment in TB meningitis (316) and some also recommend corticosteroids in addition to anti-TB treatment in TB pericarditis (312).

In previously treated TB patients, the choice of the regimen depends on the probability of the patients having MDR-TB and the availability of DST in the specific setting. The recommendation is a retreatment regimen with first-line drugs including streptomycin

(S) (2HRZES/1HRZE/5HRE) in patients returning after relapsing if the MDR-TB levels are low/medium in this specific patient group in the country-specific setting. In patients returning after failure, empirical MDR regimen is advised, which can be modified depending on the DST results (312). National TB programmes should modify or verify the assignment of relapsing and failure patients to low/medium/high likelihood of MDR according to country-specific data on the MDR-TB levels in these patients. Treatment of MDR-TB and extensively drug-resistant TB are longer and more complicated and should whenever possible be guided by the results from the DST of the Mtb isolate in each individual patient. If DST is unavailable, empirical or standard treatment regimens is advised (312, 317).

HIV testing is advocated in all presumptive TB patients, and is especially important among EPTB cases, because of the higher proportions of extrapulmonary sites involved in patients with immunosuppression. In HIV positive patients not receiving antiretroviral therapy (ART) when TB is diagnosed, ART should be initiated within 8 weeks of starting anti-TB treatment in patients with CD4 count > 50 cell/mm^3 and within 2 weeks in patients with severe immunosuppression (317).

Correct treatment regimens and adherence to treatment are of vital importance to promote cure of the individual patient, prevent emergence of drug resistance and impede transmission of TB infection. Some of the recommended strategies to achieve this are a patient-centred treatment approach, giving the patient an opportunity to be included in decisions regarding overall care and supervision of treatment, standard treatment, DOT relating to a second person directly observing the patient taking the prescribed medication and fixed-dose combination regimen (3, 312, 318).

1.7.1 Paradoxical reactions

Paradoxical reactions, defined as development of new TB lesions or worsening of existing TB disease in patients initially responding to effective anti-TB treatment (319), have been described in previous studies to occur rather frequently in HIV-uninfected EPTB patients (319, 320). In HIV positive patients, the immune reconstitution inflammatory syndrome (IRIS), often occurring during the first months of ART (321), is well recognised. As a result of immunologic recovery and restoration

of immune responses induced by ART, IRIS usually presents as a new presentation of a previously untreated subclinical infection (unmasking IRIS) or as a paradoxical exacerbation of a treated opportunistic infection (paradoxical IRIS) (322). In paradoxical TB-IRIS, HIV positive patients diagnosed with active TB, receive and typically respond to anti-TB treatment, but subsequently experience aggravation of TB signs and symptoms after commencing ART (321). The condition has been reported to be quite common in TB/HIV co-infected individuals given both anti-TB treatment and newly initiating ART (323), and a meta-analysis reported an estimated incidence of 15.7% of paradoxical worsening after initiating ART in patients with formerly diagnosed TB infection (324). Recognition of deterioration resulting from paradoxical reactions or paradoxical TB-IRIS could be difficult to distinguish from drug-resistant TB, treatment failure, adverse drug reactions or the presence of another infection or condition (325).

1.7.2 Monitoring response to treatment

The establishment of a microbiological or histological diagnosis is recommended among EPTB patients, and every effort should be made to confirm the TB diagnosis (326). However, an accurate diagnosis of EPTB is difficult and complex, and thus many EPTB cases are initiated on empirical anti-TB treatment. Monitoring response to treatment is crucial in this regard to assure correct diagnosis and appropriate disease management, and clinical response to treatment is often used to support the EPTB diagnosis (91, 327). Further, the early and timely detection of non-responders is important and attempts to uncover the reason for treatment failure such as poor compliance, drug-resistant TB or alternative diagnoses should be sought.

Several modalities have been investigated and much research is ongoing to find tools to monitor treatment response and cure among TB cases (328, 329). But still, the recommended method for monitoring treatment response among PTB patients is sputum smear microscopy at specific time points during treatment, and ideally sputum cultures among patients remaining sputum smear positive (3, 328). In EPTB cases microbiologic evaluation is usually not feasible, and clinical assessment is the recommended approach for monitoring response to treatment, especially in low-

resource settings (3, 91, 312). In real-world TB programmatic settings recording response to treatment could be challenging due to the diversity of EPTB cases, different HCPs performing the follow-up visits and absence of simple, user-friendly criteria for treatment response in EPTB. The definition of satisfactory clinical response among the various sites of EPTB infection remains to some extent unclear and there is currently no established criteria (108).

Clinical assessment of treatment response is partly subjective, and widely used indicators of improvement is disappearance of symptoms and ability to resume regular activities. Improvement of symptoms can be assessed by a symptoms count ratio (number of symptoms better or resolved compared to baseline) (330) or increase of self-reported health using standardized instruments such as the EQ-5D instruments (331), while the HCP can evaluate improvement of the patients performance status using a physician-rated functional scale like the Karnofsky Performance Status score (332-334). Objective measure such as weight gain is also a widely used marker of treatment response. Previous studies suggest weight gain to be associated with favorable treatment outcome, and unfavorable outcome with weight loss or lesser weight gain (335-338). Few studies have evaluated the association between weight and treatment outcome among EPTB patients (335, 336), and further if any weight gain is enough or should the weight gain exceed a certain percentage of body weight before taken as a marker of response to treatment.

Repeated laboratory analysis of inflammatory markers or invasive procedures are often part of suggested definitions of good treatment response (108, 330). In low-resource setting, especially in peripheral health care units, there are often scarcity of health personnel and unavailability of both laboratory and radiologic investigations, thus defining simple clinical criteria could be helpful to the HCP in pinpointing patients in need of clinical reassessment, further investigations and referral to higher levels in the health care system. There is a need to develop better tools or clinical assessment algorithms for treatment follow-up among EPTB patients, ideally not dependent on laboratory tests or invasive procedures, thus feasible in low-resource settings.

1.8 Developing new tuberculosis diagnostic tools

Important objectives in the fight against TB is the development, distribution and adaption of new TB diagnostic tools, with the potential to improve the detection of TB cases, including EPTB, by increased sensitivity and specificity of new tests compared to existing tests, that can be used in high TB burden settings. A new diagnostic tool should be thoroughly evaluated and validated and a comprehensive evidence base on test performance and user- and patient-important outcomes should be obtained, together with an assessment of the cost-effectiveness and quality of the evidence provided, before a new test is considered recommended for implementation in routine use (339, 340). A good test performance in controlled research settings needs to be complemented by evaluation of the test`s accuracy and reliability in routine diagnostic settings. In addition to the performance data under field conditions, studies providing evidence of the test`s ability to have impact on morbidity, mortality and the public health is important. Further, the feasibility of implementation, both in the routine laboratory and in the TB programmatic setting, the affordability for the health system and accessibility to the patients should be assessed. It could be challenging to adapt, implement and distribute new diagnostic tools in high burden TB settings where these tools are needed most. There is considerable ongoing research in the field of developing new TB diagnostic tools. In 2009 the New Diagnostics Working Group (NDWG) of the Stop TB Partnership published a blueprint; “Pathways to better diagnostics for tuberculosis: a blueprint for the development of TB diagnostics”. The blueprint offers a structured guide to those involved in the development of TB diagnostic tools, outlining the required phases from assessing the need, through development and evaluation of new tests, to assessing the feasibility of implementation and impact on public health in real-world settings (Figure 5) (202).

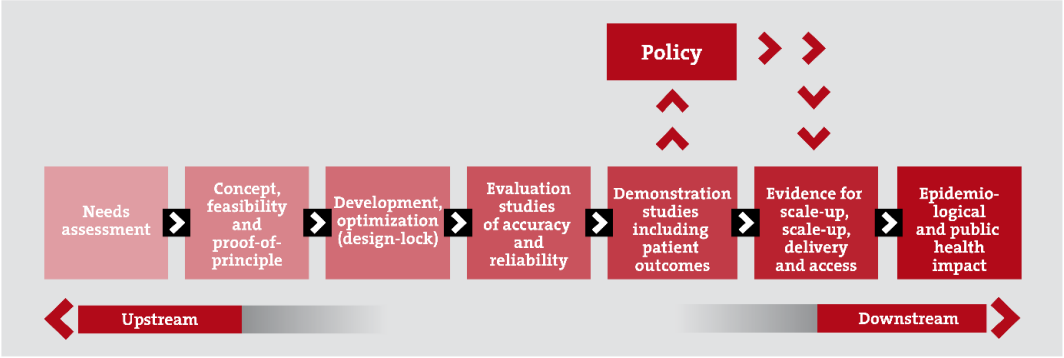

Figure 5. The pathway for developing tuberculosis diagnostics, from assessing the need to delivery. Reprinted with permission from the World Health Organization from the publication "Pathways to better diagnostics for tuberculosis: a blueprint for the development of TB diagnostics", World Health Organization Stop TB Partnership New Diagnostics Working Group (202). http://whqlibdoc.who.int/ publications/2009/9789241598811_eng.pdf. Copyright 2009.

In the first stage, one must understand the context and the problems experienced by the patients in the setting were the test is planned to be performed, including the structure of the area`s health system, the infrastructure and capacity in the routine laboratory and patients` access to the health system. This needs assessment should provide a detailed description of the specific context, the target population, at what level of the health system and diagnostic algorithm the new test should be performed, equipment and technical needs, human resources required, expected turnaround time, information on the presumed performance in real-world settings and insight regarding anticipated increase of diagnosis and access to diagnosis (202).

The next stage is developing the concept and reach a decision if a specific technical idea can be used for the required diagnostic purpose, then, decide if one should proceed with the development of a test prototype and optimization of the test through proof-of-principle studies and evaluation in the field using a partly validated test on various patient populations. Further, in the evaluation stage the performance of the new test should be assessed against gold standard techniques. First, in case-control studies or using well-characterized samples, here it is possible to use retrospectively collected data and specimens. In the later phases the performance of the test should be

evaluated in populations where such a test is typically indicated (clinical presumptive TB cases), among representative and consecutively included patients in settings or conditions of planned use, evaluating the clinical performance of the test in field settings (202). The test`s diagnostic accuracy refers to the test`s ability to correctly identify patients with a given disease and cases not having the disease (341). In diagnostic accuracy studies the test is usually assessed in comparisons to a set reference standard, and the accuracy of the test is thus referring to the extent of accordance between the reference standard and the test (341). Studies on diagnostic accuracy should be well designed and transparently reported, so others are able to assess potential bias and generalisability (342). Using tools such as "Standards for Reporting of Diagnostic Accuracy" (STARD), a reporting template for publications, (342), and "Quality Assessment of Diagnostic Accuracy Studies" (QUADAS), to assess the methodological quality of diagnostic accuracy studies (343), are recommended to improve the reporting and methodological rigor and evaluation of the quality of diagnostic accuracy studies. Further, the reproducibility (reliability) should be assessed, such as an evaluation of the test reproducibility in different field sites or laboratories, but also intra- and inter-observer, within-run and run-to-run reproducibility (202).

Diagnostic accuracy studies are required, but it can be difficult to determine the plausible impact on patient-important outcomes and public health just by evaluating how often the test classify patients accurately as diseased or non-diseased, in controlled performance trials (202, 344). It is important to demonstrate that the new test not only performs well in field conditions, but also has a valuable impact on TB morbidity and mortality, when implemented in real-world TB programmatic settings (339, 344), its influence on HCPs clinical decision-making, and detection and treatment of additional cases. At this stage, an evaluation of the feasibility of implementation of the new test in real-life settings and potential challenges to implementation is valuable. Further, to evaluate if the delivery systems, infrastructure and expertise needed for implementation is available in the specific context and are the HCPs able and willing to use the test (202, 339). It is also important to assess affordability (can the health system afford the test) and accessibility (will patients have

access to the test). The new diagnostic test is usually compared to a reference standard or other available diagnostic methods and algorithms and a cost-effectiveness analysis comparing the novel test to other alternative diagnostic tools is recommended (202). Collecting a comprehensive and solid evidence base is essential in the decision process and guide policy makers to reach a conclusion whether to adopt and implement a new diagnostic test (340).

1.9 MPT64 antigen detection test

A diagnostic test based on the detection of MtbC specific antigen MPT64 using immunostaining (MPT64 test) has been developed. This test is applicable on smears of effusions, aspirates and CSF and tissue sections from formalin-fixed, paraffin-embedded biopsies.

1.9.1 Developing the concept of the MPT64 test

A study was done for *in vivo* expression of three somatic (MPT57, Hsp65 and Mce1A) and eight secreted mycobacterial antigens (MPT64, MPT63, MPT59, MPT53, MPT51, MPT46, MPT44 and MPT32) in Mtb infected lymph nodes and lung tissues, using IHC staining with primary in-house rabbit polyclonal antibodies (345). All the secreted antigens were reported to be found in high levels in the PTB lesions, while in lymph node TB the secreted antigens were absent or only detected in low levels, except for the MPT64 antigen. The MPT64 antigen was shown to exhibit a consistent intracellular expression in tissue sections from the TB lymphadenitis cases (345). From this, the hypothesis of a special ability of MPT64 to accumulate in infected host cells (Figure 6) was established, and further the idea of developing a diagnostic test based on detection of the MPT64 antigens in paucibacillary TB lesions. Additional work supported this hypothesis and led to the development of the immunochemistry-based test using polyclonal anti-MPT64 primary antibodies to detect MPT64 antigens in various extrapulmonary specimens for the diagnosis of EPTB, i.e. the MPT64 test.

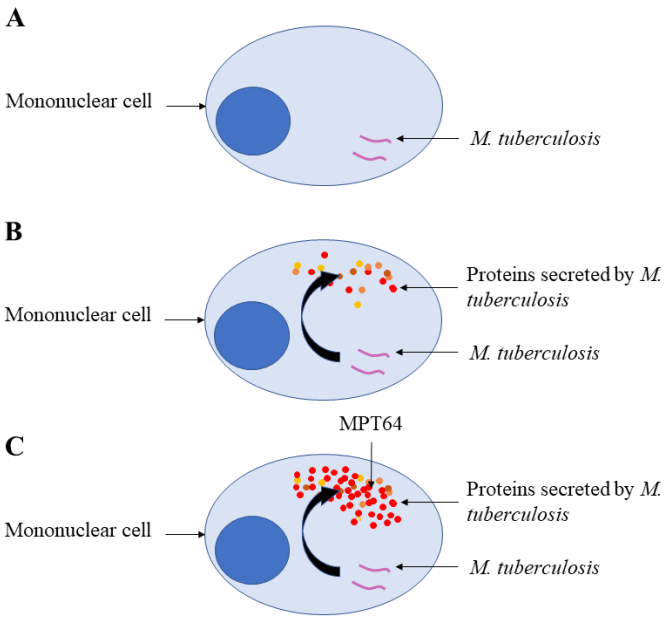

Figure 6. Schematic drawing of infected host mononuclear cell. A: Intracellular *Mycobacterium tuberculosis*. B and C: The hypothesis: *Mycobacterium tuberculosis* secretes protein MPT64 which accumulates in the infected host cell.

1.9.2 Antigen detection using anti-MPT64 primary antibody

The polyclonal anti-MPT64 primary antibody used in the MPT64 test was previously raised in-house by immunizing rabbits with purified MPT64 antigen (238, 246, 345). The specificity of the MPT64 antiserum was further enhanced by absorbing the antiserum with antigen extracts from BCGΔRD2, thus reducing cross-reactive antibodies (246). The in-house absorbed polyclonal primary antibody was utilized in subsequent studies evaluating the MPT64 test (122, 123, 247, 346, 347). The immunostaining is based on a two-step method (Figure 7). First the smear/tissue section is incubated with primary anti-MPT64 antibody attaching to and detecting MPT64 antigen if present in the sample. Then, the smear/tissue section is incubated with horseradish peroxidase (HRP) labelled dextran polymer conjugated with secondary anti-rabbit antibodies.

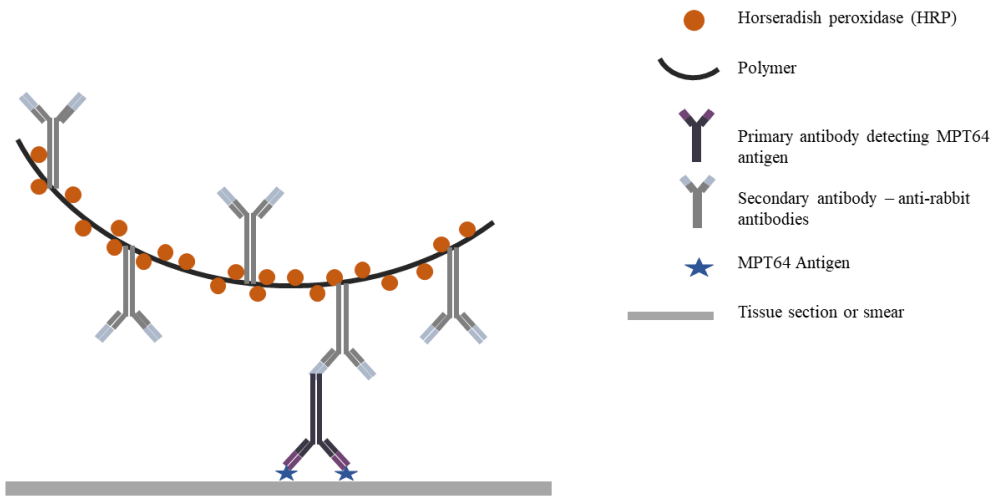

Figure 7. Illustration of the two-step method used in the immunostaining procedure.

After these steps, the smear/tissue section is incubated with substrate-chromogen solution, 3-amino-9-ethylcarbazole (AEC) containing hydrogen peroxide, this will, in the presence of HRP, produce a red-coloured oxidation product. The smear/tissue sections are further counterstained with hematoxylin before mounting. The localisation and visualisation of the antigen detected by the primary antibody will appear as reddish granular precipitates. The identification of antigens is done in the light microscope (Figure 8).

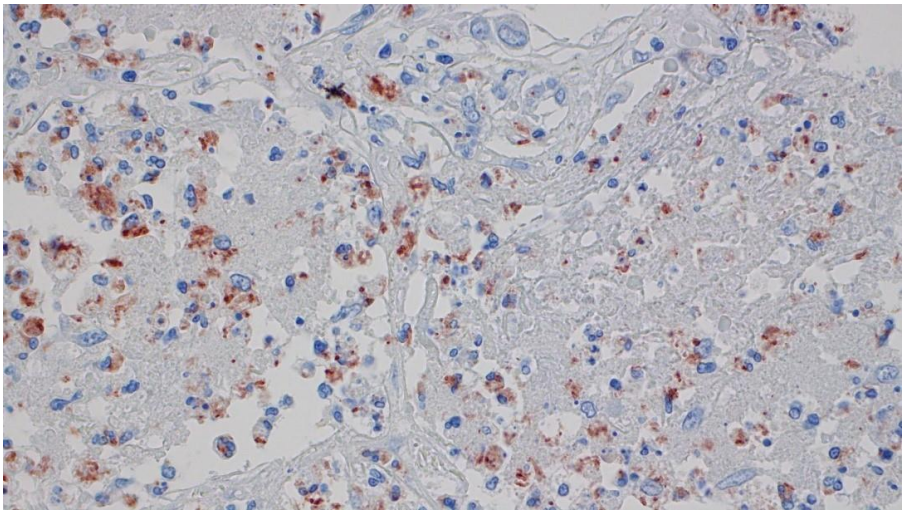

Figure 8. Immunostaining with anti-MPT64 primary antibody. Illustration of positive immunohistochemical staining with primary anti-MPT64 antibody. The tissue section is showing a high amount of reddish positive spots, and the hematoxylin blue-coloured counterstaining. Photo: Melissa Davidsen Jørstad

1.9.3 Previous studies evaluating the performance of the MTP64 test

An overview of the previous studies assessing the MPT64 test is presented in Table 1 and 2. The first study assessing the expression of mycobacterial antigen MPT64 in tissue biopsies for diagnostic purposes was published by Mustafa et al. (246). The study demonstrated the proof-of principle and assessed the diagnostic potential of the MPT64 test in the diagnosis of TB lymphadenitis using formalin-fixed biopsies (246). The novel test was compared against nested PCR for IS6110 as the gold standard and reported a sensitivity and specificity of 90% and 83%, respectively. The study also demonstrated reproducible IHC staining results by repeated staining of all samples (246). The second study, by Purohit et al., aimed to further evaluate the performance of the MPT64 test among a different study population and including other disease sites, showing consistent high sensitivity and specificity of the new diagnostic test in different tissues (122). The third study, published by Baba et al., assessed the MPT64 test in archival pleural biopsies in a high TB and HIV-endemic setting (South-Africa) (346), and the reported results agreed with previous studies.

Having evaluated the diagnostic potential of the MPT64 test in histological specimens from formalin-fixed, paraffin-embedded biopsies, Purohit et al. assessed the MPT64 test in cytological smears from effusions, CSF and FNAC from various sites (123). The sensitivity and specificity of the MPT64 test was reported using a composite reference standard (CRS) and to nested PCR as reference standard. Compared to the CRS, the sensitivity of the MPT64 test was reported to be 54-76% in the various sample materials, but 95-100% using nested PCR as gold standard (123). Another important finding described in this study was the high agreement between ICC staining of aspirates and IHC staining of biopsies from the same patients (kappa=0.85), showing that the less invasive sampling technique can be used in TB diagnosis. Additionally, all the initial studies reported that the use of primary anti-MPT64 antibodies had better performance than immunostaining with commercial anti-BCG antibodies and conventional diagnostic methods (122, 123, 246, 346). The findings described by Purohit et al. (123) was subsequently confirmed in a different setting by Tadele et al. (347). Purohit et al. extended the earlier research by assessing the diagnostic potential of the MPT64 test in additional EPTB sites showing consistently high sensitivity and specificity (247).

One of the advantages of the MPT64 test is that it does not depend on sophisticated equipment but utilizes technology and procedures often already available in routine pathology laboratories. The evaluation of the stained slides is performed using the light microscope at x10-40 magnification and the staining gives strong and sharp signals with small amount of background staining (122, 123, 247) making the interpretation quite easy. The results can be available within 1-2 working days (1 day in effusions, aspirates and CSF; 2 days, biopsies due to paraffin-embedding). Immunostaining methods are robust and have the ability to detect fragmented mycobacterial antigens (224), and thus intact bacterial cell walls are not a prerequisite. The MPT64 test is shown to have substantially higher sensitivity than Mtb culture and ZN staining, comparable sensitivity and specificity to nested PCR (122, 123, 247, 347), and advantages compared to PCR of being robust, simple, not prone to contamination and requires only minimal equipment. This makes the MPT64 test suitable for adoption in low-resource settings with rather modest laboratory facilities.

Even though the MPT64 test should be interpreted together with clinical history and other laboratory investigations, it can play a valuable role in cases where cytology/histology do not show typical features of TB disease. In addition, since the MPT64 antigen is specific for MtbC organisms, the MPT64 test can differentiate MtbC infections from NTM and other granulomatous diseases, potentially reducing overdiagnosis of TB and increasing correct diagnosis.

As described, several studies, using various EPTB specimens from different settings, have assessed the diagnostic performance of the MPT64 test against either PCR or a CRS as the gold standard, mostly in case-control study designs in relatively controlled research settings. Using case-control comparisons is known to potentially overestimate a test`s diagnostic accuracy (202). Still, the positive and consistent findings from the previous studies show that the MPT64 test may have the potential to improve the diagnosis of EPTB and warrants the assessment of the diagnostic test performance under field conditions among patient populations where such a TB diagnostic test is clinically indicated. It would also be important to assess the feasibility of implementation of the new test in routine diagnostic settings and identify potential obstacles and challenges. Expanding and building a more profound and comprehensive evidence base for the MPT64 test will ultimately assist in the decision process whether to up-scale and adopt and implement this novel test in the diagnostic algorithm in presumptive EPTB cases.

Table 1. Overview of the previous studies evaluating the MPT64 test

Author	Design	Study area	Inclusion criteria	Exclusion criteria	Number of included patients	Age group	HIV status	Sample material
Mustafa et al. 2006 (246)	Case-control, retrospective	Norway Tanzania	Cases: clinical and histologically presumptive TB Controls: other histological diagnoses than TB	Not disclosed	N=75 (Cases: n=55 Negative controls: n=16 Positive controls: n=4)	Children and adults (range 2-86 years)	Not disclosed	Formalin-fixed, paraffin-embedded biopsies Cases: lymph nodes Controls: various sites
Purohit et al. 2007 (122)	Case-control, retrospective	India Norway	Cases: histologically diagnosed TB Controls: other histological diagnoses than TB	PTB **or** use of corticosteroids **or** other immunosuppressive therapy	N=203 (Cases: n=153 Controls: n=50)	Not disclosed	All HIV negative	Formalin-fixed, paraffin-embedded biopsies Cases: abdominal (n=33), cervical lymph nodes (n=120) Controls: abdominal (n=32), cervical lymph node (n=18)
Baba et al. 2008 (346)	Case-control, retrospective	South Africa	Cases: TB diagnosis based on either 1) positive mycobacterial culture; 2) typical histological features; 3) clinical findings and response to TB treatment Controls: Pleural malignancies or hydrothorax due to trauma	Not disclosed	N= 36 (Cases: n=25 Controls: n=11)	Adults (range 19-83 years)	16/18 (89%) cases HIV positive	Formalin-fixed, paraffin-embedded biopsies Cases: pleural biopsies Controls: pleural biopsies
Purohit et al. 2012 (123)	Prospective cohort study	India	Clinical diagnosis of; 1) TB meningitis, 2) TB pleuritis, 3) TB peritonitis, 4) Cervical TB lymphadenitis	PTB **or** use of corticosteroids or other immunosuppressive therapy **or** started anti-TB treatment	Included presumptive TB cases, N=270	Children and adults (range 3-65 years)	All HIV negative	Smears: cervical lymph nodes FNAC (n =150), cerebrospinal fluid (n=27), pleural fluid (n=41), ascitic fluid (n=52) **F**ormalin-fixed, paraffin-embedded biopsies: abdominal (intestine, peritoneal or mesenteric lymph node) (n=33) and cervical lymph node (n=111).
Tadele et al. 2014 (347)	Cross-sectional study	Ethiopia	Pleural effusion or lymphadenopathy: clinical diagnoses of TB or effusion/lymphadenopathy due to other causes.	PTB on CXR **or** started anti-TB treatment **or** contraindications to thoracocentesis	N=118	Children and adults (range 3-85 years)	27/118 (23%) HIV positive	Smears: lymph node FNAC (n=55), pleural fluid (n=63)
Purohit et al. 2017 (247)	Case-control	India Norway	Presumptive TB based in cytology/histology	PTB **or** use of immunosuppressive therapy **or** < 14 years of age	N=89	≥ 15 years	Not disclosed	Smears: FNAC, various sites (n=52) Formalin-fixed, paraffin-embedded biopsies, various sites (n=37)

Abbreviations: HIV, human immunodeficiency virus; TB, tuberculosis; PTB, pulmonary tuberculosis; FNAC, fine-needle aspirate cytology; CXR, chest x-ray

Table 2. Reference standard and diagnostic validation of the MPT64 test in previous studies

Author	Reference standard	Final diagnosis based on reference standard	Sensitivity ICC/IHC*	Specificity ICC/IHC*	PPV ICC/IHC*	NPV ICC/IHC*
Mustafa et al. 2006 (246)	Nested-PCR for IS6110	Positive nested-PCR: n=40 (33/55 included as cases, 4/4 positive controls, 3/16 negative controls) Negative nested-PCR: n=35 (22/55 included as cases, 13/16 negative controls)	90%	83%	86%	88%
Purohit et al. 2007 (122)	Nested-PCR for IS6110	Positive nested-PCR: n=136 (132/153 included as cases, 4/50 negative controls) Negative nested-PCR: n=67 (21/153 included as cases, 46/50 negative controls)	TB lymphadenitis: anti-MPT64 93% anti-BCG 88% Abdominal TB: anti-MPT64 89% anti-BCG 86%	TB lymphadenitis: anti-MPT64 98% anti-BCG 86% Abdominal TB: anti-MPT64 95% anti-BCG 81%	TB lymphadenitis: anti-MPT64 99% anti-BCG 94% Abdominal TB: anti-MPT64 96% anti-BCG 86%	TB lymphadenitis: anti-MPT64 85% anti-BCG 76% Abdominal TB: anti-MPT64 87% anti-BCG 81%
Baba et al. 2008 (346)	CRS Cases: patients included as cases positive by nested-PCR for IS6110 Controls: patients included as controls negative by nested-PCR for IS6110	Cases: n=16 Controls: n=9	All cases: anti-MPT64 81% anti-BCG 56% HIV positive cases: anti-MPT64 90% anti-BCG 70%	anti-MPT64 100% anti-BCG 78%	Not disclosed	Not disclosed
Purohit et al. 2012 (123)	CRS Cases: Pleural, abdominal or meningeal TB, one of the two criteria; 1) AFB on ZN staining and/or positive mycobacterial culture and/or positive nested-PCR, 2) good response to treatment at follow-up after 8 weeks of anti-TB treatment in presumptive TB cases based on (i) cytology with lymphocytic predominance and protein level > 3 g/dl or (ii) granulomatous inflammation seen in tissue sections. TB lymphadenitis one of the following criteria; 1) AFB on ZN staining, 2) positive mycobacterial culture (aspirate or biopsy), 3) positive nested-PCR for IS6110 (aspirate or biopsy), 4) good response to treatment at follow-up after 8 weeks of anti-TB treatment. Controls: negative ZN stain and negative mycobacterial culture and negative nested-PCR for IS6110 and cytology/histology not suggestive of TB (Nested PCR for IS6110 was also used as reference standard for diagnostic validation)	Based on CRS Cases: n=190 Controls: n=80	CRS as reference standard Overall: anti-MPT64 67% anti-BCG 59% PCR as reference standard Overall: anti-MPT64 96% anti-BCG 87%	CRS as reference standard Overall: anti-MPT64 95% anti-BCG 80% PCR as reference standard Overall: anti-MPT64 96% anti-BCG 88%	PCR as reference standard Overall: anti-MPT64 95% anti-BCG 86%	PCR as reference standard Overall: anti-MPT64 97% anti-BCG 88%

Continuation of table 2

Author	Reference standard	Final diagnosis based on reference standard	Sensitivity ICC/IHC*	Specificity ICC/IHC*	PPV ICC/IHC*	NPV ICC/IHC*
Tadele et al. 2014 (347)	CRS Cases: Clinical suspicion of TB and one of the following; 1) AFB on ZN stain, 2) positive mycobacterial culture, 3) positive PCR for IS1081, 4) Cytomorphological features suggestive of TB Controls: Other clinical diagnosis than TB and negative ZN stain and negative mycobacterial culture and negative PCR for IS1081 and cytomorphological features not suggestive of TB (PCR for IS1081was also used as reference standard for diagnostic validation)	Cases: n=51 (pleuritis, n=26; lymphadenitis, n=25) Controls: n=67	CRS as reference standard Overall: anti-MPT64 75% PCR as reference standard Overall: anti-MPT64 88%	CRS as reference standard Overall: anti-MPT64 90% PCR as reference standard Overall: anti-MPT64 90%	CRS as reference standard Overall: anti-MPT64 84% PCR as reference standard Overall: anti-MPT64 82%	CRS as reference standard Overall: anti-MPT64 82% PCR as reference standard Overall: anti-MPT64 93%
Purohit et al. 2017 (247)	CRS Cases: final diagnosis of TB based on either criterion; 1) AFB on ZN stain, 2) positive mycobacterial culture, 3) positive nested-PCR for IS6110 Controls: negative ZN stain and negative mycobacterial culture and negative nested-PCR for IS6110 (Nested PCR for IS6110 was also used as reference standard for diagnostic validation)	Based on CRS Cases: n=51 Controls: n=38	CRS as reference standard Overall: anti-MPT64 100% anti-BCG 100% PCR as reference standard Overall: anti-MPT64 100% anti-BCG 100%	CRS as reference standard Overall: anti-MPT64 97% anti-BCG 84% PCR as reference standard Overall: anti-MPT64 97% anti-BCG 84%	PCR as reference standard Overall: anti-MPT64 98% anti-BCG 88%	PCR as reference standard Overall: anti-MPT64 100% anti-BCG 100%

* Sensitivity, specificity, positive predictive value and negative predictive value: the results of immunocytochemical/immunohistochemical staining using either in-house polyclonal anti-MPT64 primary antibody (anti-MPT64) or using commercial anti-BCG primary antibody (anti-BCG) (Dako) compared to reference standard.

Abbreviations: ICC, immunocytochemistry; IHC, immunohistochemistry; PPV, positive predictive value; NPV, negative predictive value; PCR, polymerase chain reaction; TB, tuberculosis; BCG, bacillus Calmette-Guérin; CRS, composite reference standard; HIV, human immunodeficiency virus; AFB, acid-fast bacilli; ZN, Ziehl-Nee

2. RESEARCH AIMS

The primary aim of this thesis was to improve the diagnosis and management of EPTB cases in a low-resource setting at a referral hospital in Zanzibar.

Secondary aims:

- To implement the MPT64 test in routine diagnostics and evaluate the test for the diagnosis of EPTB as compared with the routine tests and GeneXpert® MTB/RIF assay (paper I and unpublished data).

- To study the health care seeking pathways by presumptive EPTB patients and identify factors associated with diagnostic delay, and to assess the impact of anti-TB treatment on self-rated health among EPTB patients (paper II).

- To describe the clinical presentation of EPTB among patients, and to describe the effect of anti-TB treatment on clinical parameters. Further, to assess if simple clinical parameters, without laboratory support, could be used to reliably evaluate treatment response (paper III).

3. MATERIALS AND METHODS

3.1 Study setting

This study was performed at the main referral hospital in Zanzibar, Mnazi Mmoja Hospital (MMH). Zanzibar which comprises the two main islands, Unguja and Pemba, and some smaller inhabited and uninhabited islands, is situated off the coast of Tanzania mainland. In 1964, Zanzibar merged with Tanganyika to form The United Republic of Tanzania, but Zanzibar retained its own government and has substantial independence in internal affairs, whereas defense and foreign affairs are managed by a central government. Administratively, Zanzibar is divided into five regions, three in Unguja and two in Pemba, and each region is further divided in two districts.

The population in Zanzibar is 1.3 million inhabitants, and 42.5% of the population is <15 years of age (348). Life expectancy increased from 57 years in 2002 to 65.2 years in 2012 and infant mortality rate declined from 61 in 2004/05 to 46 per 1000 live births in 2012 (349). The HIV prevalence is 1% in the adult population aged 15-49 years, 1.1% HIV positives among women and 0.9% among men (350). According to a national survey in 2012, the estimated prevalence of bacteriologically confirmed PTB in the adult population is 124 per 100 000 (291). Although there has been an increase in TB cases notifications from 350 in 2000 to 855 notified TB cases in 2015 (351), the notified cases is far below the estimates. EPTB accounted for 23-30% of the new TB cases notified between 2013-2015, the TB treatment success rate in the same period was 87-91%, HIV testing was performed among 93-96% of the registered TB patients and 14-18% of the patients tested were HIV positive (351).

3.1.1 The health care system in Zanzibar

The Ministry of Health (MoH) regulates, governs and coordinates all health-related issues in the region. Health services are provided through operating departments of the MoH and specialized programs such as malaria, TB and leprosy control programmes. Besides public health facilities governed by the MoH, other health facilities are under the armed forces. In addition, the private sector, development partners, faith-based and non-governmental organizations support the public health sector (352). The

geographical distribution of the various health facilities is shown in figure 9. Most of the private health facilities are located in urban areas, whereas there is a fair distribution of public health facilities across Zanzibar. Ninety-five percent of the Zanzibari citizens live within 5 km of a public health facility (352).

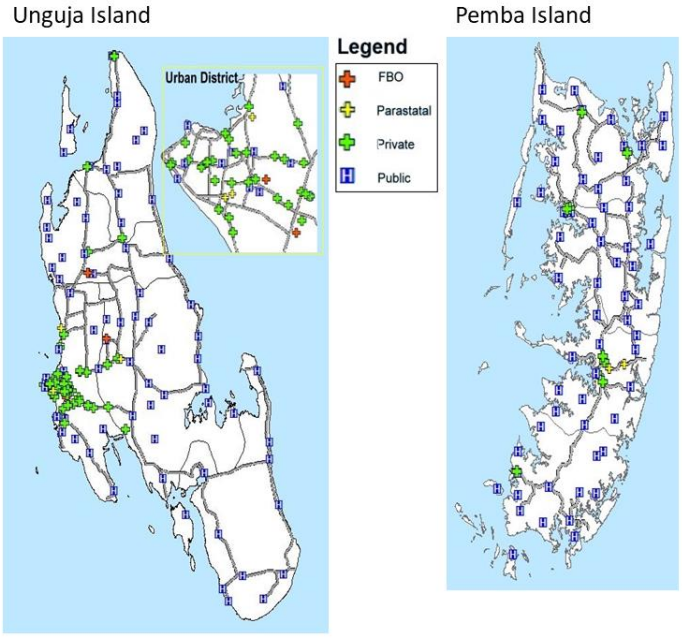

Figure 9. Distribution of health care facilities in Zanzibar. In 2013/14, there were 71 private clinics/dispensaries (11 in Pemba, 60 in Unguja) and 4 private hospitals (all at Unguja) (353). In addition, but not shown in the figure, there were 25 private pharmacies and 335 over-the -counter drug shops (170 in Pemba, 165 in Unguja). Abbreviation: FBO, faith-based organization. Figure from «Zanzibar Health Sector Strategic Plan III 2013/14-2018/19 » (352), reprinted with permission from the Ministry of Health, Revolutionary Government of Zanzibar.

In the public sector the health care system is divided in primary, secondary and tertiary level of health care (Figure 10) (352). At the primary level, the primary health care units (PHCU) deliver basic outpatient services, such as treatment of minor injuries and common diseases, maternal and child health care and health promotion activities, both at the facility and the community-level. The PHCU+ provides in addition basic laboratory, delivery, dispensing and dental services. The PHCU/PHCU+ refer to primary health care centres (PHCC) or district hospitals, which again are supported by

MMH. MMH, with a capacity of 400 beds, provides tertiary level of health care to all districts, and primary and secondary services to some districts.

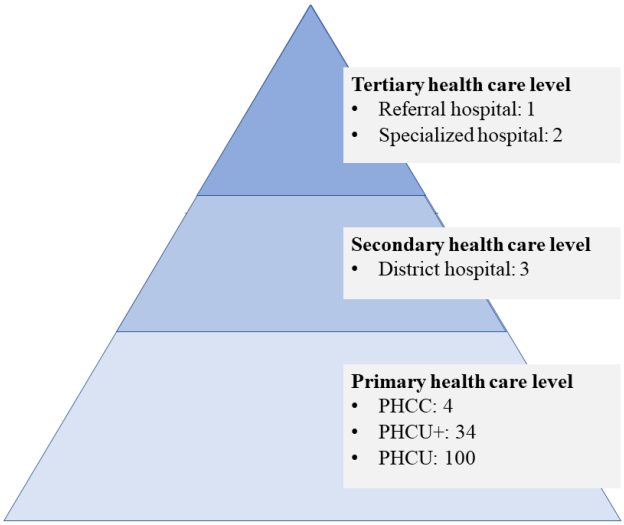

Figure 10. Levels of the public health care system in Zanzibar. The tertiary level comprises 1 referral hospital (Mnazi Mmoja Hospital) and the 2 special hospitals, Mwembeladu maternity home (34 bed capacity) and Kidonge Chekundu psychiatric hospital (110 bed capacity), all located in Unguja Island (352). The secondary level consists of 3 district hospitals (80-120 bed capacity) situated in Pemba Island, these hospitals have inpatient medical, basic surgical and diagnostic services, in addition to providing outpatient health care (352). The primary health care centres, deliver outpatient services, basic inpatient care (30 beds) and diagnostic services such as ultrasound and x-rays. Abbreviations: PHCC, primary health care centre; PHCU, primary health care unit.

3.1.2 Zanzibar Integrated HIV, Tuberculosis and Leprosy Programme

The Zanzibar Tuberculosis and Leprosy Programme (ZTLP) was established by the MoH in 1987 (351). In 2012, the ZTLP was officially joined with Zanzibar AIDS Control Programme to form the Zanzibar Integrated HIV, TB and Leprosy Programme (ZIHTLP) (351). The ZIHTLP facilitates and coordinates all activities related to HIV, TB and leprosy prevention, treatment and control in Zanzibar. The TB services are integrated and provided by the public health sector at all three levels. Anti-TB treatment, free of charge for the patients, is given according to international recommended guidelines (351). Every public health facility offers treatment given

under DOT, as do a few private health facilities which has an agreement with the MoH to provide these services. The patient can choose to receive anti-TB treatment under facility-based or community-based DOT and a treatment supporter of their own chose. Most of the registered TB patients in Zanzibar are choosing treatment as community-based DOT (351). TB case-finding is still predominantly passive, relaying on patients contacting the health care system, with exceptions of contact tracing around smear positive PTB patients and screening of HIV positive patients, prisoners and people staying in sober living houses.

3.1.3 Tuberculosis diagnostic capacity in Zanzibar

In 2015 there were 52 diagnostic sites (33 in Unguja and 19 in Pemba) performing AFB smear microscopy (351) and one laboratory providing mycobacterial culture. The implementation process of establishing a reference laboratory performing TB solid culture and identification of Mtb at the Public Health Laboratory-Ivo de Carneri (PHL-IdC) was started in 2007 (148). Before this, if requiring mycobacterial culture and DST, the specimen was sent to the Central Tuberculosis Reference Laboratory (CTRL), Muhimbili Hospital, Dar es Salaam. In 2010, the PHL-IdC was recognized as the national reference laboratory for mycobacterial culture in Zanzibar (148). During the study period, if species identification and DST were required, the positive cultures had to be sent from PHL-IdC to CTRL. The Xpert assay was introduced at MMH in early 2014, and used mainly for Mtb detection in sputum specimens (351). The laboratory at MMH is the only public laboratory performing cytological and histological evaluation of body fluids, aspirates and biopsies. In the year before the current study, the PHL-IdC only received few specimens for mycobacterial culturing sampled from extrapulmonary sites. Thus, prior to the present study, the diagnosis of EPTB in Zanzibar relied almost entirely on clinical presumptive EPTB based on the patients` medical history and signs and symptoms suggestive of EPTB, supported in some patients by diagnostic imaging, mostly x-ray and ultrasound, and cytology/histology results.

3.2 Study design and care delivery pathway

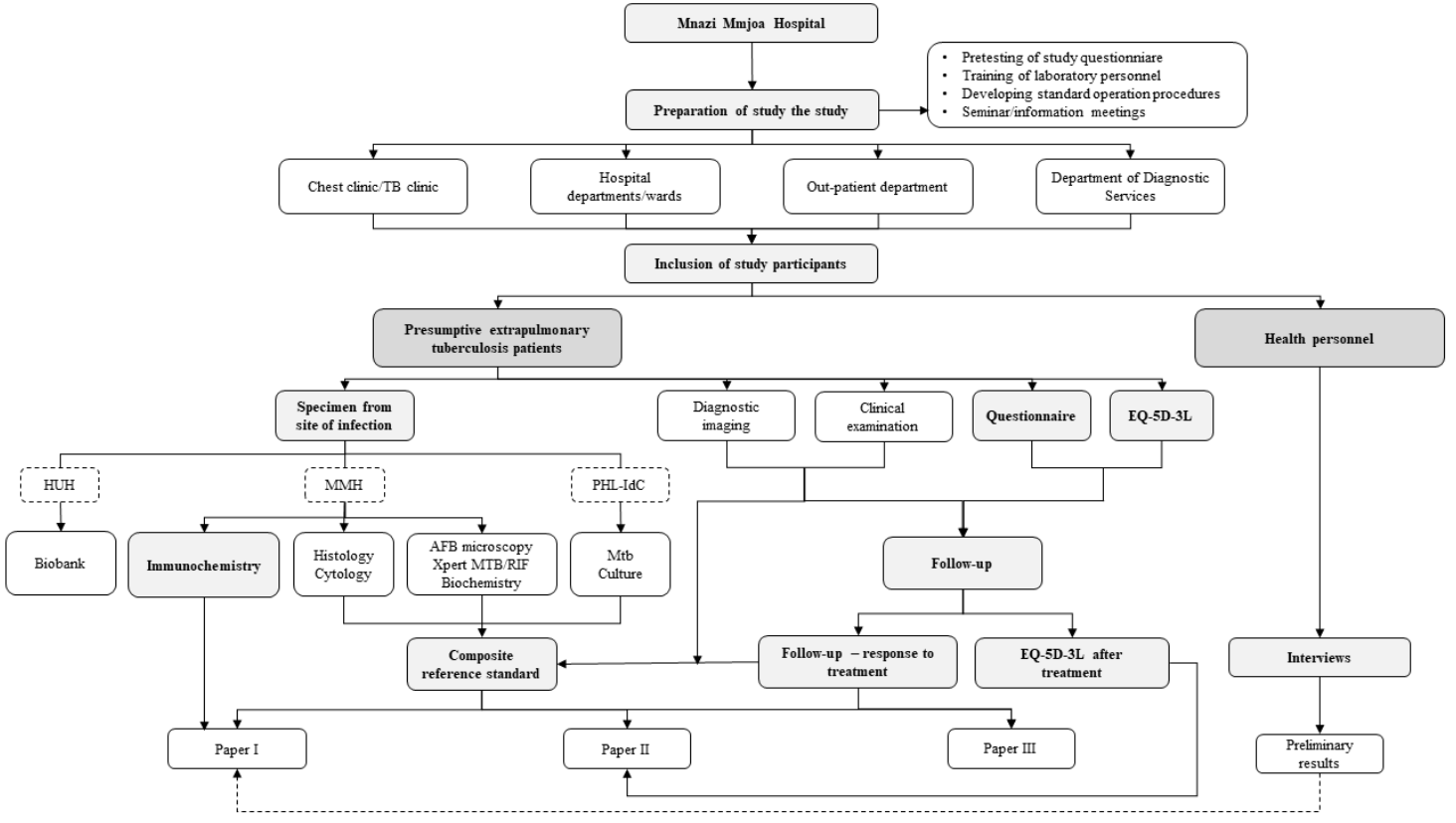

Figure 11. Study design and care delivery pathway. Abbreviations: TB, tuberculosis; HUH, Haukeland University Hospital; MMH, Mnazi Mmoja Hospital; PHL-IdC, Public Health Laboratory-Ivo de Carneri; AFB, acid fast bacilli; Mtb; *Mycobacterium tuberculosis*.

This was a prospective cohort study, consecutively including presumptive EPTB patients from various in- and outpatient departments at MMH hospital. The cohort was examined and interviewed at baseline, before the decision to start anti-TB treatment was reached, and followed until a local clinician decided to initiate anti-TB treatment or an alternative diagnosis was given. The patient group commencing anti-TB treatment was followed until the treatment was completed. A CRS was used to categorize the patients as TB, non-TB and uncategorized cases. Further local health personnel at MMH were interviewed in-depth by the PhD-fellow to get an understanding of the facilitators and challenges associated with the implementation of the MPT64 test and sustainability in the future.

3.2.1 Preparation of the study

The semi-structured study questionnaire (Appendix A) was developed after a literature review of other studies deliberating on similar topics. Most of the questions included were adapted or drawn from previous studies covering the same field of interest. The first English version was translated to Swahili, and then the first Swahili version back to English. The two English versions were then compared, reviewed and discussed with two experts familiar with the research subject and construct of interest to further assess the content validity. Both language versions were evaluated by two bilingual Zanzibaris and the Swahili version was tested in a pilot-study in May 2014 among inpatients at MMH. This, to further accommodate the language according to the study setting and identify questions that were ambiguous or poorly understood by the respondents.

Standard operating procedures (SOP) for sample processing, storage, ICC and IHC staining (Appendix B) were developed, tested, optimized and adjusted to the specific setting prior to the inclusion of study samples. Two laboratory technologists at MMH were trained in performing the immunostaining technique, and the pathologist in the evaluation of the test and generation of reports. This training was done in collaboration with Muhimbili National Hospital, Dar es Salaam, The United Republic of Tanzania, and HUH, Bergen, Norway. This contributed towards strengthening the local (south-

south) collaboration. After the first training session the PhD-fellow provided continuous training and supervision of the laboratory technologists during the study period to optimize the staining procedure in this specific setting and assuring the quality.

As EPTB can affect all organs we expected to find presumptive EPTB patients hospitalized in the various departments/wards, and in the outpatient Chest/TB clinic, fine-needle aspiration clinic and other outpatient departments. Information meetings were held for the clinicians in the different wards regarding the current study, the new diagnostic test, which specimens to collect and containers/tubes to use for the collected specimens and where to send the specimens. This information was repeated several times to the various wards during the study, to account for new staff members and rotation of clinician to other wards.

AFB microscopy and cytological/histological evaluation of tissue/fluid samples were already established at MMH before the study. Mtb detection in sputum using the Xpert assay was also in place and was expanded to include extrapulmonary specimens during the study period. The extrapulmonary specimens were stored, prepared and analysed according to the WHO recommended protocol (167). The national TB reference laboratory, PHL-IdC, scarcely cultured extrapulmonary specimens prior to the study. An agreement was therefore established for transportation and culturing of extrapulmonary specimens during the current study.

3.3 Data collection

3.3.1 Study participants and patient interviews

Presumptive EPTB cases from various in- and outpatient departments at MMH were invited to participate in the study. The inclusions criteria were; clinical presumptive EPTB and written informed consent and a specimen sampled and sent for laboratory investigations from the presumptive site of EPTB infection. The exclusion criterion was anti-TB treatment during the previous year. Those fulfilling the criteria, were consecutively enrolled during thirteen months from August 2014 until the end of August 2015.

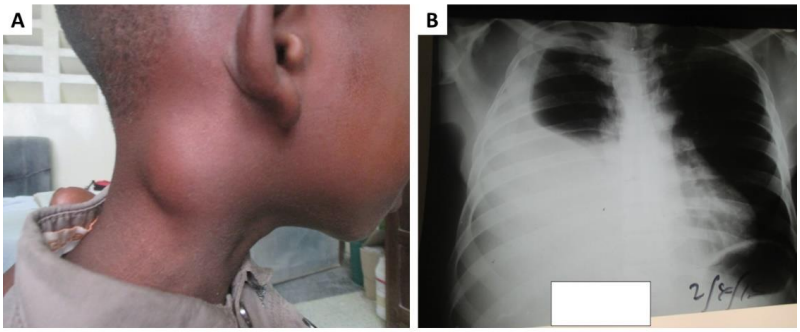

Figure 12. Study participants: presumptive EPTB patients. Picture A, presumptive TB lymphadenitis; Picture B, chest x-ray of a patient with presumptive TB pleuritis. Photo: Melissa Davidsen Jørstad.

At the time of inclusion all patients were interviewed face-to-face at the MMH using the semi-structured questionnaire. All interviews were conducted in the local language, Swahili by two local clinicians (study clinicians), fluent in both Swahili and English. The interviewers had received prior training in the data collection by the PhD-fellow. If possible, the interviews were performed in a private room. The answers were handwritten by the interviewer in the patient`s study folder. A separate folder was used for each patient throughout the study. TB registers, TB treatment cards and patient medical records were used to countercheck some of the information given.

The EQ-5D 3 level version (EQ-5D-3L), developed by the EuroQol Group (331), was used to assess the self-rated health status of the included adult EPTB patients before and after anti-TB treatment to evaluate the impact of treatment on self-reported health. The instrument was provided in Swahili and instructions were written in Swahili, and in addition, orally explained by the study clinician.

Figure 13. Patient interviews at the time of inclusion. Picture C, Dr Hasnu Makame Mwazini, one of the study clinicians performing a patient interview (reprinted with permission). Photo: Melissa Davidsen Jørstad.

At baseline a full physical examination was performed and recorded in a standardized form in the patient's study folder. Lymphadenopathy was drawn onto a figure (Figure 14), and number and size of the lymph node enlargements, determined using an eye estimate or measuring tape when available, were carefully recorded. The physical examination was conducted by one of the study clinicians together with the PhD-fellow.

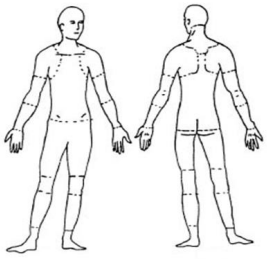

Figure 14. Schematic drawing used for documentation of lymphadenopathy at the time of inclusion.

3.3.2 Biological specimens and sample processing

At inclusion biological specimens were collected from the presumptive sites of EPTB infection. Effusions and CSF were aspirated aseptically into sterile tubes. FNAC was performed using 23-g needle from palpable lymph nodes. Surgical tissue biopsies were immediately divided equally, in the operating room, in two halves, one half was

fixated in 4 % phosphate-buffered formaldehyde solution and the other half stored in 0.9% saline for mycobacterial culture.

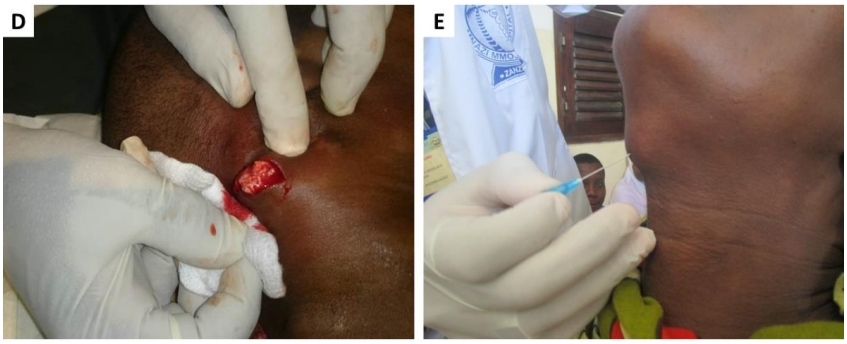

Figure 15. Collection of biological specimens at the time of inclusion. Picture D, surgical biopsy; Picture E, fine needle aspiration cytology from a lymph node. Photo: Melissa Davidsen Jørstad.

Smears from fine-needle aspirates were prepared bedside, two for ICC staining (air-dried and alcohol fixated before transportation to the laboratory), and one each for AFB microscopy and cytology. Further, sterile 0.9% saline solution (2 ml) was used to rinse the needle and the fluid divided equally in two tubes, for mycobacterial culture and the Xpert assay. The formalin-fixed biopsies were embedded into paraffin blocks, and 5µm thick sections were obtained from the blocks using a sliding microtome. Effusions, CSF and pus/abscess material were sent directly to the laboratory in tubes, each specimen was divided in three to four tubes (or collected in three to four tubes if enough material), for mycobacterial culture, Xpert assay, microbiological culture, Gram stain or other special stains depending on the clinical presentation, cytology and ICC staining. Smears for cytology and ICC staining were made from the sediment after the tube had been processed by centrifugation.

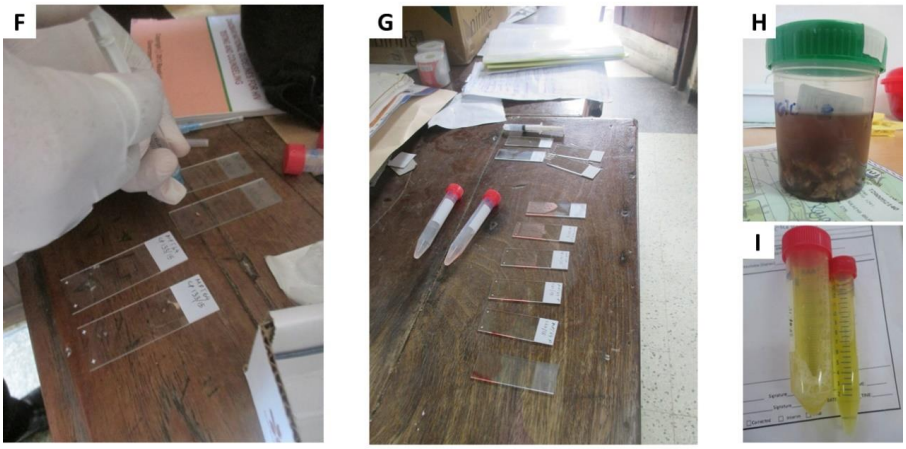

Figure 16. **Sample processing**. Picture F and G, smears from fine-needle aspirates prepared bedside; Picture H, formalin-fixed biopsy, Picture I, pleural effusion. Photo: Melissa Davidsen Jørstad.

3.3.3 Diagnostic laboratory procedures

3.3.3.1 Acid fast bacilli microscopy

The ZN staining technique was used to detect AFB by microscopy in smears and tissue sections from biopsies according to the standard SOPs at MMH.

3.3.3.2 Mycobacterial Culture

Specimens for mycobacterial culture was transported to PHL-IdC, on Pemba Island. At PHL-IdC the samples were centrifuged at 5040 rpm for 15 minutes, the supernatant was discarded and 100 µl of the sediment was inoculated into LJ medium. The remaining sediment was stored and examined after 1-2 days for contamination. If contaminated, the specimen was decontaminated with 4% sodium hydroxide for 15 minutes, the suspension neutralized with phosphate buffer, centrifuged at 5040 rpm for 15 minutes, and 100 µl of the sediment inoculated on LJ medium. The inoculated tubes were incubated at 36.1°C and examined for growth of mycobacteria weekly for 8 weeks. For tubes identified as positive (creamy colonies), the presence of mycobacteria was confirmed by ZN smear. Positive cultures were sent to the CTRL, Muhimbili National Hospital, Dar es Salaam, for species identification and DST. The

specimens were stored at MMH at 4°C and shipped by air to PHL-IdC. Since transportation of extrapulmonary samples from MMH to PHL-IdC was not generally done in routine TB diagnostics some specimens were not sent for culture, further, shipment was occasionally delayed, and a few samples were sent for culture but for unknown reasons results were missing.

3.3.3.3 Xpert® MTB/RIF

Before the current study, the Xpert assay was used in routine TB diagnostics preferably for detection of Mtb in sputum specimen. During the study, SOPs for processing of extrapulmonary samples was developed by the laboratory personnel at MMH based on the WHO recommend protocol (167). The Xpert assay was performed in aspirates and fluid samples, not in biopsies. Shortly, Xpert MTB/RIF sample reagent was added to the specimen container in a 2:1 ratio. The container was shaken for ≥ 10 seconds, incubated at room temperature for 10 min, shaken again for ≥ 10 seconds and incubated for 5 min. Then, 2 ml of the processed sample was transferred to the Xpert MTB/RIF cartridge. The cartridge was loaded into and the specimen analyzed by the GeneXpert instrument according to instructions from the manufacturer. For CSF, equivalent volume of sample reagent was added if CSF volume was 1-5 ml, if CSF volume was < 1 ml, sample reagent was added to a total volume of 2 ml. The sample mixture (2 ml) was then added to the Xpert MTB/RIF cartridge and loaded into the instrument. As recommended, the specimens were stored at 4°C and processed within 7 days (167). Due to scarcity in the availability of cartridges for some time periods during the study, sputum samples had to be prioritized and thus many of the extrapulmonary samples were not examined with the Xpert assay.

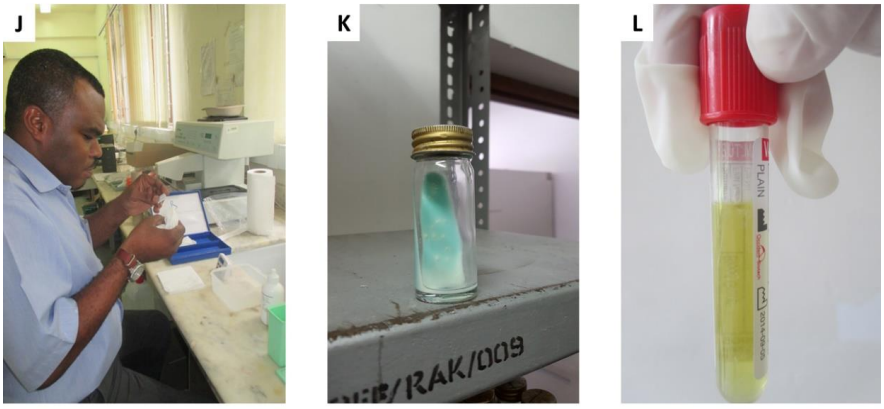

Figure 17. **Diagnostic procedures.** Picture J, technologist performing immunostaining (reprinted with permission); Picture K, mycobacterial culture on Lowenstein-Jensen medium; Picture L, supernatant of pleural effusion. Protein and glucose concentrations were estimated in the supernatant. Photo: Melissa Davidsen Jørstad.

3.3.3.4 Histology/cytology

The smears from FNAC, effusions and CSF, and tissue sections from biopsies were stained as per routine with Papanicolaou staining and haematoxylin and eosin staining, respectively. The cytological and histological evaluation was done by the experienced local pathologist. The results from cytology/histology were evaluated as morphological consistent with mycobacterial infection based on specific criteria (Table 3 and 4). In effusions and CSF, smears showing a lymphocytic predominance was noted as suggestive of TB.

Table 3. **Histomorphological features taken as consistent with TB in tissue sections from biopsies**

Granulomas without necrosis
Granulomas with necrosis
Poorly formed granulomas with necrosis
Necrotic material • Suppurative inflammation with necrotic background • Necrosis without granulomas, but with suggestive inflammatory component present • Necrosis only

Table 4. Cytomorphological features taken as consistent with TB in fine-needle aspirates

Granulomatous inflammation without necrosis
Granulomatous inflammation with necrosis
Necrotic material • Suppurative inflammation with necrosis • Lymphoid cells and necrosis • Necrosis only

3.3.3.5 Immunostaining – the MPT64 test

We assessed the reproducibility of the immunostaining procedures in the specific study setting before the inclusion of study specimens. Slides from the same specimen were stained on consecutive days by two laboratory technologists and the slides assessed by different observers (run-to-run reproducibility, inter- and intra-observer reproducibility). Further, several slides from the same specimen was stained in one run (within-run reproducibility). During the study, the laboratory technologists performed the immunostaining two times a week. Cohen`s κ was calculated to determine the inter-observer agreement between the local pathologist at MMH and two independent observers at HUH, Bergen, Norway. Slides were sent to HUH and evaluated separately by two of the PhD-fellow's supervisors (T.M. and L.S.), who both were blinded for the results of the immunostaining performed at MMH.

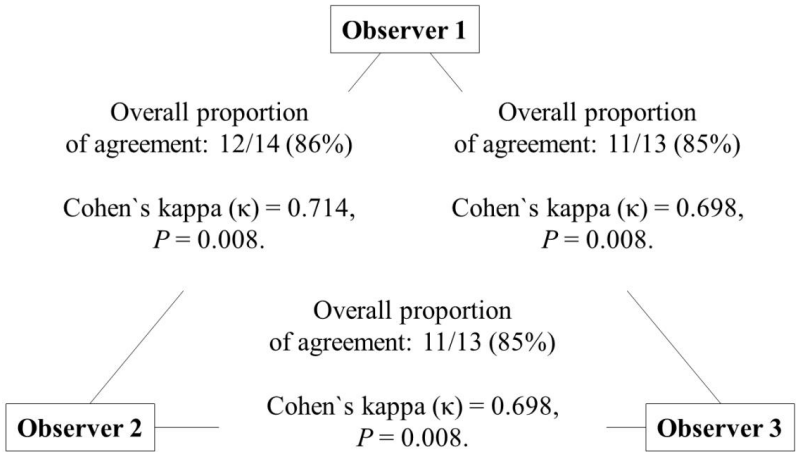

Figure 18. **Inter-observer agreement.** Immunostaining was evaluated by three independent observers. Fourteen slides were assessed by observer 1 and observer 2. Observer 3 evaluated 13 of the same slides.

Two controls were included in each run of the immunostaining. The positive control used were tissue sections form a lung biopsy (Mtb culture positive) provided by the Department of Pathology, HUH, Norway, previously showing consistently positive results with the MTP64 test. As negative control, we used the same specimen omitting the primary antibody in the staining procedure, thus assuring absence of nonspecific binding of the secondary antibody.

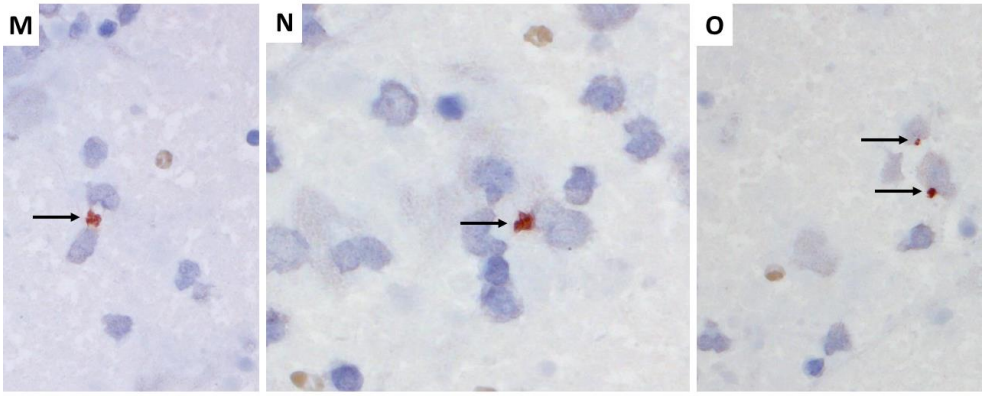

Figure 19. **The MPT64 test - positive signals.** The pictures demonstrate positive signals in FNAC smears from lymph nodes. The MPT64 antigens were stained red-coloured and seen as intracytoplasmic granular spots or lying extracellular in necrotic area. Photo: Melissa Davidsen Jørstad.

3.3.3.6 Turnaround time of immunostaining and Mtb culture

The time-to-results of immunostaining and mycobacterial culture were documented (Figure 20). The total median time, from specimen collection until the results were available to the clinician was 7.5 days (interquartile range (IQR) 6-13 days) for immunostaining and 64 days (IQR 62-69 days) for mycobacterial culture.

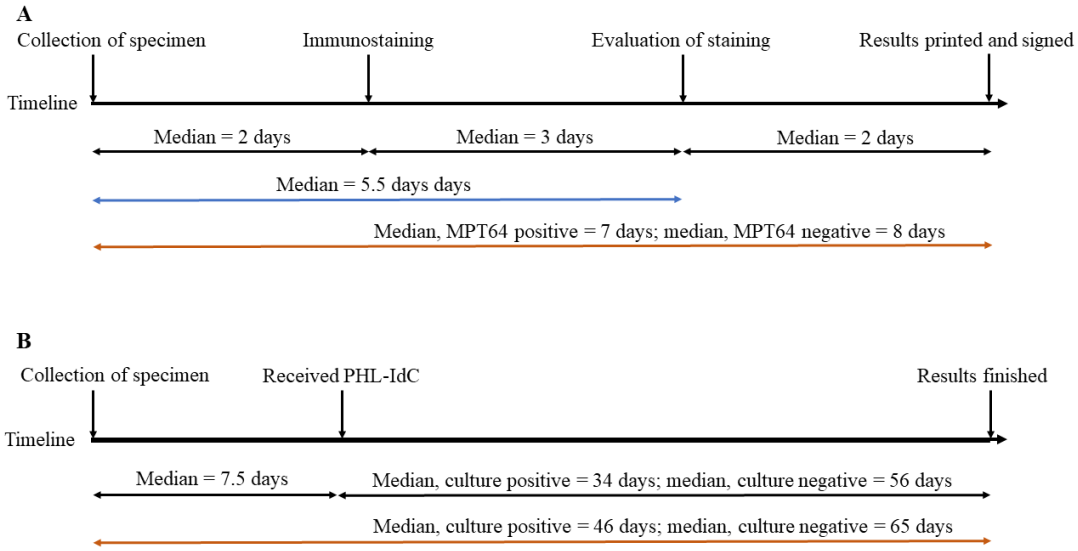

Figure 20. Turnaround time for the MPT64 test and *Mycobacterium tuberculosis* culture. A: MPT64 test. B: Mycobacterial culture. Abbreviation: PHL-IdC, Public Health Laboratory-Ivo de Carneri.

3.3.4 Clinical follow-up of patients

All patients were followed until a decision was reached by the local clinician to initiate anti-TB treatment. The alternative diagnoses were noted among those not starting anti-TB treatment. The patients commencing anti-TB treatment were followed on at least two occasion; 1) after the intensive phase of treatment; 2) after treatment was completed. At follow-up visits the patients were examined by one of the study clinicians together with the PhD-fellow. A medical history was taken, and a physical examination was conducted. All reported symptoms and signs and objective findings were described in the patient's study folder, including weight, lymph nodes measures

and results of diagnostic imaging if available. Weight changes between baseline and follow-up visits were recorded. The longest diameter of the largest lymph node swelling was used when evaluating regression of lymph nodes at two intervals; 1) between baseline and 2 months of anti-TB treatment, 2) between 2 months of anti-TB treatment and treatment completion. Adult patients on anti-TB treatment were asked to complete the EQ-5D-3L at treatment completion.

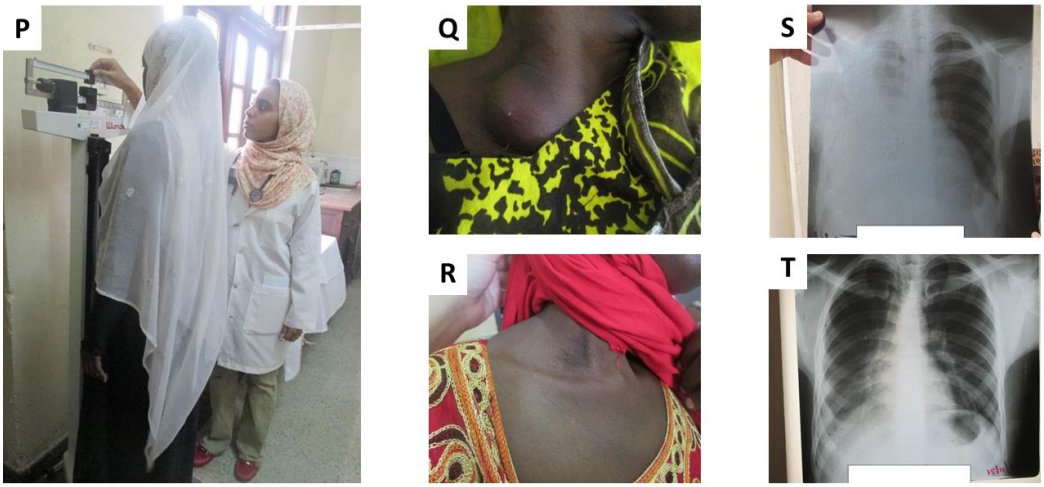

Figure 21. Patient follow-up during treatment. Picture P, documentation of weight at follow-up (reprinted with permission); Picture Q shows a patient with TB lymphadenitis before the initiation of anti-TB treatment and picture R presents the same patient after 6 months of treatment. Picture S shows a chest x-ray in a TB pleuritis case at the time of commencing anti-TB treatment and picture T is a chest x-ray in the same patient after 4 months of anti-TB treatment. Photo: Melissa Davidsen Jørstad.

3.3.5 In-depth interviews of health personnel

Local health personnel working in the National TB programme and various departments at the MMH were purposively selected for in-depth interviews. This sampling strategy was chosen to cover the spectrum of health care workers being involved in the implementation of the new diagnostic test. In-depth interviews were performed as audio-recorded, face-to-face interviews, using a semi-structured interview guide. The interview guide was developed, based on field experience of the

PhD-fellow and discussion with co-authors, and comprised open and close-ended questions. The interviewer could change the order of the questions and rephrase or clarify confusing questions or ask follow-up questions not included in the interview guide to further investigate topics introduced by the respondent. All interviews were conducted in English by the PhD-fellow. The duration of each interview was between 30-60 minutes. The interviews were carried out in various private offices at the study site.

A qualitative researcher transcribed the interviews verbatim in English. When the transcriber experienced unclear conversation, the transcriber left an open field in the sentence. The audio-recordings and transcripts were double-checked by the interviewer, and together with consulting the field notes, unclear words/phrases were filled. The transcribed data was read, and re-read, together with listening to the audio-recordings to get familiar with the data. One interview was coded manually by the PhD-fellow using OpenCode version 4.02 and a list of codes was developed from the first interview. Two other co-authors read the first interview and assessed the developed codes, followed by a thorough discussion of the content and codes in the research team to resolve ambiguities. Subsequently, the remaining interviews were coded, and the coding list were revised and expanded if new key issues emerged from reading the transcripts.

3.4 Definitions

TB case definition

The study participants were categorized after the study as TB cases (confirmed, probable or possible) or non-TB cases using a CRS as described in paper I. The combination of the various diagnostic criteria defining each CRS category is presented in table 5. Patients not fulfilling the definition as a TB or non-TB case were defined as uncategorized cases and excluded from further analyses in paper I and III. In paper II, a less strict approach was used, and the uncategorized cases in paper I and III were defined as TB cases if the local clinician had decided to initiate a full course of anti-

TB treatment based on clinical presumptive EPTB and as a non-TB case if anti-TB treatment had not been initiated.

Table 5. Algorithm for defining categories of the composite reference standard

	Results							
CRS category	**Symptoms/ signs**	**Culture**	**Xpert**	**AFB smear**	**Radiology[a]**	**Histology/ cytology[b]**	**Concomitant PTB**	**Follow-up[c]**
Confirmed TB	+	+	+/-	+/-	+/-	+/-	+/-	*
	+	+/-	+	+/-	+/-	+/-	+/-	*
Probable TB	+	-	-	+	+/-	+/-	-	+
	+	-	-	+/-	+	+/-	-	+
	+	-	-	+/-	+/-	+	-	+
	+	-	-	+	+/-	+/-	+	*
	+	-	-	+/-	+	+/-	+	*
	+	-	-	+/-	+/-	+	+	*
Possible TB	+	-	-	+	+/-	+/-	-	**
	+	-	-	+/-	+	+/-	-	**
	+	-	-	+/-	+/-	+	-	**
	+	-	-	-	-	-	-	+
Non-TB	+	-	-	+/-	+/-	+/-	-	***

[a] Positive: presence of pleural/peritoneal effusion, signs of skeletal TB, infiltrates/nodules consistent with tuberculosis.

[b] Positive: cytology/histology shows morphological features consistent with tuberculosis, effusion/CSF shows lymphocytosis on fluid cytology and protein level > 3 g/dl (> 1 g/l for CSF).

[c] Response to antituberculosis treatment evaluated at 2-3 months and treatment completion – response to treatment is marked with a positive sign.

* Response to treatment not included in this definition.

** Patients died or were lost to follow.

*** Patients improving without anti-TB treatment or cytology/histology concluded other diagnosis than TB or alternative diagnosis concluded by the local clinician or non-response to anti-TB treatment.

Abbreviations: CRS, composite reference standard; Xpert, Xpert® MTB/RIF assay, AFB, acid fast bacilli; PTB, pulmonary tuberculosis; TB, tuberculosis

Definitions of delay (Paper II)

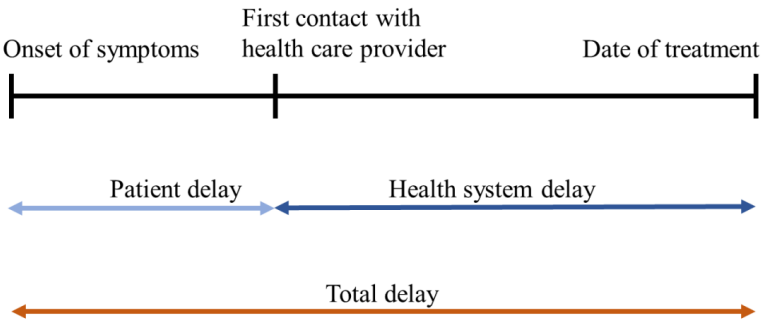

Figure 22. Chart showing the different delays. All modern health facilities, both in the public and the private sector, and registered pharmacies were defined as health care providers, thus excluding non-formal health care providers such as over-the counter drug stores and traditional healers.

3.5 Statistical analysis

The Chi-square test was used in all papers to compare group differences in categorical variables. In paper I, cross-tabulations were used to evaluate sensitivity and specificity with 95% confidence intervals, further, positive predictive and negative predictive values and accuracy. In paper II and III, non-parametric tests were used to evaluate group differences of continuous variables, the Mann-Whitney test for two-group comparison, the Kruskal-Wallis test for more than two groups and the Wilcoxon-signed rank test when comparing related samples. The significance level (α) was set to 0.05 in all analyses. In paper II and III, due to multiple testing, Bonferroni correction was applied to the significance level (α = 0.05/number of tests).

3.6 Ethical considerations

Ethical clearance was secured from the Regional Committee for Medical and Health Research Ethics (REK), Western-Norway (2014/46/REK vest) and from the local authorities in Zanzibar, the Zanzibar Medical Research and Ethics Committee (ZAMREC) (ZAMREC/0001/MAY/014) before the recruitment of study participant as

mentioned in papers I-III. The study participants gave written consent before being included in the study, and were additionally asked to sign a separate consent for the transfer of collected biological samples and de-identified data to HUH, Bergen, Norway. The samples were stored in an approved biobank (Project number NSD no 12032) for future research studies. A material transfer agreement for the transferal of biological material was established between MMH and HUH, and a permission to transport the biological samples was received from MoH, Revolutionary Government of Zanzibar. Permission to publish data from the project was also obtained from ZAMREC.

For in-depth interviews, all participants provided informed written consent before the interview. The participants were given the opportunity to read through the transcripts of their interview and give comments and/or corrections. Further, the participants will be given the possibility of reading the final manuscript before it is submitted for publication and give comments.

4. SUMMARY OF RESULTS

4.1 MPT64 antigen detection test improves routine diagnosis of extrapulmonary tuberculosis in a low-resource setting: A study from the tertiary care hospital in Zanzibar (Paper I)

Jorstad MD, Marijani M, Dyrhol-Riise AM, Sviland L, Mustafa T. PLoS One. 2018; 13(5):e0196723.

In total, 132 patients with presumptive EPTB were included at MMH and categorized as TB cases (confirmed TB, n = 12; probable TB, n = 34; possible TB, n=18), non- TB cases (n = 62) and as uncategorized cases (n = 6). Only categorized patients were included in the analyses. The specimens were FNAC from lymph nodes (n = 66), effusions (pleural, n=31; ascites, n=16; pericardial, n=1), CSF (n = 8), biopsies (n = 21) and pus/abscess material (n = 2).

Among specimens collected from the TB cases the MPT64 test showed the highest proportion of positive results (45/69, 65%), compared to ZN staining (8/69, 12%), culture (8/60, 13%) and the Xpert assay (6/38, 16%). Further, the MPT64 test demonstrated 81% positivity in specimens subjected to all four diagnostic tests. The MPT64 test was positive in 6/8 (75%) culture positive samples and in all Xpert assay and/or ZN positive samples. Compared to the CRS the MPT64 test showed an overall sensitivity and specificity of 69% and 95%, respectively, and the accuracy was 82%. The best test performance was found in TB lymphadenitis, with sensitivity, specificity and accuracy of 79%, 97% and 88%. Further, the sensitivity was significantly higher in paediatric TB compared to TB in adults (sensitivity, 100% versus 58%).

Cytomorphological features consistent with TB were present in only 56% of the TB lymphadenitis FNAC specimens (n = 34). In TB specimens without cytomorphology consistent with TB, the MPT64 test, ZN staining, culture, and the Xpert assay was positive in 12/15 (80%), 1/15 (7%), 1/15 (7%) and 2/10 (20%), respectively.

4.2 Implementation of the MPT64 test for diagnosing extrapulmonary tuberculosis: facilitators and challenges

(partly included in paper I, and unpublished)

A total of 9 in-depth interviews were conducted. All the informants were male, and the age range was from 28 to 53 years

The data analysis of this study is not yet completed. The preliminary results show that the participants identified the need for improving the diagnosis of EPTB. Lack of trained personnel to perform invasive sampling, costs involved in laboratory investigations, procurement and availability of reagents and equipment, and challenges associated with changing the practice of empirical treatment were identified as some of the main challenges associated with the long-term sustainability of the test. Regular information and education in Swahili, improving the collaboration and communication between the hospital wards, the TB programme and the laboratory, guideline on what samples to collect and where to send the samples when EPTB is a differential diagnosis, and short turnaround time of the diagnostic test, were some of the facilitators mentioned by the study participants.

4.3 Diagnostic delay in extrapulmonary tuberculosis and impact on patient morbidity: A study from Zanzibar (Paper II)

Jorstad MD, Aßmus J, Marijani M, Sviland L, Mustafa T. *PLoS One.* 2018;13(9):e0203593

Of 146 identified patients presenting with clinical presumptive EPTB at MMH, 132 patients were included, and categorized as TB cases (n = 69) and non-TB cases (n = 63). We evaluated care seeking pathways and behaviour among these patients. Most patients reported MMH (30%), PHCUs (24%) or private clinics/dispensaries (17%) as their first contact with a HCP after the onset of the current illness. Forty-six percent reported self-medication before care-seeking. When excluding cases only visiting MMH, repeated visits to the same health care level before being referred to MMH was reported among 45/99 (45%) of the patients. Among the TB cases, 56/69 (81%) reported repeated consultations at MMH, 31/69 (48%) described ≥ 4 visits to any HCPs and 18/69 (26%) had been in contacting with ≥ 3 different HCPs before anti-TB treatment was initiated.

In TB cases, the median total, patient and health system delay were, 62 days (IQR, 31-126 days), 14 days (IQR, 5-28 days) and 34 days (IQR, 19-76 days), respectively. In comparison to other extrapulmonary sites of disease, TB lymphadenitis cases reported both a significantly longer patient and total delay. A delay exceeding > 6 months was reported among 26% of the lymphadenitis patients. A shorter health system delay was experienced by patients initially seeking care at MMH and among patients with ≤ 3 health care visits.

The general PTB knowledge among the respondents was relatively good, but EPTB knowledge was low, only one respondent answered an extrapulmonary site of TB disease.

Among the adult TB cases 39/47 (83%) experienced that the ongoing illness affected their working capacity. The median reported days of reduced capacity or stopped working altogether was 60 days (IQR, 21-90 days). Assessing the self-rated health status before and after anti-TB treatment showed that even though the TB

lymphadenitis patients reported better EQ VAS scores (lymphadenitis, median 79%; other sites, median 50%) and lesser problems on the EQ-5D descriptive system at baseline as compared to patients with other disease sites, both groups reported significantly higher EQ VAS scores (lymphadenitis, median 96%; other sites, median 94%) and described lesser problems after treatment compared to baseline.

4.4 Evaluation of treatment response in extrapulmonary tuberculosis in a low-resource setting (Paper III)

Jorstad MD, Dyrhol-Riise AM, Aßmus J, Marijani M, Sviland L, Mustafa T. *BMC Infectious Diseases2019; 19:426*

In this study the 62 non-TB cases and 64 TB cases were included. The presumptive site of EPTB infection were lymphadenitis (n = 67), pleuritis (n = 31), peritonitis (n = 16), meningitis (n = 8), spondylitis/osteomyelitis (n = 2), pericarditis (n = 1) and mastitis (n = 1). Local symptoms were described by all patients, while constitutional symptoms were reported by 73% of the patients. There was no difference between TB and non-TB patients regarding the type and frequencies of constitutional symptoms.

Among TB cases, 52/64 (81%) patients were followed during the course treatment. After 2 months of anti-TB treatment an overall clinical improvement was reported in all patients, and regression of lymphadenopathy and pleural effusion was reported in the majority of patients. The weight increased with ≥5% in 36/49 (73%) after 2 months of treatment, and in 38/46 (83%) of the TB patients after finishing treatment.

We suggest that a combination of three clinical parameters; clinical improvement, weight gain and regression of objective findings, can be incorporated in a simple assessment tool and used in the evaluation of treatment response in EPTB patients. Among the TB patients 47/48 (98%) fulfilled ≥2 parameters at 2 months as compared to 0 or 1 parameter among non-TB patients (n=7). A simple tool to assess treatment response is feasible for implementation in low-resource settings with the potential of improved patient management in this group of patients.

5. DISCUSSION

5.1 Methodological considerations

5.1.1 Study design, study population and follow-up period

This study was designed as a prospective cohort study, where the study participants were consecutively included, followed and observed during anti-TB treatment or until alternative diagnoses were concluded. The enrolled study subjects were categorized as TB or non-TB patients based on a set of criteria, the CRS. The STARD checklist giving guidelines for the reporting of diagnostic accuracy studies (342) were followed as closely as possible in presenting the evaluation of the performance of the MPT64 test (paper I). Further, data collected at baseline and follow-up visits, such as weight, regression of clinical findings and responses using the EQ-5D-3L instrument as a measure of self-reported health status were presented (Paper II and III). Even though the study was designed as a prospective cohort, some data gathered at baseline using the study questionnaire included retrospective data regarding variables such as health care seeking pathways and symptoms onset. One of the strengths of our prospective cohort study design is that all the patients went through the same interview (using the study questionnaire), clinical examination and laboratory investigations irrespective of their final diagnosis as TB or non-TB cases. The limitation with this design is that it was time consuming and some of the study subjects were lost to follow or died before follow-up.

5.1.1.1 Recruitment of patients

Even though our recruitment site was MMH, our aim was to invite all patients presenting with presumptive EPTB at Unguja Island, based on the knowledge during the project planning that all patients with presumptive EPTB at Unguja Island were referred to MMH. However, during the study, we experienced that many patients were not referred to MMH and anti-TB treatment was started outside the hospital, at peripheral PHCU and PHCC. We applied and received the approval from ZAMREC to include patients also from two PHCC, one in the north and one in the south at Unguja island, one private hospital and one military hospital, but logistic challenges made it

difficult to send samples from these sites. As one of the inclusion criteria was collection of a biological specimen from the presumptive site of TB infection, patients without specimens for laboratory investigations were excluded from the study. This led to study participants only being recruited from the various wards and outpatient departments at MMH, and we did not attain our goal of inviting all presumptive EPTB patients at Unguja Island. As a consequence our sample size is smaller than expected.

At MMH, including patients from the fine needle aspirate outpatient clinic and patients who had been referred to the TB clinic were done without major difficulties. It was more challenging to trace and include patients admitted in the hospital. The study researcher went around the hospital several times a week consulting the hospital clinicians if there were any presumptive EPTB patients admitted. In addition, several information meetings regarding the current study and when to suspect EPTB were given on several occasions to the hospital staff. Still, some patients were missed due to various reasons, such as biological samples from the presumptive site of infection were not collected or patients being started on treatment prior to collection of specimens for investigations.

5.1.1.2 Follow-up visits

In our study design, the follow-up of patients commencing anti-TB treatment was planned after the intensive phase (2 months), and completion of treatment. However, it is recommended that that the clinical treatment response should be evaluated after one month among patients starting anti-TB treatment without histology suggestive of TB or bacteriological confirmed TB (91). Since the ZIHTLP offers TB treatment services as DOTS, we assumed that we could obtain information regarding clinical response to treatment after one month from the TB register or TB treatment cards. This proved to be challenging as the information available in the TB register and treatment cards among EPTB cases were limited. In addition, most patients in this setting were choosing community-based DOTS (351), as a result, many of the patients did not visit the hospital for routine follow-up during treatment. Thus, the follow-up data is available for only 2 and 6 months. This is a limitation of the study design and we should have included more frequent follow-ups or organized the follow-up visits

differently, potentially as home visits, at least monthly for the first 3 months of treatment. This would have given the opportunity to evaluate the simple clinical parameters as an assessment tool for response to treatment (paper III) at an earlier time point.

At the time of inclusion, medical history, including direct questions covering symptoms from various organ systems and the onset of symptoms, were included in the study questionnaire. Physical examination was reported using a standardized form formatted as check boxes. At follow-up visits standardized questions or examination forms were not used, and signs, symptoms and findings from the physical examination were instead documented as a continuous text in the patients' study folder. Due to lack of standardized questionnaires and forms during follow-up visits some details of the clinical information could have been missed, and this potentially avoided using such tools. Further, the comparison of symptoms and findings from clinical examinations at baseline and follow-up visits would have been easier using standardized questionnaires and forms at all visits.

5.1.2 Imperfect reference standard and composite reference standard

When evaluating the performance of a new diagnostic test, the results from the new test frequently are compared to a gold standard that label a patient as diseased or non-diseased (354). Ideally, the new diagnostic test should be evaluated against a perfect gold standard, but gold standards are seldom absolute predictors of disease. Using an imperfect reference standard when evaluating a new diagnostic test, potentially lead to misclassification of patients (187, 354), and bias the measures of performance of the new test (354, 355). Culture is still regarded as the gold standard when developing and evaluating new TB diagnostic tools, but is known to be a suboptimal reference standard in the diagnosis of EPTB (10, 187). Given the assumption that the new test rightly identifies TB infection in a culture-negative sample, the result from the new test would be regarded as false positive and underestimate the true specificity of the new test (187). A CSR, combines the result of various imperfect component tests or clinical criteria to define absence or presence of disease (187, 355, 356). This may

reclassify patients evaluated as false positive (classified as non-TB using only culture) as true positives and increase the estimate of the new tests' specificity (187).

To assess the performance of our new test in field conditions, the MPT64 test was evaluated by comparison with a CRS (table 5), dividing the patients in TB and non-TB cases. The CRS used in this project was composed of laboratory diagnostics available in the current setting, imaging and clinical findings and follow-up of patients at certain time points assessing response to treatment or improvement without anti-TB treatment or other specific non-tuberculous therapy. The TB cases was further divided according to the level of certainty of TB disease as confirmed, probable and possible TB cases, representing high, medium and lower certainty of the true TB diagnosis respectively. In the study design all patients were intended to receive the same component tests of the CRS, which would be interpreted and combined in a fixed way for all patients to be categorized according to the CRS criteria. However, diverse challenges led to some samples not being subjected to all the various laboratory component tests, some patients died before follow-up or were lost-to-follow, which may have had an impact on the accuracy of the CRS and on the estimated performance of the new test under evaluation. Further, patients with clinical presumptive EPTB responding to anti-TB treatment were defined as possible TB cases, this does not necessarily give a correct diagnosis, as some patients may have alternative infections responding to anti-TB treatment and thus incorrectly classified as TB cases. There were limited possibilities to perform various investigations to distinguish TB from other infections to secure specificity. On the other hand, poor treatment response can be due to drug resistant TB, poor compliance or other intercurrent conditions, potentially leading to the misclassification of true TB cases.

5.1.3 Reliability and validity

5.1.3.1 The study questionnaire

The content validity of the study questionnaire was properly evaluated, however the reliability of the questionnaire was not assessed. The test-retest reliability (stability) of the study questionnaire, that is assessing whether the same respondent would produce consistent answers if the questionnaire was administered repeatedly at two distinct

occasions, or the inter-rater reliability, referring to the consistency of re-interviewing the same respondents by two distinct interviewers (357), was not evaluated. This can be seen as a limitation. The test-retest and inter-rater reliability can depend on the complexity or ambiguity of the questions and the interviewer`s skills. However, all the interviews were conducted by one of two well trained medical doctors. In addition, most of the questions included in the questionnaire were relatively simple and close-ended, offering specific feedback regarding focused areas, limiting the possible responses and making the statistical analysis easier. Still, some open-ended questions were included to give the researcher the opportunity of more insight into some areas.

5.1.3.2 Information bias

In our study, self-reported data during interviews using the study questionnaire, promote the possibility of interviewer and self-reporting bias, both forms of information bias. Interviewer bias, is where the interviewer through behavioural and personal characteristics and interviewing technique and skills can influence the interview process and the answers given by the respondents (358). The interviewers in our study were both local medical doctors, familiar with the study and subject areas, who received training prior to conducting the face-to-face interviews. Further, as the study questionnaire was mainly structured, where the interviewers asked standardized questions, committed to the settled structure and order of the questions with minimal opportunity to alter the content we tried to minimize the possibility of interviewer bias. Self-reporting bias, is a common problem in observational study designs (359). Using face-to-face interviews and questionnaires to collect data, the respondents may give information that they perceive as more socially acceptable, i.e. social desirability bias (358, 359). This can specially influence subjects perceived as sensitive or shameful. Assessing the questions in the questionnaire, self-reported HIV status and visiting traditional healers can fall into this category. In the cohort the HIV status was known in 99/132 (75%) (information retrieved from TB register books and patient hospital files), 22/99 (22%) were HIV positive and 77/99 (78%) HIV negative. Among these, 16/99 (16%) reported being HIV positive, 48/99 (48%) being HIV negative and 35/99 (35%) did not know their HIV status. In addition to patients being hesitant to divulge their HIV status, an alternative and plausible explanation for the discrepancy between

self-reported HIV status and actual HIV status can be due to some patients being tested for HIV after the study baseline interviews were performed. Another potential sensitive subject is consulting traditional healers instead of or in addition to modern health care facilities. Zanzibar has a long historical use of various forms of traditional medicine, and reports have stated that most of the population use traditional medicine and seek traditional health care for prevention and treatment of various diseases (360, 361). In 2012 it was estimated that around 800 traditional healers' practiced in Zanzibar (361). Among our cohort, 12/132 (9%) of the respondents reported having visited a traditional healer during the care seeking period. This is a lower proportion than reported in two studies from the Tanzanian mainland. In one study, including smear positive PTB patients from 6 districts in various regions, 20% of the respondents described seeing a traditional healer prior to receiving their diagnosis (311). Whereas a study in Mwanza region, among smear positive PTB cases, reported that 38.9% of the respondents had first consulted a traditional healer after symptom onset (309). It is thus plausible to assume that the responses regarding traditional healers among our cohort can be biased, conceivably due to some respondents being reluctant to disclose consulting traditional healers to the professional HCPs.

Another form of self-reporting bias is recall bias, where the respondents provide erroneously responses due to a dependence on her/his ability to remember past events, resulting in recall error (359). The range of the recall period and acute versus insidious onset of symptoms can influence recall bias, where a shorter recall period is preferred, and an acute onset of symptoms is apparently easier to remember than subtle symptom onset. In the study questionnaire, the information requested regarding variables such as symptoms, symptom onset and duration, first HCP consulted and number of HCPs visited, where the data was used to estimate the various delays and assess health seeking trajectories (Paper II), relied to a great extent on the study participants recalling previous events. Measures to reduce recall bias can be to use validated and well-structured instruments for data collection, gain information from alternative sources or helping the patients remember the date of events by using a local calendar with holidays and happenings. Among the study cohort, information gathered in the questionnaires was counterchecked using the patients` hospital files and health books

(each patient usually has their own notebook where the clinician write handwritten notes during visits to outpatient clinics or PHCU), TB treatment cards and laboratory reports, when these sources were available. Still, there is a possibility of recall bias influencing the results. Other means of minimising recall bias are including patients with new onset of symptoms, but this was not feasible in the current project since many patients had been to various peripheral health care units before being referred to MMH.

5.1.3.3 Selection bias

We aimed to include patients with all forms of EPTB disease from the whole island, however we had fewer presumptive TB patients as mentioned in section 5.1.1.1 adding a selection bias. Furthermore, all forms of EPTB were not included in the study population. Only 13 of the included patients had presumptive bone, pericardial or neurological TB, and none had genitourinary tract TB. This could be due to the inclusion criteria of only including those patients with a representative biological sample. Another challenge was that we quickly experienced that some patients could not afford the laboratory investigations and diagnostic imaging included in a routine diagnostic work-up. It was thus decided that the research project would fund all the investigations and diagnostic work-up of the invited study participant. To overcome the ethical issue of patients feeling pressured to participate in the study to get all expenses covered, we chose to fund all invited patients, regardless of their decision to participate or not. We identified and invited 146 patients, where 132 were enrolled in the study. Among those excluded, 3 patients had been given empirical anti-TB treatment the last year, in 6 a representative sample was not obtained and 5 patients did not consent to participate. Patients lost to follow can also introduce bias if they somehow differ from the patients remaining in the study. Among the 64 patients defined as TB cases in Paper I and III, 10 patients died before follow-up (2 culture positive EPTB patients died before starting anti-TB treatment, 8 died during anti-TB treatment) and 2 patients were lost to follow.

5.1.3.4 External validity

Since the inclusion of the study participants only took place at one hospital, we potentially missed the patients only visiting other health care units. MMH is primarily

a third level referral hospital, and even though the hospital was also providing primary and secondary health care services to some of the district during the project period, one cannot disregard that there is a possibility that patients being referred to the hospital had a more advanced disease state than patients seen and treated in peripheral health care units. One the other hand, one could assume that some patients were referred to MMH for diagnostics, who before the study would have been started on empirical treatment outside the hospital. This could have been due to the information given to TB programme health personnel regarding the current TB project.

To get an impression whether our included study participants represented the intended study population it would be valuable to know how many of the anticipated study population were included in the study and evaluate eventual differences among the included and not included patients. In this respect, finding the number of patients with presumptive EPTB, where another diagnosis was concluded, and thus not initiating anti-TB treatment (Group A in figure 23), was not doable. On the other hand, it was possible to receive an overview of the presumptive EPTB patients initiating anti-TB treatment and by that registered in the TB register during the inclusion period. In retrospect, a review of the manually written TB registration books from Unguja TB districts was performed. Available features among the TB patients not included (Group C, figure 23) and the TB cases included in the study were compared (Group D, figure 23).

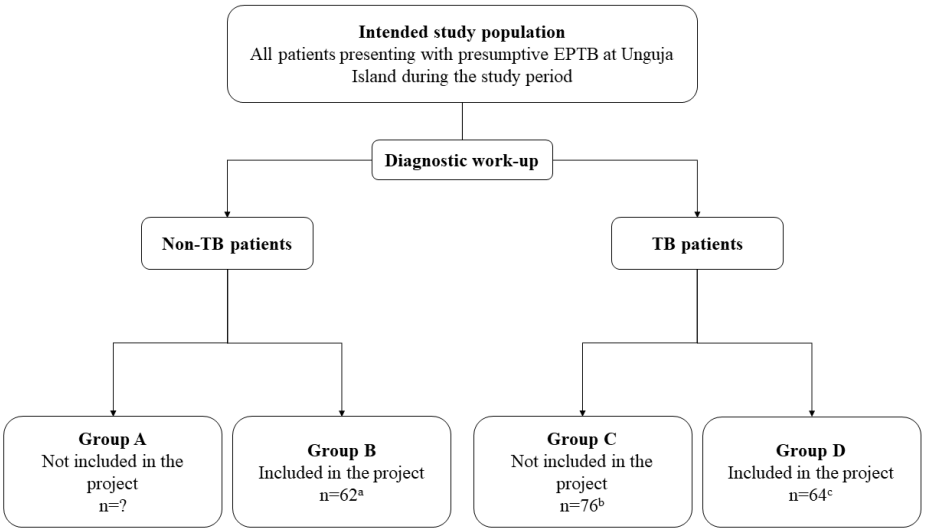

Figure 23. The intended study population. [a]Patients defined as non-TB cases in paper I and III. [b]TB cases not included in the project, only reported as EPTB cases in TB registration books at Unguja Island during the project period. [c]Patients defined as TB cases in paper I and III. Abbreviations: EPTB, extrapulmonal tuberculosis; TB, tuberculosis.

In the current project period, 140 patients were registered as EPTB patients in the TB registration books at Unguja Island. Of these, 76 cases were not included in the study (Group C in figure 23). The median age was similar among the study participants and patients not included in the study (included cases, median age 25 years, IQR 7.5-38; not included cases, median age 25.5 years, IQR 6-46.8). The two groups did not differ significantly with regard to sex, HIV status or residence, but there was a significant difference in the site of infection. Among the included patients the most frequent registered sites of infection were lymphadenitis (53%), pleuritis (31%) and abdominal TB (9%), whereas among the TB patients not included in the study, no site registered (43%) and pleuritis (38%) were most commonly reported.

The patients included in this project are thus a subgroup of patients presenting with presumptive EPTB at Unguja Island. Consequently, the study results may not necessarily be generalizable to all the EPTB patients in Zanzibar and should be interpreted in the given context.

5.1.3.5 Controls for immunostaining

In the current study, we used biopsy tissue sections as controls for both the IHC and the ICC staining procedures due to limited numbers of cell smears which could be used as controls. Using biopsy tissue sections as controls for ICC staining is suboptimal as tissue sections and smears from fluid material have different sample processing and fixation steps, leading to some variation between the IHC and ICC staining protocols. In addition to only having tissue sections as controls, some of the staining procedures were run without controls due to limited access. Our intention was to use material from our own study samples or specimens from the local laboratory as controls during the study. However, we faced challenges in having access to controls as few non-sputum samples had been sent for Mtb culture prior to the study, further, only a minority of the study samples were reported as Mtb culture positive. Among the 8 Mtb culture positive study samples none were applicable as a control specimen. The given context is a limitation of the study, and may potentially have led to improper interpretation of the staining in some runs of immunostaining. However, we are reasonably confident about the authenticity of the staining method based on correlation of staining results with other diagnostic methods and the CRS.

5.1.3.6 Reliability of immunostaining

We assured the run-to-run, within-run, intra-observer and inter-observer reproducibility of the immunostaining prior to the inclusion of patients` study material. Furthermore, inter-observer agreement between pathologists was assessed on the study material, showing substantial agreement as shown in figure 18, section 3.3.3.5. It was difficult to assess run-to-run or within-run reproducibility on study samples due to limited number of slides being prepared from each specimens.

5.1.3.7 Data management

False data entry is another potential source of error. Answers in the questionnaire and EQ-5D-3L and results from the physical examination and laboratory reports were all hand-written and subsequently coded and entered into Excel spreadsheets. To limit the possible error, data cleaning was performed to detect and correct inaccurate or invalid codes, using the primary data set (patients study folders) as reference. All laboratory

data and answers form the EQ-5D-3L were entered twice and cross-checked. Further, double entry was performed in 10% of the study questionnaires and cross-checked showing 3/2769 (1.1‰) typing errors.

5.1.4 Statistical considerations

The sample size in all three papers are relatively small, this reduces the statistical power of the analyses and increases the risk of type II errors, i.e. not rejecting the null hypothesis when it is false, believing there is no effect in our population, when, truly there is. Further, the risk of Type I error, i.e. rejection of the null hypothesis when it is true, believing there actual is an effect in our population, increases with multiple testing performed. In paper II and III we used the Bonferroni correction of the significance level, using a significance level of α/m, where α was the set overall significance level and m the number of tests, to adjust for the possible effect of multiple comparison. On the other hand, using the Bonferroni adjustment to counteract the effect of multiple testing may have been too conservative.

5.2 Discussion of the main results

5.2.1 Improving the laboratory diagnosis of extrapulmonary tuberculosis in a low-resource setting

We have showed that the MPT64 test is implementable in a low-resource, routine diagnostic setting. The immunostaining can be performed by local laboratory technologists and the local pathologist is able to evaluate the stained slides. The performance of the new test in this routine diagnostic setting is comparable with prior studies evaluating the MPT64 test in more controlled contexts, particularly for TB lymphadenitis (123, 347). We found a better overall performance of the MPT64 test compared to Mtb culture, ZN staining and the Xpert assay. The high performance of the MPT64 test among TB lymphadenitis cases and in paediatric patients could have the most important impact on an improved and more rapid diagnosis of EPTB in this setting.

TB lymphadenitis, together with TB pleuritis, are the most common forms of EPTB (12, 82, 83). In TB endemic areas, TB lymphadenitis is a prevalent cause of persistent peripheral lymphadenopathy in children. Enlarged lymph nodes is a typical clinical presentation of EPTB in paediatric patients, responsible for around 50% of extrathoracic TB in children (93, 362-365). Biopsy and subsequent Mtb culture, ZN staining and histopathological examination of the tissue sections is considered one of the most important methods for diagnosing EPTB (124). However, biopsy requires manpower and facilities, often lacking in low-resource settings. FNAC from lymph nodes is a cost-effective, simple and safe diagnostic modality. It can be performed in out-patient clinics, requires minimal unsophisticated equipment and provides representative specimens from lymph nodes for Mtb culture, ZN staining and material for cytological evaluation (366, 367). It is the diagnostic modality advised in paediatric patients with persistent enlarged lymph nodes (365, 368), and has an undeniable role in the diagnosis of EPTB in all age groups (286, 369-371). Even though considered the gold standard for TB diagnosis, Mtb culture has its challenges in resource-constrained settings such as the need of highly specialized laboratories, trained personnel and long turnaround time for results. Further, ZN staining of FNAC specimens from lymph nodes shows suboptimal yield (123, 285). The cytomorphological picture can be suggestive of TB disease (285, 287, 372) but it is not confirmatory as the morphological features found in TB disease are not specific and other conditions may present similar features (288). In addition, immunocompromised TB patients often present with atypical cytological features, and even FNAC from immunocompetent patients can show other morphological features such as suppurative acute lymphadenitis (285). Relying just on cytological examination can thus lead to both over- and under-treatment. In our study participants categorized as TB lymphadenitis cases, 19/34 (56%) had FNAC showing a cytomorphological picture consistent with TB, whereas the MPT64 test was positive in 26/34 (76%) of these cases. The immunostaining using primary anti-MPT64 antibody showed superior sensitivity among TB lymphadenitis cases in FNAC specimens compared to the Xpert assay, Mtb culture and ZN staining, and excellent specificity, being negative in all non-TB cases (n=32). Positive immunostaining was noted in all FNAC smears sampled from

confirmed TB lymphadenitis cases (Mtb culture positive and/or Mtb detected by the Xpert assay). FNAC from lymph nodes is also helpful in distinguishing TB from metastatic lesions or lymphoma (373, 374). Among our cohort, suspected malignancy was noted in 10/66 (15%) of the patients included with peripheral lymphadenopathy. Combining cytological evaluation and Mtb culture of FNAC samples from lymph nodes with a sensitive and rapid diagnostic technique can contribute considerably to effective management of TB lymphadenitis. We believe the MPT64 test could be a valuable confirmatory add-on test in presumptive TB lymphadenitis cases. It can differentiate Mtb infection from NTM and other etiologies in samples showing granulomatous inflammation, and further indicate TB infection in samples potentially discarded as TB due to cytomorphological inconsistent with TB.

Many patients in developing countries live in remotes areas far from hospitals and reference laboratories and the referral system from low-level to higher level of health care are also often weak. We acknowledge the importance of diagnostic tests being available at health care units in proximity to patients` residence. The new MPT64 test needs to be performed in a histopathology laboratory but uses only procedures and technology already available in most routine pathology laboratories. The possibility of collecting the sample at other health care units and sending the specimen to centralized units, such as MMH, for examination, as is done for sputum samples examined with the Xpert assay, could be possible in this setting. Nurses or other medical personnel in peripheral health care units can be trained in the correct technique to perform FNAC of peripheral lymph nodes (366, 373), this can be particularly helpful in resource-constrained settings. FNAC and other sample material can be collected and slides prepared and fixated at other health care units using very basic laboratory equipment. The slides can then be transported to MMH for immunostaining and cytological examination. In addition, material can be collected in tubes and sent for Mtb culturing and the Xpert assay. Adapting the new diagnostic technology to the local infrastructure is crucial, increasing the opportunity for success and accessibility for patients not able to travel to MMH.

We found a significantly higher sensitivity of the MPT64 test in children compared to adults. Most of the specimens among the paediatric TB cases were FNAC from lymph nodes (FNAC from lymph nodes, n=11; pleural effusion, n=3; ascites, n=1; pericardial effusion, n=1). The sensitivity of the MPT64 test in children was higher compared to adults in FNAC samples from peripheral lymph nodes (100% sensitivity versus 65% sensitivity). In the recent years, childhood TB has received increased attention (375). Estimates indicate that only a portion of active paediatric TB cases are notified (376). The challenge of establishing a bacteriologically confirmed diagnosis, due to difficulty in acquiring adequate samples for laboratory investigations and the lower diagnostic yield of traditional diagnostic methods, such as smear microscopy and Mtb culture, is one of the reasons why many active TB cases in children go unreported (110, 375). Our test can contribute towards microbiological confirmation of TB lymphadenitis in children by using a relatively less invasive procedure of FNAC. We acknowledge that more studies with a larger sample size are needed to further explore these results.

A limitation of the study is that not all samples were subjected to each of the various laboratory tests, this can have affected the estimated diagnostic performance of the component tests of the CRS and the MPT64 test. Mtb culture was performed in 86%, ZN staining in 99% and the Xpert assay in only 50% of the samples, respectively. The low number of samples analysed with the Xpert assay was due to limited number of Xpert cartridges which had to be prioritized for sputum specimens. Further, there was lack of equipment for sample processing of biopsies, thus, the Xpert assay was not performed in any of the biopsies. We still included the results of the Xpert assay in our paper, but the results should be interpreted in the clarified context. Before the study, specimens from extrapulmonary sites of infection was not usually sent for Mtb culture. We got an agreement with the ZIHTLP and PHL-IdC and were able to send specimens for culture. The low performance of mycobacterial culture in our study could possibly partly be explained by reduced viability of bacilli during storage and transportation to PHL-IdC at Pemba Island, as the samples was received at PHL-IdC with a median of 7.5 days (IQR 5-13 days) after sample collection. Further, dividing the samples into separate tubes may lead to uneven distribution of bacilli, and the possible impact on the performance of the different laboratory tests cannot be disregarded.

Generally, it is thought that more accurate, rapid and preferably easily implementable diagnostic tests have the potential to improve patient management and decrease disease burden by reducing diagnostic delay and increase case detection. The ability of a new test to convey these benefits is not necessarily evident based purely on studies of test performance in routine diagnostics. We were not able to evaluate the potential impact on delay as the study was not designed for such analysis. To do this we would have had to include two separate arms in our study design, were patients were randomized to receive or not receive the new diagnostic test. We still argue that the MPT64 test has the potential to improve the diagnosis of EPTB in Zanzibar, and conceivably also in other low-resource settings. However, we acknowledge that there are several challenges which need to be addressed to ensure the sustainability of the MPT64 test in this setting, such as feasible procurement of equipment and reagents, continuous training and supervision of laboratory personnel performing the immunostaining and the evaluation of the test results and establishment of external quality control programs. In addition, a cost-effectiveness analysis is a critical aid and undoubtedly needed in the decision process whether to adopt the new test in a diagnostic algorithms. It should be emphasized that the MPT64 test must be interpreted in conjunction with the patient's clinical history, results of clinical examination, imaging and other established routine laboratory diagnostic tests. The MPT64 test should thus be regarded as an add-on test to the already established diagnostic procedures, and by that should not replace other investigations such as cytology/histology, which is invaluable also in the diagnosis of other conditions.

5.2.2 Diagnostic delay and impact of treatment on self-rated health status

This is the first project assessing the diagnostic delay, health care seeking pathways and EPTB knowledge among presumptive EPTB patients in Zanzibar. The small sample size limits the possibility of stratifying the results according to all the distinct sites of EPTB disease. Still, we believe that these findings could help HCPs working with TB patients in Zanzibar identifying potential interventions and strategies to improve EPTB case finding and possibly reduce the diagnostic delay among EPTB patients in this setting.

5.2.2.1 Diagnostic delay and health care seeking pathways

As an example of the delay experienced and health care seeking pathways followed, we present one of the patients included, a young child with culture-confirmed TB lymphadenitis (Figure 24). The guardian described local signs and symptoms which had been present for almost 2 years. The first documented visit to a HCP was 654 days prior to the study baseline interview. The patient had 8 documented visits to the same public health care unit prior to referral to MMH, and during these visits had been given multiple antibiotic treatments, without obvious improvement. There was no documentation that any microbiological investigations had been performed despite incision and drainage of the enlarged lymph nodes/abscesses were documented on two previous health care visits.

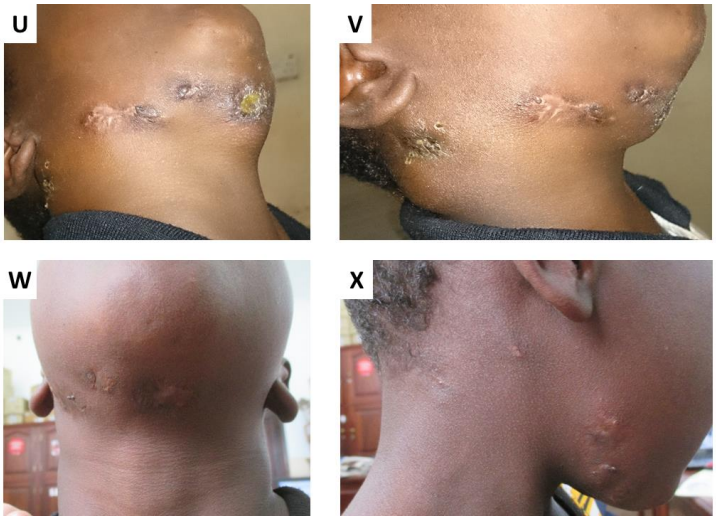

Figure 24. TB lymphadenitis patient. Picture U and V, before anti-TB treatment; Picture W and X, after anti-TB treatment.

This study participant illustrates our main findings showing that many EPTB cases experience a delay exceeding two months from symptom onset until treatment is initiated. Significantly longer patient and total delay were described among TB lymphadenitis cases, with ¼ of the TB lymphadenitis patients experiencing an overall delay > 6 months. In concordance with studies from diverse settings we found health system delay as the main contributing factor towards total delay (301, 305, 308, 377,

378). Among the TB patients in our study, 80% experienced longer health system delay than patient delay. This is contrast to others reporting patient delay as the primary contributor (306, 307, 379). We also noted that when adopting cut-offs for acceptable/unacceptable delay used by others (unacceptable; patient delay >30 days, health system delay >14 days) (380, 381), unacceptable patients delay was noted among 24% of the TB cases, whereas 83% of the TB patients experienced unacceptable health system delay. A longer health system delay was noted among TB cases consulting other HCPs than MMH at first visit and in patients with > 3 visits to any HCP. Thirty-eight percent of the TB patients reported ≥5 visits to any HCP before starting anti-TB treatment. Others have also reported health system delay associated with number of HCPs visited before TB diagnosis (308). When evaluating the care seeking pathways, we found that the majority of patients first visited a public HCP, and many had repeated visits to the same health care level before referral to MMH. In a systematic review assessing diagnostic and treatment delay in TB, repeated consultations within the same level of health care, which resulted in unspecific antibiotic treatment and not being referred to specialized TB health care services, was concluded as the core problem causing delay (300). Recognition of presumptive EPTB cases requires adequate training of HCPs, and for correct diagnosis, both identification of the presumptive EPTB patients and effective and accessible diagnostic tools are vital. In Zanzibar, during the study period, many peripheral health care units were performing ZN staining and sputum smear microscopy, but to our impression, extrapulmonary material was not examined at these sites and rarely sent for Mtb culture. Further, cytological and histological examinations were only performed at MMH. There were few sites performing X-ray, and computer tomography was only available at MMH. Thus, the health system delay reported in our study does not inevitably reflect HCPs not recognizing possible EPTB cases, but can also reflect shortage of facilities and personnel performing various invasive procedures, inadequate diagnostic tools and knowledge regarding available diagnostic possibilities, insufficient follow-up routines and incomplete algorithms on when to refer presumptive EPTB to higher levels of health care. To achieve a reduction of health system delay we believe that continuous education in EPTB disease and repetitive

training in early recognition of the various EPTB clinical presentations to HCPs at all levels, but especially at the primary health care level, both in the public and private sectors, are essential. In addition, the HCPs should be given regular information regarding the currently available EPTB diagnostics in the specific setting. As 58% of the patients reported a course of antibiotics given at first visit, proper scheduled follow-up of these patients and establishment of timely algorithms including recommended diagnostics and when to refer presumptive EPTB cases to higher level health care facilities with capacity and expertise to perform invasive diagnostic procedures could be valuable. We recommend developing such diagnostic and referral algorithms for presumptive EPTB patients specifically for the current setting. Finally, the development of new and improved diagnostic tools, implementable, feasible and sustainable in low-resource settings is of uttermost importance, in combination with strengthening and supporting TB laboratory personnel and facilities.

5.2.2.2 Extrapulmonary tuberculosis knowledge

Among the cohort, the knowledge regarding EPTB was remarkably low. Most had heard of PTB disease prior to the study, but only one respondent mentioned an extrapulmonary site and none reported other local symptoms than respiratory symptoms. The reality that most public and patients` educational TB campaigns only include cardinal symptoms of PTB, can lead to the population being unaware of EPTB and its symptoms. The findings among our cohort of scarce knowledge regarding EPTB represents a cause of concern, as awareness and knowledge of signs and symptoms could have impact on patients` care seeking action, but also identifies a possible area of intervention. Higher TB knowledge has been shown associated with care seeking behaviour (382), and lack of awareness has been reported by patients as one of the reasons for delayed care seeking (383). We believe that enhancing the knowledge of EPTB as a disease and increasing the awareness of signs and symptoms suggestive of EPTB in the general population are valuable. This can be done by systematically incorporating information regarding EPTB in the already ongoing public campaigns covering PTB disease. This can potentially contribute towards a reduced diagnostic delay among EPTB patients in this setting.

5.2.2.3 Improvement of self-rated health after treatment

Although EPTB patients are no threat to a community in terms of transmission of disease, still, these patients can suffer continuing disability, reduced quality of life, higher morbidity and mortality due to late diagnosis, loss of income and economic constraints. We thus wanted to get an impression among the study cohort on the impact of EPTB disease on regular activities, work capacity and general health status as reported by the patients. Instruments assessing self-reported health-related quality of life (HRQoL) are generally classified as either disease-specific or as generic tools which are applicable to diverse conditions (384). Most studies assessing HRQoL among TB patients have used generic instruments in cross-sectional study designs to evaluate impairment of health associated with TB, fewer studies have used prospective longitudinal study designs reporting the effect of anti-TB treatment on quality of life (384-386). Some TB disease-specific instruments have been developed (384, 387-389). Few studies have included EPTB patients. Differentiating EPTB from PTB appear important when assessing health status and quality of life, in addition, the data should also be stratified by site of EPTB disease (385). To the best of our knowledge, no TB-specific instrument translated into Swahili has been published and validated. In our study, we included questions in the study questionnaire covering work capacity, in addition, we chose to administer one version of the EQ-5D instruments, the EQ-5D-3L, to measure self- perceived health status and potential changes after anti-TB treatment. The standardised EQ-5D is an easy, generic measure of self-reported health status or HRQoL, developed to value and describe health. It has been reported to be reliable and valid in diverse populations and conditions (390), and has been used among TB patients (391). It is a frequently used instrument to value health utility and measure quality of life for economic evaluations (392). Further, it is a short questionnaire, quick to complete, simple to understand and translated and validated in Swahili (Swahili version for Tanzania, validated for Haydom, Manyara Region, © EuroQoL Group 1990). Most of the adult TB patients, irrespective of site of TB infection, reported at the time of inclusion that they had reduced work capacity or had entirely stopped working due to the current disease, with a median of 60 days (IQR, 21-90 days). The number and proportion of TB patients reporting problems at baseline

within the five dimensions of the EQ-5D descriptive system were substantially larger before compared to after completing anti-TB treatment (Table 6). At baseline, patients with TB lymphadenitis described lesser problems than patients with other sites of TB infection in all five dimensions, still, both patient groups, reported considerably lesser problems at treatment completion. This was also reflected in the EQ visual analogue scale (EQ VAS).

Table 6. Number of patients and proportions reporting levels within the five dimensions of the EQ-5D descriptive system before and after anti-TB treatment, n (%)[a]

	Dimensions in the EQ-5D descriptive system									
	Mobility		Self-care		Usual activities		Pain/discomfort		Anxiety/depression	
	Before	After	Before	After	Before	After	Before	After	Before	After
Level 1 no problems	18 (58%)	29 (94%)	19 (61%)	29 (94%)	11 (35%)	30 (97%)	10 (32%)	29 (94%)	16 (52%)	31 (100%)
Level 2 some problems	9 (29%)	2 (6%)	9 (29%)	2 (6%)	11 (35%)	1 (3%)	21 (68%)	2 (6%)	15 (48%)	0
Level 3 extreme problems	4 (13%)	0	3 (10%)	0	9 (29%)	0	0	0	0	0

[a] Thirty-one adult TB patients completed the EQ-5D form both before anti-TB treatment and at treatment completion.

To give an example, an adult TB patient from the cohort, described onset of symptoms 2 months prior to the baseline interview, with gradual increasing weakness and pain of the lower extremities and pain in the lumbar region during the last 2-3 weeks. The patient was totally confined to bed when included in the study and had not been working since the onset of symptoms. The computer tomography showed involvement of vertebrae Th12, L1 and L2, in addition bilateral psoas abscesses and abscess in the right musculus erector spinae. The patient reported extreme problems in the three first dimensions of the EQ-5D descriptive system (mobility, self-care and usual activities) and some problems in the last two dimensions (pain/discomfort and anxiety/depression), and rated his health to 20% of best imaginable health on the EQ VAS before commencing anti-TB treatment. After anti-TB treatment, the patient reported working as normal and described no problems in either dimension of the EQ-5D descriptive system and rated his health in the EQ VAS to 100%. The computer

tomography after treatment showed no significant vertebrae collapse or canal stenosis and complete resolution of the abscesses.

Our findings give a clear impression that completing anti-TB treatment has an impact on the quality of life among EPTB cases. Showing improvement in self-reported health status with treatment is valuable and gives considerable motivation to reduce the diagnostic delay among EPTB patients. Further, it would be interesting to assess how TB affects the health status before treatment and potential residual deficits or impact on quality of life after treatment when compared to the health status of the general population. This can be done by comparing problems reported in the EQ-5D instrument among the patient group to problems reported in a sample from the general population in the current setting (393, 394). However, such reference scores have not been derived in Zanzibar or the Tanzanian mainland, and such a comparison was thus not possible. Despite this, in addition to the small sample size, we still argue that our findings imply that early and correct treatment of all forms of EPTB could have important impact on the morbidity, quality of life and the economic situation for afflicted patients and families.

5.2.3 Evaluation of treatment response

We showed that among patients presenting to MMH with presumptive EPTB the clinical presentation alone could not separate the TB and the non-TB cases. In the current setting empirical treatment is often initiated, emphasizing the importance of monitoring treatment response among these patients. Clinical assessment is still the recommended approach to evaluate improvement and response to treatment among EPTB patients (3, 91, 312), and should include resolution of constitutional and local symptoms, overall improved performance status and weight gain (395). However, the criteria for what constitutes adequate clinical treatment response in the heterogeneous disease EPTB is presently not established. We wanted to assess if a combination of simple clinical parameters can predict good treatment response. The included study participants starting anti-TB treatment were followed during treatment and clinical findings, weight, symptoms reported and general condition as evaluated by the clinician were noted.

We included regression of objective clinical findings, weight gain and improvement of symptoms as the three clinical parameters for assessing treatment response. Among the patients categorized as TB cases by the CRS, 47/48 (98%) patients satisfied ≥2 parameters after 2 months of anti-TB treatment. Regression of peritoneal and pleural effusion were noted in 3/3 (100%) abdominal TB and in 14/15 (93%) TB pleuritis cases at 2 months, respectively. Among the 34 TB lymphadenitis cases, 32 patients were seen after 2 months of anti-TB treatment, and partial or full regression of lymph node swellings were reported in 29/32 (91%) patients. Complete resolution of lymphadenopathy was noted in 24/28 (86%) cases at treatment completion. Among the remaining 4 patients (all confirmed TB cases) residual palpable lymph nodes were noted after 6 months of treatment. Three of these were followed further and no enlarged lymph nodes were described after 3 months in 2 patients and 6 months in 1 patient. Other studies have reported enlargement and appearance of new nodes during treatment and residual lymphadenopathy at treatment completion without necessarily indicating relapse or treatment failure (396-398). Still, paradoxical reactions and residual lymphadenopathy can complicate the clinical assessment.

Both ≥5% weight gain and any weight gain were evaluated as a potential clinical parameter. A weight gain ≥5% was reported in 12/13 (92%) of the paediatric TB cases and among 24/36 (67%) of the adult TB patients, and any weight gain was noted in 46/48 (96%) of the TB cases after the intensive phase of anti-TB treatment. Including any weight gain as one of the clinical markers of treatment response is simpler, still, using a certain percentage weight gain, such as ≥5%, can be more sensitive in capturing non-responders or identifying patients requiring closer follow-up. More studies are needed to describe the association between treatment outcome and weight gain in EPTB patients. Weight gain in relation to the commencement of ART could bias the effect of weight gain measured as a result of effective anti-TB treatment. In the current cohort, only 7 HIV positive patients were followed during anti-TB treatment. Among these, 4/7 patients were already receiving ART, while 3/7 initiated ART after starting anti-TB treatment.

Improvement of symptoms were the third clinical parameter included when assessing treatment response. This was done by evaluating symptoms described in the questionnaire at baseline with symptoms reported by the patients at follow-up visits. In our study we only assessed if the patients overall reported improvement of symptoms. In retrospect we acknowledge that if we had used a consistent form for documentation of symptoms also at the follow-up visits, it would have given us the opportunity to calculate the symptoms count ratio (number of symptoms reported by the patients as improved at follow-up divided by the number of symptoms reported at baseline). Including a symptoms count ratio above a certain level, as Wilson et al. included as one of the parameters in their criteria of response to treatment among HIV infected adults with smear negative PTB and EPTB (330), could have reduced the potential bias when assessing improvement of symptoms. Incorporating results of the symptoms count ratio, self-rated health using the EQ-5D instruments (331) and the clinicians evaluation of the performance status using the Karnofsky Performance Status score (332, 334), can make a treatment response assessment tool potentially more user friendly. Further studies are required to establish clinically relevant criteria or cut-off points to define treatment response.

One of the limitations of this study in addition to the small sample size is the inclusion of mainly lymphadenitis, pleuritis and peritoneal TB patients, and few or no cases with other forms of EPTB disease. We recognize that these simple clinical parameters should ideally be evaluated on a sub-group level divided according to the site of EPTB disease. In addition, we did not have a comparable control group, as only 7 patients initiating anti-TB treatment and categorized as non-TB cases were followed during treatment. The non-TB cases did however fulfil only 0 or 1 of the clinical parameters after the intensive phase of anti-TB treatment. Still, we believe that a combination of simple clinical parameters, without the need of laboratory support, can be developed into and used as an easy tool for monitoring of treatment response early during anti-TB treatment in EPTB patients. Further, such an assessment tool can be incorporated systematically into standard follow-up protocols, attached to the patients TB treatment cards or in TB registers, and assist the HCP in low-resource settings in timely detection of non-responders in need of clinical reassessment or closer follow-up.

6. CONCLUSIONS

- The immunochemistry-based MPT64 test for EPTB is implementable in a routine diagnostic laboratory in a low-resource setting.
- The MPT64 test was sensitive and specific for the diagnosis of EPTB in this setting, especially in TB lymphadenitis and paediatric TB. The overall performance of the MPT64 test was better compared to both the Xpert assay and conventional diagnostic methods, with potential to improve the diagnosis of EPTB in low-resource settings.
- Many EPTB patients experienced delays exceeding two months from the onset of symptoms until the initiation of anti-TB treatment. The longest delay was reported among the TB lymphadenitis patients. The major contributor to the overall delay was health system delay.
- The majority of the adult TB patients reported reduced work capacity due to the ongoing illness and improved self-rated health status after treatment using the EQ-5D-3L instrument. This indicates that reducing diagnostic delay, early case detection and initiation of treatment is important among EPTB patients.
- Strengthening the EPTB knowledge and awareness among the general population and HCPs at all levels can be valuable interventions for improved management of EPTB in this setting.
- Monitoring of treatment response among EPTB patients often starting empirical treatment by using a combination of simple clinical parameters can be a useful aid to HCPs in low-resource settings.

7. FUTURE PERSPECTIVES

Proper diagnosis and appropriate treatment of all individuals with all forms of TB are incorporated as one of the key components of the End TB strategy. Intensified research and development of new and improved diagnostic tool, also for presumptive EPTB, are needed, to reach these goals.

We have shown that our new diagnostic test for the diagnosis of EPTB is implementable and doable in a low-resource setting and has the prospect to be a part of improving the diagnosis of EPTB. However, the MPT64 test needs to be implemented and validated in other routine diagnostic settings, to evaluate the feasibility and performance in various setting. Our research group is involved in the implementation and validation of this test in a multicentre study, where the MPT64 test are currently being assessed at two sites at the mainland of Tanzania (Mbeya Zonal Referral Hospital, Mbeya, and Muhimbili National Hospital, Dar es Salaam), in Pakistan (Gulab Devi Hospital) and in India (Ujjain Charitable Hospital).

Showing that the MPT64 test is implementable, and further, has a good diagnostic performance in various settings is not enough. The sustainability of the MPT64 test needs to be evaluated. Will the test be performed in routine diagnostics even after an ongoing project? We tried to get an understanding of the possible facilitators and barriers for the implementation and sustainability of a new diagnostic test for diagnosing EPTB in our study setting, by performing a qualitative study interviewing health personnel working at MMH and in the national TB programme. The data analysis from this study is ongoing. Just as important are cost-effectiveness analyses, which should be performed not only in Zanzibar, but in different settings. This will all be valuable and necessary when evaluating where our new test fits in the diagnostic algorithm for EPTB.

We believe that developing an easy assessment tool for monitoring of treatment response can be highly useful especially in low-resource settings to support HCPs in the follow-up of EPTB patients. Further studies are needed for the development, selection of clinical parameters to be included and establishment of relevant cut-off

points for the distinct parameters among EPTB patients, separated and evaluated according to the site of EPTB disease. The validity and reliability of the assessment tool must be tested in larger sample sizes and in various settings.

8. REFERENCES

1. World Health Organization. Top 10 causes of death [Available from: http://www.who.int/gho/mortality_burden_disease/causes_death/top_10/en/.
2. World Health Organization. Global tuberculosis report 2019. WHO, Geneva Switzerland. 2019;WHO/CDS/TB/2019.15.
3. TB CARE I. International standards for tuberculosis care, Edition 3. TB CARE I, The Hague. 2014.
4. World Health Organization, Stop TB partnership. The Stop TB Strategy: Building on and enhancing the DOTS to meet the TB-related Millennium Development Goals. 2006.
5. World Health Organization. The End TB strategy 2014 [Available from: https://www.who.int/tb/strategy/end-tb/en/.
6. Sharma SK, Mohan A. Extrapulmonary tuberculosis. Indian J Med Res. 2004;120(4):316-53.
7. Harries AD, Hargreaves NJ, Gausi F, Kwanjana JH, Salaniponi FM. High early death rate in tuberculosis patients in Malawi. Int J Tuberc Lung Dis. 2001;5(11):1000-5.
8. Nassikas N, Yang H, Forson A, Kwarteng E, Kwara A. Factors associated with mortality in extrapulmonary tuberculsis patients at a teaching hospital in Ghana. Ghana Medical Journal. 2015;49(4).
9. Chakravorty S, Sen MK, Tyagi JS. Diagnosis of extrapulmonary tuberculosis by smear, culture, and PCR using universal sample processing technology. J Clin Microbiol. 2005;43(9):4357-62.
10. Vadwai V, Boehme C, Nabeta P, Shetty A, Alland D, Rodrigues C. Xpert MTB/RIF: a new pillar in diagnosis of extrapulmonary tuberculosis? J Clin Microbiol. 2011;49(7):2540-5.
11. Adada H, Valley MA, Nour SA, Mehta J, Byrd RP, Jr., Anderson JL, et al. Epidemiology of extra-pulmonary tuberculosis in the United States: high rates persist in the post-HIV era. Int J Tuberc Lung Dis. 2014;18(12):1516-21.
12. Sandgren A, Hollo V, van der Werf MJ. Extrapulmonary tuberculosis in the European Union and European Economic Area, 2002 to 2011. Euro Surveill. 2013;18(12).
13. Kruijshaar ME, Abubakar I. Increase in extrapulmonary tuberculosis in England and Wales 1999-2006. Thorax. 2009;64(12):1090-5.
14. te Beek LA, van der Werf MJ, Richter C, Borgdorff MW. Extrapulmonary tuberculosis by nationality, The Netherlands, 1993-2001. Emerg Infect Dis. 2006;12(9):1375-82.
15. Elliott AM, Halwiindi B, Hayes RJ, Luo N, Tembo G, Machiels L, et al. The impact of human immunodeficiency virus on presentation and diagnosis of tuberculosis in a cohort study in Zambia. J Trop Med Hyg. 1993;96(1):1-11.
16. Memish ZA, Bamgboye EA, Abuljadayel N, Smadi H, Abouzeid MS, Al Hakeem RF. Incidence of and risk factors associated with pulmonary and extra-pulmonary tuberculosis in Saudi Arabia (2010-2011). PLoS One. 2014;9(5):e95654.
17. Yang Z, Kong Y, Wilson F, Foxman B, Fowler AH, Marrs CF, et al. Identification of risk factors for extrapulmonary tuberculosis. Clin Infect Dis. 2004;38(2):199-205.
18. Gonzalez OY, Adams G, Teeter LD, Bui TT, Musser JM, Graviss EA. Extra-pulmonary manifestations in a large metropolitan area with a low incidence of tuberculosis. Int J Tuberc Lung Dis. 2003;7(12):1178-85.
19. Jones BE, Young SM, Antoniskis D, Davidson PT, Kramer F, Barnes PF. Relationship of the manifestations of tuberculosis to CD4 cell counts in patients with human immunodeficiency virus infection. Am Rev Respir Dis. 1993;148(5):1292-7.
20. Kumar P, Sharma N, Sharma NC, Patnaik S. Clinical profile of tuberculosis in patients with HIV Infection/AIDS. Indian J Chest Dis Allied Sci. 2002;44(3):159-63.
21. Sharma SK, Mohan A, Gupta R, Kumar A, Gupta AK, Singhal VK, et al. Clinical presentation of tuberculosis in patients with AIDS: an Indian experience. Indian J Chest Dis Allied Sci. 1997;39(4):213-20.
22. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, et al. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. N Engl J Med. 2001;345(15):1098-104.

23. Sotgiu G, Falzon D, Hollo V, Kodmon C, Lefebvre N, Dadu A, et al. Determinants of site of tuberculosis disease: An analysis of European surveillance data from 2003 to 2014. PLoS One. 2017;12(11):e0186499.
24. Qian X, Nguyen DT, Lyu J, Albers AE, Bi X, Graviss EA. Risk factors for extrapulmonary dissemination of tuberculosis and associated mortality during treatment for extrapulmonary tuberculosis. Emerg Microbes Infect. 2018;7(1):102.
25. Ohene SA, Bakker MI, Ojo J, Toonstra A, Awudi D, Klatser P. Extra-pulmonary tuberculosis: A retrospective study of patients in Accra, Ghana. PLoS One. 2019;14(1):e0209650.
26. Kourbatova EV, Leonard MK, Jr., Romero J, Kraft C, del Rio C, Blumberg HM. Risk factors for mortality among patients with extrapulmonary tuberculosis at an academic inner-city hospital in the US. Eur J Epidemiol. 2006;21(9):715-21.
27. Cambau E, Drancourt M. Steps towards the discovery of Mycobacterium tuberculosis by Robert Koch, 1882. Clin Microbiol Infect. 2014;20(3):196-201.
28. Banuls AL, Sanou A, Anh NT, Godreuil S. Mycobacterium tuberculosis: ecology and evolution of a human bacterium. J Med Microbiol. 2015;64(11):1261-9.
29. Lawn SD, Zumla AI. Tuberculosis. Lancet. 2011;378(9785):57-72.
30. Muller B, Durr S, Alonso S, Hattendorf J, Laisse CJ, Parsons SD, et al. Zoonotic Mycobacterium bovis-induced tuberculosis in humans. Emerg Infect Dis. 2013;19(6):899-908.
31. Glaziou P, Floyd K, Raviglione MC. Global Epidemiology of Tuberculosis. Semin Respir Crit Care Med. 2018;39(3):271-85.
32. Tuberculosis - A Comprehensive Clinical Reference. Shchaaf HS, Zumla A, editors: Saunders Elsevier; 2009.
33. Davies BH. Infectivity of tuberculosis. Thorax. 1980;35(7):481-2.
34. Behr MA, Warren SA, Salamon H, Hopewell PC, Ponce de Leon A, Daley CL, et al. Transmission of Mycobacterium tuberculosis from patients smear-negative for acid-fast bacilli. Lancet. 1999;353(9151):444-9.
35. Pai M, Behr MA, Dowdy D, Dheda K, Divangahi M, Boehme CC, et al. Tuberculosis. Nat Rev Dis Primers. 2016;2:16076.
36. Cardona PJ. A spotlight on liquefaction: evidence from clinical settings and experimental models in tuberculosis. Clin Dev Immunol. 2011;2011:868246.
37. Esmail H, Barry CE, 3rd, Young DB, Wilkinson RJ. The ongoing challenge of latent tuberculosis. Philos Trans R Soc Lond B Biol Sci. 2014;369(1645):20130437.
38. Jasenosky LD, Scriba TJ, Hanekom WA, Goldfeld AE. T cells and adaptive immunity to Mycobacterium tuberculosis in humans. Immunol Rev. 2015;264(1):74-87.
39. Barry CE, 3rd, Boshoff HI, Dartois V, Dick T, Ehrt S, Flynn J, et al. The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. Nat Rev Microbiol. 2009;7(12):845-55.
40. Pai M, Denkinger CM, Kik SV, Rangaka MX, Zwerling A, Oxlade O, et al. Gamma interferon release assays for detection of Mycobacterium tuberculosis infection. Clin Microbiol Rev. 2014;27(1):3-20.
41. Mack U, Migliori GB, Sester M, Rieder HL, Ehlers S, Goletti D, et al. LTBI: latent tuberculosis infection or lasting immune responses to M. tuberculosis? A TBNET consensus statement. Eur Respir J. 2009;33(5):956-73.
42. Dye C, Scheele S, Dolin P, Pathania V, Raviglione MC. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. JAMA. 1999;282(7):677-86.
43. Getahun H, Chaisson RE, Raviglione M. Latent Mycobacterium tuberculosis Infection. N Engl J Med. 2015;373(12):1179-80.
44. Comstock GW, Livesay VT, Woolpert SF. The prognosis of a positive tuberculin reaction in childhood and adolescence. Am J Epidemiol. 1974;99(2):131-8.
45. Vynnycky E, Fine PE. The natural history of tuberculosis: the implications of age-dependent risks of disease and the role of reinfection. Epidemiol Infect. 1997;119(2):183-201.
46. Sutherland I. Recent studies in the epidemiology of tuberculosis, based on the risk of being infected with tubercle bacilli. Adv Tuberc Res. 1976;19:1-63.

47. Wiker HG, Mustafa T, Bjune GA, Harboe M. Evidence for waning of latency in a cohort study of tuberculosis. BMC Infect Dis. 2010;10:37.
48. Wallis RS, Broder M, Wong J, Lee A, Hoq L. Reactivation of latent granulomatous infections by infliximab. Clin Infect Dis. 2005;41 Suppl 3:S194-8.
49. Solovic I, Sester M, Gomez-Reino JJ, Rieder HL, Ehlers S, Milburn HJ, et al. The risk of tuberculosis related to tumour necrosis factor antagonist therapies: a TBNET consensus statement. Eur Respir J. 2010;36(5):1185-206.
50. Ai JW, Ruan QL, Liu QH, Zhang WH. Updates on the risk factors for latent tuberculosis reactivation and their managements. Emerg Microbes Infect. 2016;5:e10.
51. Hussein M, Mooij J. Tuberculosis and chronic renal disease. Saudi J Kidney Dis Transpl. 2002;13(3):320-30.
52. Harries AD, Lin Y, Satyanarayana S, Lonnroth K, Li L, Wilson N, et al. The looming epidemic of diabetes-associated tuberculosis: learning lessons from HIV-associated tuberculosis. Int J Tuberc Lung Dis. 2011;15(11):1436-44, i.
53. Dobler CC, Flack JR, Marks GB. Risk of tuberculosis among people with diabetes mellitus: an Australian nationwide cohort study. BMJ Open. 2012;2(1):e000666.
54. Marais BJ, Lonnroth K, Lawn SD, Migliori GB, Mwaba P, Glaziou P, et al. Tuberculosis comorbidity with communicable and non-communicable diseases: integrating health services and control efforts. Lancet Infect Dis. 2013;13(5):436-48.
55. Bates MN, Khalakdina A, Pai M, Chang L, Lessa F, Smith KR. Risk of tuberculosis from exposure to tobacco smoke: a systematic review and meta-analysis. Arch Intern Med. 2007;167(4):335-42.
56. Hnizdo E, Murray J. Risk of pulmonary tuberculosis relative to silicosis and exposure to silica dust in South African gold miners. Occup Environ Med. 1998;55(7):496-502.
57. Cowie RL. The epidemiology of tuberculosis in gold miners with silicosis. Am J Respir Crit Care Med. 1994;150(5 Pt 1):1460-2.
58. Turner RD, Chiu C, Churchyard GJ, Esmail H, Lewinsohn DM, Gandhi NR, et al. Tuberculosis Infectiousness and Host Susceptibility. J Infect Dis. 2017;216(suppl_6):S636-S43.
59. Achkar JM, Jenny-Avital ER. Incipient and subclinical tuberculosis: defining early disease states in the context of host immune response. J Infect Dis. 2011;204 Suppl 4:S1179-86.
60. Esmail H, Lai RP, Lesosky M, Wilkinson KA, Graham CM, Coussens AK, et al. Characterization of progressive HIV-associated tuberculosis using 2-deoxy-2-[(18)F]fluoro-D-glucose positron emission and computed tomography. Nat Med. 2016;22(10):1090-3.
61. Young DB, Gideon HP, Wilkinson RJ. Eliminating latent tuberculosis. Trends Microbiol. 2009;17(5):183-8.
62. Tailleux L, Schwartz O, Herrmann JL, Pivert E, Jackson M, Amara A, et al. DC-SIGN is the major Mycobacterium tuberculosis receptor on human dendritic cells. J Exp Med. 2003;197(1):121-7.
63. Wolf AJ, Linas B, Trevejo-Nunez GJ, Kincaid E, Tamura T, Takatsu K, et al. Mycobacterium tuberculosis infects dendritic cells with high frequency and impairs their function in vivo. J Immunol. 2007;179(4):2509-19.
64. Hossain MM, Norazmi MN. Pattern recognition receptors and cytokines in Mycobacterium tuberculosis infection--the double-edged sword? Biomed Res Int. 2013;2013:179174.
65. Mihret A. The role of dendritic cells in Mycobacterium tuberculosis infection. Virulence. 2012;3(7):654-9.
66. Cooper AM. Cell-mediated immune responses in tuberculosis. Annu Rev Immunol. 2009;27:393-422.
67. van Crevel R, Ottenhoff TH, van der Meer JW. Innate immunity to Mycobacterium tuberculosis. Clin Microbiol Rev. 2002;15(2):294-309.
68. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. J Exp Med. 1993;178(6):2249-54.

69. Giacomini E, Iona E, Ferroni L, Miettinen M, Fattorini L, Orefici G, et al. Infection of human macrophages and dendritic cells with Mycobacterium tuberculosis induces a differential cytokine gene expression that modulates T cell response. J Immunol. 2001;166(12):7033-41.
70. Pagan AJ, Ramakrishnan L. The Formation and Function of Granulomas. Annu Rev Immunol. 2018;36:639-65.
71. Liao W, Lin JX, Leonard WJ. IL-2 family cytokines: new insights into the complex roles of IL-2 as a broad regulator of T helper cell differentiation. Curr Opin Immunol. 2011;23(5):598-604.
72. Kleinnijenhuis J, Oosting M, Joosten LA, Netea MG, Van Crevel R. Innate immune recognition of Mycobacterium tuberculosis. Clin Dev Immunol. 2011;2011:405310.
73. Bozzano F, Marras F, De Maria A. Immunology of tuberculosis. Mediterr J Hematol Infect Dis. 2014;6(1):e2014027.
74. Ramakrishnan L. Revisiting the role of the granuloma in tuberculosis. Nat Rev Immunol. 2012;12(5):352-66.
75. Adams DO. The structure of mononuclear phagocytes differentiating in vivo. II. The effect of Mycobacterium tuberculosis. Am J Pathol. 1975;80(1):101-16.
76. Adams DO. The structure of mononuclear phagocytes differentiating in vivo. I. Sequential fine and histologic studies of the effect of Bacillus Calmette-Guerin (BCG). Am J Pathol. 1974;76(1):17-48.
77. Williams GT, Williams WJ. Granulomatous inflammation--a review. J Clin Pathol. 1983;36(7):723-33.
78. Ehlers S, Schaible UE. The granuloma in tuberculosis: dynamics of a host-pathogen collusion. Front Immunol. 2012;3:411.
79. Adigun R, Murray J. Necrosis, Cell (Liquefactive, Coagulative, Caseous, Fat, Fibrinoid, and Gangrenous). StatPearls. Treasure Island (FL)2019.
80. Di Perri G, Cazzadori A, Vento S, Bonora S, Malena M, Bontempini L, et al. Comparative histopathological study of pulmonary tuberculosis in human immunodeficiency virus-infected and non-infected patients. Tuber Lung Dis. 1996;77(3):244-9.
81. Jetley S, Jairajpuri ZS, Pujani M, Khan S, Rana S. Tuberculosis 'The Great Imitator': A usual disease with unusual presentations. Indian J Tuberc. 2017;64(1):54-9.
82. Peto HM, Pratt RH, Harrington TA, LoBue PA, Armstrong LR. Epidemiology of extrapulmonary tuberculosis in the United States, 1993-2006. Clin Infect Dis. 2009;49(9):1350-7.
83. Garcia-Rodriguez JF, Alvarez-Diaz H, Lorenzo-Garcia MV, Marino-Callejo A, Fernandez-Rial A, Sesma-Sanchez P. Extrapulmonary tuberculosis: epidemiology and risk factors. Enferm Infecc Microbiol Clin. 2011;29(7):502-9.
84. Lin JN, Lai CH, Chen YH, Lee SS, Tsai SS, Huang CK, et al. Risk factors for extra-pulmonary tuberculosis compared to pulmonary tuberculosis. Int J Tuberc Lung Dis. 2009;13(5):620-5.
85. Sreeramareddy CT, Panduru KV, Verma SC, Joshi HS, Bates MN. Comparison of pulmonary and extrapulmonary tuberculosis in Nepal- a hospital-based retrospective study. BMC Infect Dis. 2008;8:8.
86. El Sahly HM, Teeter LD, Pan X, Musser JM, Graviss EA. Mortality associated with central nervous system tuberculosis. J Infect. 2007;55(6):502-9.
87. Sunnetcioglu A, Sunnetcioglu M, Binici I, Baran AI, Karahocagil MK, Saydan MR. Comparative analysis of pulmonary and extrapulmonary tuberculosis of 411 cases. Ann Clin Microbiol Antimicrob. 2015;14:34.
88. Karstaedt AS. Extrapulmonary tuberculosis among adults: experience at Chris Hani Baragwanath Academic Hospital, Johannesburg, South Africa. S Afr Med J. 2013;104(1):22-4.
89. Noertjojo K, Tam CM, Chan SL, Chan-Yeung MM. Extra-pulmonary and pulmonary tuberculosis in Hong Kong. Int J Tuberc Lung Dis. 2002;6(10):879-86.
90. Golden MP, Vikram HR. Extrapulmonary tuberculosis: an overview. Am Fam Physician. 2005;72(9):1761-8.

91. World Health Organization. Improving the diagnosis and treatment of smear-negative pulmonary and extrapulmonary tuberculosis among adults and adolescents. Recommendations for HIV-prevalent and resource-constrained settings 2006 [cited 2019 13th of May]. Available from: http://www.who.int/tb/publications/2006/tbhiv_recommendations.pdf.
92. Jha BC, Dass A, Nagarkar NM, Gupta R, Singhal S. Cervical tuberculous lymphadenopathy: changing clinical pattern and concepts in management. Postgrad Med J. 2001;77(905):185-7.
93. Marais BJ, Gie RP, Schaaf HS, Hesseling AC, Enarson DA, Beyers N. The spectrum of disease in children treated for tuberculosis in a highly endemic area. Int J Tuberc Lung Dis. 2006;10(7):732-8.
94. Purohit MR, Mustafa T, Morkve O, Sviland L. Gender differences in the clinical diagnosis of tuberculous lymphadenitis--a hospital-based study from Central India. Int J Infect Dis. 2009;13(5):600-5.
95. Shriner KA, Mathisen GE, Goetz MB. Comparison of mycobacterial lymphadenitis among persons infected with human immunodeficiency virus and seronegative controls. Clin Infect Dis. 1992;15(4):601-5.
96. Levine H, Szanto PB, Cugell DW. Tuberculous pleurisy. An acute illness. Arch Intern Med. 1968;122(4):329-32.
97. Light RW. Update on tuberculous pleural effusion. Respirology. 2010;15(3):451-8.
98. Ruan SY, Chuang YC, Wang JY, Lin JW, Chien JY, Huang CT, et al. Revisiting tuberculous pleurisy: pleural fluid characteristics and diagnostic yield of mycobacterial culture in an endemic area. Thorax. 2012;67(9):822-7.
99. Valdes L, Alvarez D, San Jose E, Penela P, Valle JM, Garcia-Pazos JM, et al. Tuberculous pleurisy: a study of 254 patients. Arch Intern Med. 1998;158(18):2017-21.
100. Luzze H, Elliott AM, Joloba ML, Odida M, Oweka-Onyee J, Nakiyingi J, et al. Evaluation of suspected tuberculous pleurisy: clinical and diagnostic findings in HIV-1-positive and HIV-negative adults in Uganda. Int J Tuberc Lung Dis. 2001;5(8):746-53.
101. Benzagmout M, Boujraf S, Chakour K, Chaoui Mel F. Pott's disease in children. Surg Neurol Int. 2011;2:1.
102. Garg RK, Somvanshi DS. Spinal tuberculosis: a review. J Spinal Cord Med. 2011;34(5):440-54.
103. Pigrau-Serrallach C, Rodriguez-Pardo D. Bone and joint tuberculosis. Eur Spine J. 2013;22 Suppl 4:556-66.
104. Leibert E, Schluger NW, Bonk S, Rom WN. Spinal tuberculosis in patients with human immunodeficiency virus infection: clinical presentation, therapy and outcome. Tuber Lung Dis. 1996;77(4):329-34.
105. Anley CM, Brandt AD, Dunn R. Magnetic resonance imaging findings in spinal tuberculosis: Comparison of HIV positive and negative patients. Indian J Orthop. 2012;46(2):186-90.
106. Sharma MP, Bhatia V. Abdominal tuberculosis. Indian J Med Res. 2004;120(4):305-15.
107. Manohar A, Simjee AE, Haffejee AA, Pettengell KE. Symptoms and investigative findings in 145 patients with tuberculous peritonitis diagnosed by peritoneoscopy and biopsy over a five year period. Gut. 1990;31(10):1130-2.
108. Sharma V, Singh H, Mandavdhare HS. Defining 'satisfactory response' to therapy in abdominal tuberculosis : A work in progress. Infect Disord Drug Targets. 2018.
109. Kilpatrick ME, Girgis NI, Yassin MW, Abu el Ella AA. Tuberculous meningitis--clinical and laboratory review of 100 patients. J Hyg (Lond). 1986;96(2):231-8.
110. Marais BJ. Tuberculosis in children. J Paediatr Child Health. 2014;50(10):759-67.
111. Donald PR, Schoeman JF. Tuberculous meningitis. N Engl J Med. 2004;351(17):1719-20.
112. Thwaites GE, Duc Bang N, Huy Dung N, Thi Quy H, Thi Tuong Oanh D, Thi Cam Thoa N, et al. The influence of HIV infection on clinical presentation, response to treatment, and outcome in adults with Tuberculous meningitis. J Infect Dis. 2005;192(12):2134-41.
113. Berenguer J, Moreno S, Laguna F, Vicente T, Adrados M, Ortega A, et al. Tuberculous meningitis in patients infected with the human immunodeficiency virus. N Engl J Med. 1992;326(10):668-72.

114. Katrak SM, Shembalkar PK, Bijwe SR, Bhandarkar LD. The clinical, radiological and pathological profile of tuberculous meningitis in patients with and without human immunodeficiency virus infection. J Neurol Sci. 2000;181(1-2):118-26.
115. Sharma SK, Mohan A, Sharma A. Challenges in the diagnosis & treatment of miliary tuberculosis. Indian J Med Res. 2012;135(5):703-30.
116. Mert A, Arslan F, Kuyucu T, Koc EN, Ylmaz M, Turan D, et al. Miliary tuberculosis: Epidemiologicaland clinical analysis of large-case series from moderate to low tuberculosis endemic Country. Medicine (Baltimore). 2017;96(5):e5875.
117. Hussain SF, Irfan M, Abbasi M, Anwer SS, Davidson S, Haqqee R, et al. Clinical characteristics of 110 miliary tuberculosis patients from a low HIV prevalence country. Int J Tuberc Lung Dis. 2004;8(4):493-9.
118. Kim JY, Park YB, Kim YS, Kang SB, Shin JW, Park IW, et al. Miliary tuberculosis and acute respiratory distress syndrome. Int J Tuberc Lung Dis. 2003;7(4):359-64.
119. Maartens G, Willcox PA, Benatar SR. Miliary tuberculosis: rapid diagnosis, hematologic abnormalities, and outcome in 109 treated adults. Am J Med. 1990;89(3):291-6.
120. Malbruny B, Le Marrec G, Courageux K, Leclercq R, Cattoir V. Rapid and efficient detection of Mycobacterium tuberculosis in respiratory and non-respiratory samples. Int J Tuberc Lung Dis. 2011;15(4):553-5.
121. Hillemann D, Rusch-Gerdes S, Boehme C, Richter E. Rapid molecular detection of extrapulmonary tuberculosis by the automated GeneXpert MTB/RIF system. J Clin Microbiol. 2011;49(4):1202-5.
122. Purohit MR, Mustafa T, Wiker HG, Morkve O, Sviland L. Immunohistochemical diagnosis of abdominal and lymph node tuberculosis by detecting Mycobacterium tuberculosis complex specific antigen MPT64. Diagn Pathol. 2007;2:36.
123. Purohit MR, Mustafa T, Wiker HG, Sviland L. Rapid diagnosis of tuberculosis in aspirate, effusions, and cerebrospinal fluid by immunocytochemical detection of Mycobacterium tuberculosis complex specific antigen MPT64. Diagn Cytopathol. 2012;40(9):782-91.
124. Purohit M, Mustafa T. Laboratory Diagnosis of Extra-pulmonary Tuberculosis (EPTB) in Resource-constrained Setting: State of the Art, Challenges and the Need. J Clin Diagn Res. 2015;9(4):EE01-6.
125. Uddin MK, Chowdhury MR, Ahmed S, Rahman MT, Khatun R, van Leth F, et al. Comparison of direct versus concentrated smear microscopy in detection of pulmonary tuberculosis. BMC Res Notes. 2013;6:291.
126. World Health Organization. Fluorescent light-emitting diode (LED) microscopy for diagnosis of tuberculosis, Policy statement. WHO, Geneva Switzerland. 2011;WHO/HTM/TB/2011.8.
127. Kunkel A, Abel Zur Wiesch P, Nathavitharana RR, Marx FM, Jenkins HE, Cohen T. Smear positivity in paediatric and adult tuberculosis: systematic review and meta-analysis. BMC Infect Dis. 2016;16:282.
128. Monkongdee P, McCarthy KD, Cain KP, Tasaneeyapan T, Nguyen HD, Nguyen TN, et al. Yield of acid-fast smear and mycobacterial culture for tuberculosis diagnosis in people with human immunodeficiency virus. Am J Respir Crit Care Med. 2009;180(9):903-8.
129. Perkins MD, Roscigno G, Zumla A. Progress towards improved tuberculosis diagnostics for developing countries. Lancet. 2006;367(9514):942-3.
130. Bahr NC, Tugume L, Rajasingham R, Kiggundu R, Williams DA, Morawski B, et al. Improved diagnostic sensitivity for tuberculous meningitis with Xpert((R)) MTB/RIF of centrifuged CSF. Int J Tuberc Lung Dis. 2015;19(10):1209-15.
131. Jarvis JN, Meintjes G, Williams A, Brown Y, Crede T, Harrison TS. Adult meningitis in a setting of high HIV and TB prevalence: findings from 4961 suspected cases. BMC Infect Dis. 2010;10:67.
132. Bhigjee AI, Padayachee R, Paruk H, Hallwirth-Pillay KD, Marais S, Connoly C. Diagnosis of tuberculous meningitis: clinical and laboratory parameters. Int J Infect Dis. 2007;11(4):348-54.
133. Aljafari AS, Khalil EA, Elsiddig KE, El Hag IA, Ibrahim ME, Elsafi ME, et al. Diagnosis of tuberculous lymphadenitis by FNAC, microbiological methods and PCR: a comparative study. Cytopathology. 2004;15(1):44-8.

134. Thwaites GE, Chau TT, Farrar JJ. Improving the bacteriological diagnosis of tuberculous meningitis. J Clin Microbiol. 2004;42(1):378-9.
135. Chaidir L, Annisa J, Dian S, Parwati I, Alisjahbana A, Purnama F, et al. Microbiological diagnosis of adult tuberculous meningitis in a ten-year cohort in Indonesia. Diagn Microbiol Infect Dis. 2018;91(1):42-6.
136. Amer S, Hefnawy AE, Wahab NA, Okasha H, Baz A. Evaluation of different laboratory methods for rapid diagnosis of tuberculous pleurisy. Int J Mycobacteriol. 2016;5(4):437-45.
137. Prasanthi K, Kumari AR. Efficacy of fluorochrome stain in the diagnosis of pulmonary tuberculosis co-infected with HIV. Indian J Med Microbiol. 2005;23(3):179-81.
138. Kivihya-Ndugga LE, van Cleeff MR, Githui WA, Nganga LW, Kibuga DK, Odhiambo JA, et al. A comprehensive comparison of Ziehl-Neelsen and fluorescence microscopy for the diagnosis of tuberculosis in a resource-poor urban setting. Int J Tuberc Lung Dis. 2003;7(12):1163-71.
139. Anthony RM, Kolk AH, Kuijper S, Klatser PR. Light emitting diodes for auramine O fluorescence microscopic screening of Mycobacterium tuberculosis. Int J Tuberc Lung Dis. 2006;10(9):1060-2.
140. Steingart KR, Henry M, Ng V, Hopewell PC, Ramsay A, Cunningham J, et al. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. Lancet Infect Dis. 2006;6(9):570-81.
141. Annam V, Kulkarni MH, Puranik RB. Comparison of the modified fluorescent method and conventional Ziehl-Neelsen method in the detection of acidfast bacilli in lymphnode aspirates. Cytojournal. 2009;6:13.
142. Bagdia M, Bijwe S, Hirani N, Joshi A, Chowdhary A, Agrawal M, et al. Lab diagnosis of extra pulmonary tuberculosis: Comparison of histopathology, cytology, ZeihlNeelsen stain and light emission diode microscopy with culture and nucleic acid amplification tests. International Journal of Current Research and Review. 2018;10(8):15-9.
143. Marais BJ, Brittle W, Paincyk K, Hesseling AC, Beyers N, Wasseman E, et al. Use of light-emitting diode fluorescence microscopy to detect acid-fast bacilli in sputum. Clinical Infectious Diseases. 2008;47:203-7.
144. Toman K. What are the main causes of false-positive and false-negative sputum smears? In: Frieden T, editor. Toman`s Tuberculosis: case detection, treatment, and monitoring - questions and answers. 2nd edition. WHO, Geneva, Switzerland2004. p. 23-7.
145. Muricy EC, Lemes RA, Bombarda S, Ferrazoli L, Chimara E. Differentiation between Nocardia spp. and Mycobacterium spp.: Critical aspects for bacteriological diagnosis. Rev Inst Med Trop Sao Paulo. 2014;56(5):397-401.
146. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, et al. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am J Respir Crit Care Med. 2007;175(4):367-416.
147. World Health Organization. Systematic screening for active tuberculosis: principles and recommendations. WHO, Geneva, Switzerland. 2013;WHO/HTM/TB/2013.04.
148. Paglia MG, Bevilacqua N, Haji HS, Vairo F, Girardi E, Nicastri E, et al. Improvement of tuberculosis laboratory capacity on Pemba Island, Zanzibar: a health cooperation project. PLoS One. 2012;7(8):e44109.
149. Asmar S, Drancourt M. Rapid culture-based diagnosis of pulmonary tuberculosis in developed and developing countries. Front Microbiol. 2015;6:1184.
150. Gil-Setas A, Torroba L, Fernandez JL, Martinez-Artola V, Olite J. Evaluation of the MB/BacT system compared with Middlebrook 7H11 and Lowenstein-Jensen media for detection and recovery of mycobacteria from clinical specimens. Clin Microbiol Infect. 2004;10(3):224-8.
151. Watterson SA, Drobniewski FA. Modern laboratory diagnosis of mycobacterial infections. J Clin Pathol. 2000;53(10):727-32.
152. Richter E, Rusch-Gerdes S, Hillemann D. Evaluation of the GenoType Mycobacterium Assay for identification of mycobacterial species from cultures. J Clin Microbiol. 2006;44(5):1769-75.

153. Arora J, Kumar G, Verma AK, Bhalla M, Sarin R, Myneedu VP. Utility of MPT64 Antigen Detection for Rapid Confirmation of Mycobacterium tuberculosis Complex. J Glob Infect Dis. 2015;7(2):66-9.
154. Kim SJ. Drug-susceptibility testing in tuberculosis: methods and reliability of results. Eur Respir J. 2005;25(3):564-9.
155. Pfyffer GE, Wittwer F. Incubation time of mycobacterial cultures: how long is long enough to issue a final negative report to the clinician? J Clin Microbiol. 2012;50(12):4188-9.
156. Saito S, Howard AA, Reid MJ, Elul B, Scardigli A, Verkuijl S, et al. TB diagnostic capacity in sub-Saharan African HIV care settings. J Acquir Immune Defic Syndr. 2012;61(2):216-20.
157. Davies PD, Pai M. The diagnosis and misdiagnosis of tuberculosis. Int J Tuberc Lung Dis. 2008;12(11):1226-34.
158. Negi SS, Khan SF, Gupta S, Pasha ST, Khare S, Lal S. Comparison of the conventional diagnostic modalities, bactec culture and polymerase chain reaction test for diagnosis of tuberculosis. Indian J Med Microbiol. 2005;23(1):29-33.
159. Wu X, Tan G, Gao R, Yao L, Bi D, Guo Y, et al. Assessment of the Xpert MTB/RIF Ultra assay on rapid diagnosis of extrapulmonary tuberculosis. Int J Infect Dis. 2019;81:91-6.
160. Purohit MR, Mustafa T, Sviland L. Detection of Mycobacterium tuberculosis by polymerase chain reaction with DNA eluted from aspirate smears of tuberculous lymphadenitis. Diagn Mol Pathol. 2008;17(3):174-8.
161. Goloubeva V, Lecocq M, Lassowsky P, Matthys F, Portaels F, Bastian I. Evaluation of mycobacteria growth indicator tube for direct and indirect drug susceptibility testing of Mycobacterium tuberculosis from respiratory specimens in a Siberian prison hospital. J Clin Microbiol. 2001;39(4):1501-5.
162. Chihota VN, Grant AD, Fielding K, Ndibongo B, van Zyl A, Muirhead D, et al. Liquid vs. solid culture for tuberculosis: performance and cost in a resource-constrained setting. Int J Tuberc Lung Dis. 2010;14(8):1024-31.
163. Hepple P, Novoa-Cain J, Cheruiyot C, Richter E, Ritmeijer K. Implementation of liquid culture for tuberculosis diagnosis in a remote setting: lessons learned. Int J Tuberc Lung Dis. 2011;15(3):405-7.
164. Muyoyeta M, Schaap JA, De Haas P, Mwanza W, Muvwimi MW, Godfrey-Faussett P, et al. Comparison of four culture systems for Mycobacterium tuberculosis in the Zambian National Reference Laboratory. Int J Tuberc Lung Dis. 2009;13(4):460-5.
165. Hanna BA, Ebrahimzadeh A, Elliott LB, Morgan MA, Novak SM, Rusch-Gerdes S, et al. Multicenter evaluation of the BACTEC MGIT 960 system for recovery of mycobacteria. J Clin Microbiol. 1999;37(3):748-52.
166. World Health Organization. Use of liquid TB culture and drug susceptibility testing (DST) in low- and medium-income settings. Summary report of the Expert Group Meeting on the Use of Liquid Culture Media, Geneva, 26 March 2007. WHO, Geneva Switzerland. 2007.
167. World Health Organization. Xpert MTB/RIF implementation manual: technical and operational 'how-to'; practical considerations. WHO, Geneva, Switzerland. 2014;WHO/HTM/TB/2014.1.
168. Dinnes J, Deeks J, Kunst H, Gibson A, Cummins E, Waugh N, et al. A systematic review of rapid diagnostic tests for the detection of tuberculosis infection. Health Technol Assess. 2007;11(3):1-196.
169. Fakruddin M, Mannan KS, Chowdhury A, Mazumdar RM, Hossain MN, Islam S, et al. Nucleic acid amplification: Alternative methods of polymerase chain reaction. J Pharm Bioallied Sci. 2013;5(4):245-52.
170. Kim SS, Chung SM, Kim JN, Lee MA, Ha EH. Application of PCR from the fine needle aspirates for the diagnosis of cervical tuberculous lymphadenitis. J Korean Med Sci. 1996;11(2):127-32.
171. Goel MM, Budhwar P, Goel M, Tiwari V, Jain A. Nucleic acid amplification of Mycobacterium tuberculosis complex DNA from archival fine needle aspiration smear scrapings vs. fresh fine needle aspirates of tuberculous lymphadenitis. Acta Cytol. 2006;50(4):393-7.

172. Azov AG, Koch J, Hamilton-Dutoit SJ. Improved diagnosis of mycobacterial infections in formalin-fixed and paraffin-embedded sections with nested polymerase chain reaction. APMIS. 2005;113(9):586-93.
173. Daley P, Thomas S, Pai M. Nucleic acid amplification tests for the diagnosis of tuberculous lymphadenitis: a systematic review. Int J Tuberc Lung Dis. 2007;11(11):1166-76.
174. Pai M, Flores LL, Hubbard A, Riley LW, Colford JM, Jr. Nucleic acid amplification tests in the diagnosis of tuberculous pleuritis: a systematic review and meta-analysis. BMC Infect Dis. 2004;4:6.
175. Pai M, Flores LL, Pai N, Hubbard A, Riley LW, Colford JM, Jr. Diagnostic accuracy of nucleic acid amplification tests for tuberculous meningitis: a systematic review and meta-analysis. Lancet Infect Dis. 2003;3(10):633-43.
176. Altez-Fernandez C, Ortiz V, Mirzazadeh M, Zegarra L, Seas C, Ugarte-Gil C. Diagnostic accuracy of nucleic acid amplification tests (NAATs) in urine for genitourinary tuberculosis: a systematic review and meta-analysis. BMC Infect Dis. 2017;17(1):390.
177. Solomons RS, van Elsland SL, Visser DH, Hoek KG, Marais BJ, Schoeman JF, et al. Commercial nucleic acid amplification tests in tuberculous meningitis--a meta-analysis. Diagn Microbiol Infect Dis. 2014;78(4):398-403.
178. Blakemore R, Story E, Helb D, Kop J, Banada P, Owens MR, et al. Evaluation of the analytical performance of the Xpert MTB/RIF assay. J Clin Microbiol. 2010;48(7):2495-501.
179. Helb D, Jones M, Story E, Boehme C, Wallace E, Ho K, et al. Rapid detection of Mycobacterium tuberculosis and rifampin resistance by use of on-demand, near-patient technology. J Clin Microbiol. 2010;48(1):229-37.
180. Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, et al. Rapid molecular detection of tuberculosis and rifampin resistance. N Engl J Med. 2010;363(11):1005-15.
181. Lawn SD, Nicol MP. Xpert(R) MTB/RIF assay: development, evaluation and implementation of a new rapid molecular diagnostic for tuberculosis and rifampicin resistance. Future Microbiol. 2011;6(9):1067-82.
182. World Health Organization. Policy Statement: Automated Real-Time Nucleic Acid Amplification Technology for Rapid and Simultaneous Detection of Tuberculosis and Rifampicin Resistance: Xpert MTB/RIF System. WHO, Geneva, Switzerland. 2011;WHO/HTM/TB/2011.4.
183. Chang K, Lu W, Wang J, Zhang K, Jia S, Li F, et al. Rapid and effective diagnosis of tuberculosis and rifampicin resistance with Xpert MTB/RIF assay: a meta-analysis. J Infect. 2012;64(6):580-8.
184. Steingart KR, Schiller I, Horne DJ, Pai M, Boehme CC, Dendukuri N. Xpert(R) MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. Cochrane Database Syst Rev. 2014(1):CD009593.
185. Lawn SD, Mwaba P, Bates M, Piatek A, Alexander H, Marais BJ, et al. Advances in tuberculosis diagnostics: the Xpert MTB/RIF assay and future prospects for a point-of-care test. Lancet Infect Dis. 2013;13(4):349-61.
186. Tortoli E, Russo C, Piersimoni C, Mazzola E, Dal Monte P, Pascarella M, et al. Clinical validation of Xpert MTB/RIF for the diagnosis of extrapulmonary tuberculosis. Eur Respir J. 2012;40(2):442-7.
187. 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.
188. World Health Organization. Automated real-time nucleic acid amplification technology for rapid and simultaneous detection of tuberculosis and rifampicin resistance: Xpert MTB/RIF system for the diagnosis of pulmonary and extrapulmonary TB in adults and children. WHO, Geneva, Switzerland. 2013;WHO/HTM/TB/2013.16.
189. World Health Organization. WHO Meeting Report of a Technical Expert Consultation: Non-inferiority analysis of Xpert MTB/RIF Ultra compared to Xpert MTB/RIF. WHO, Geneva, Switzerland. 2017;WHO/HTM/TB/2017.04.

190. Chakravorty S, Simmons AM, Rowneki M, Parmar H, Cao Y, Ryan J, et al. The New Xpert MTB/RIF Ultra: Improving Detection of Mycobacterium tuberculosis and Resistance to Rifampin in an Assay Suitable for Point-of-Care Testing. MBio. 2017;8(4).
191. Dorman SE, Schumacher SG, Alland D, Nabeta P, Armstrong DT, King B, et al. Xpert MTB/RIF Ultra for detection of Mycobacterium tuberculosis and rifampicin resistance: a prospective multicentre diagnostic accuracy study. Lancet Infect Dis. 2018;18(1):76-84.
192. Bisognin F, Lombardi G, Lombardo D, Re MC, Dal Monte P. Improvement of Mycobacterium tuberculosis detection by Xpert MTB/RIF Ultra: A head-to-head comparison on Xpert-negative samples. PLoS One. 2018;13(8):e0201934.
193. Bahr NC, Nuwagira E, Evans EE, Cresswell FV, Bystrom PV, Byamukama A, et al. Diagnostic accuracy of Xpert MTB/RIF Ultra for tuberculous meningitis in HIV-infected adults: a prospective cohort study. Lancet Infect Dis. 2018;18(1):68-75.
194. Perez-Risco D, Rodriguez-Temporal D, Valledor-Sanchez I, Alcaide F. Evaluation of the Xpert MTB/RIF Ultra Assay for Direct Detection of Mycobacterium tuberculosis Complex in Smear-Negative Extrapulmonary Samples. J Clin Microbiol. 2018;56(9).
195. Atherton RR, Cresswell FV, Ellis J, Skipper C, Tadeo KK, Mugumya G, et al. Detection of Mycobacterium tuberculosis in urine by Xpert MTB/RIF Ultra: A useful adjunctive diagnostic tool in HIV-associated tuberculosis. Int J Infect Dis. 2018;75:92-4.
196. Donovan J, Thu DDA, Phu NH, Dung VTM, Quang TP, Nghia HDT, et al. Xpert MTB/RIF Ultra versus Xpert MTB/RIF for the diagnosis of tuberculous meningitis: a prospective, randomised, diagnostic accuracy study. Lancet Infect Dis. 2020;20(3):299-307.
197. Zhang M, Xue M, He JQ. Diagnostic accuracy of the new Xpert MTB/RIF Ultra for tuberculosis disease: A preliminary systematic review and meta-analysis. Int J Infect Dis. 2020;90:35-45.
198. World Health Organization. WHO consolidated guidelines on tuberculosis. Module 3: diagnosis – rapid diagnostics for tuberculosis detection. WHO, Geneva Switzerland. 2020.
199. Small PM, Pai M. Tuberculosis diagnosis--time for a game change. N Engl J Med. 2010;363(11):1070-1.
200. Banada PP, Sivasubramani SK, Blakemore R, Boehme C, Perkins MD, Fennelly K, et al. Containment of bioaerosol infection risk by the Xpert MTB/RIF assay and its applicability to point-of-care settings. J Clin Microbiol. 2010;48(10):3551-7.
201. Trebucq A, Enarson DA, Chiang CY, Van Deun A, Harries AD, Boillot F, et al. Xpert(R) MTB/RIF for national tuberculosis programmes in low-income countries: when, where and how? Int J Tuberc Lung Dis. 2011;15(12):1567-72.
202. World Health Organization. Pathways to better diagnostics for tuberculosis: a blueprint for the development of TB diagnostics by the new diagnostics working group of the Stop TB partnership. Geneva, Switzerland; 2009.
203. Mudaliar AV, Kashyap RS, Purohit HJ, Taori GM, Daginawala HF. Detection of 65 kD heat shock protein in cerebrospinal fluid of tuberculous meningitis patients. BMC Neurol. 2006;6:34.
204. Boehme C, Molokova E, Minja F, Geis S, Loscher T, Maboko L, et al. Detection of mycobacterial lipoarabinomannan with an antigen-capture ELISA in unprocessed urine of Tanzanian patients with suspected tuberculosis. Trans R Soc Trop Med Hyg. 2005;99(12):893-900.
205. Peter JG, Theron G, van Zyl-Smit R, Haripersad A, Mottay L, Kraus S, et al. Diagnostic accuracy of a urine lipoarabinomannan strip-test for TB detection in HIV-infected hospitalised patients. Eur Respir J. 2012;40(5):1211-20.
206. Krambovitis E, McIllmurray MB, Lock PE, Hendrickse W, Holzel H. Rapid diagnosis of tuberculous meningitis by latex particle agglutination. Lancet. 1984;2(8414):1229-31.
207. Carabias E, Palenque E, Serrano R, Aguado JM, Ballestin C. Evaluation of an immunohistochemical test with polyclonal antibodies raised against mycobacteria used in formalin-fixed tissue compared with mycobacterial specific culture. APMIS. 1998;106(3):385-8.

208. Goel MM, Budhwar P. Species-specific immunocytochemical localization of Mycobacterium tuberculosis complex in fine needle aspirates of tuberculous lymphadenitis using antibody to 38 kDa immunodominant protein antigen. Acta Cytol. 2008;52(4):424-33.
209. Flores LL, Steingart KR, Dendukuri N, Schiller I, Minion J, Pai M, et al. Systematic review and meta-analysis of antigen detection tests for the diagnosis of tuberculosis. Clin Vaccine Immunol. 2011;18(10):1616-27.
210. Hamasur B, Bruchfeld J, Haile M, Pawlowski A, Bjorvatn B, Kallenius G, et al. Rapid diagnosis of tuberculosis by detection of mycobacterial lipoarabinomannan in urine. J Microbiol Methods. 2001;45(1):41-52.
211. Lawn SD. Point-of-care detection of lipoarabinomannan (LAM) in urine for diagnosis of HIV-associated tuberculosis: a state of the art review. BMC Infect Dis. 2012;12:103.
212. Mutetwa R, Boehme C, Dimairo M, Bandason T, Munyati SS, Mangwanya D, et al. Diagnostic accuracy of commercial urinary lipoarabinomannan detection in African tuberculosis suspects and patients. Int J Tuberc Lung Dis. 2009;13(10):1253-9.
213. Reither K, Saathoff E, Jung J, Minja LT, Kroidl I, Saad E, et al. Low sensitivity of a urine LAM-ELISA in the diagnosis of pulmonary tuberculosis. BMC Infect Dis. 2009;9:141.
214. Minion J, Leung E, Talbot E, Dheda K, Pai M, Menzies D. Diagnosing tuberculosis with urine lipoarabinomannan: systematic review and meta-analysis. Eur Respir J. 2011;38(6):1398-405.
215. Lawn SD, Kerkhoff AD, Vogt M, Wood R. Diagnostic accuracy of a low-cost, urine antigen, point-of-care screening assay for HIV-associated pulmonary tuberculosis before antiretroviral therapy: a descriptive study. Lancet Infect Dis. 2012;12(3):201-9.
216. Gounder CR, Kufa T, Wada NI, Mngomezulu V, Charalambous S, Hanifa Y, et al. Diagnostic accuracy of a urine lipoarabinomannan enzyme-linked immunosorbent assay for screening ambulatory HIV-infected persons for tuberculosis. J Acquir Immune Defic Syndr. 2011;58(2):219-23.
217. Correia-Neves M, Froberg G, Korshun L, Viegas S, Vaz P, Ramanlal N, et al. Biomarkers for tuberculosis: the case for lipoarabinomannan. ERJ Open Res. 2019;5(1).
218. World Health Organization. Lateral flow urine lipoarabinomannan assay (LF-LAM) for the diagnosis of active tuberculosis in people living with HIV. Policy update 2019. WHO, Geneva Switzerland. 2019;WHO/CDS/TB/2019.16.
219. Kwizera R, Cresswell FV, Mugumya G, Okirwoth M, Kagimu E, Bangdiwala AS, et al. Performance of Lipoarabinomannan Assay using Cerebrospinal fluid for the diagnosis of Tuberculous meningitis among HIV patients. Wellcome Open Res. 2019;4:123.
220. Dheda K, Van-Zyl Smit RN, Sechi LA, Badri M, Meldau R, Symons G, et al. Clinical diagnostic utility of IP-10 and LAM antigen levels for the diagnosis of tuberculous pleural effusions in a high burden setting. PLoS One. 2009;4(3):e4689.
221. World Health Organization. The use of lateral flow urine lipoarabinomannan assay (LF-LAM) for the diagnosis and screening of active tuberculosis in people living with HIV. Policy Guidance. WHO, Geneva; Switzerland. 2015;WHO/HTM/TB/2015.25.
222. Sumi S, Radhakrishnan VV. Evaluation of immunohistochemistry with a panel of antibodies against recombinant mycobacterial antigens for the diagnosis of tuberculous lymphadenitis. International Journal of Medicine and Medical Sciences. 2009;Vol 1.(5) (May):pp. 215-9.
223. Barbolini G, Bisette A, Colizzi V, Damiani G, Migaldi M, Vismara D. Immunohistological analysis of mycobacterial antigens by monocloncal antibodies in tuberculosis and mycobacteriosis. Hum Pathol. 1989;20:1078–83.
224. Ulrichs T, Lefmann M, Reich M, Morawietz L, Roth A, Brinkmann V, et al. Modified immunohistological staining allows detection of Ziehl-Neelsen-negative Mycobacterium tuberculosis organisms and their precise localization in human tissue. J Pathol. 2005;205(5):633-40.
225. Mukherjee A, Kalra N, Beena KR. Immunohistochemical detection of mycobacterial antigen in tuberculous lymphadenitis. Indian J Tuberc. 2002;49:213-6.
226. Kohli R, Punia RS, Kaushik R, Kundu R, Mohan H. Relative value of immunohistochemistry in detection of mycobacterial antigen in suspected cases of tuberculosis in tissue sections. Indian J Pathol Microbiol. 2014;57(4):574-8.

227. Padmavathy I, Lakshmana Rao L, Ramanadhan., Shakila. Mycobaterial antigen in tissues in diagnosis of cutanous tuberculosis. Indian J Tuberc. 2005; 52:31-5.
228. Ahmed NY. Anti-BCG Immunohistochemical Detection of Mycobacteria in Formalin-Fixed Paraffin-Embedded Tissue Samples if Granulomatous Lymphadenitis. Journal of University of Babylon, Pure and Applied Sciences. 2018;29(9).
229. Wiley EL, Mulhollan TJ, Beck B, Tyndall JA, Freeman RG. Polyclonal antibodies raised against Bacillus Calmette-Guerin, Mycobacterium duvalii, and Mycobacterium paratuberculosis used to detect mycobacteria in tissue with the use of immunohistochemical techniques. Am J Clin Pathol. 1990;94(3):307-12.
230. Humphrey DM, Weiner MH. Mycobacterial antigen detection by immunohistochemistry in pulmonary tuberculosis. Hum Pathol. 1987;18(7):701-8.
231. Prapanna P, Srivastava R, Arora VK, Singh N, Bhatia A, Kaur IR. Immunocytochemical detection of mycobacterial antigen in extrapulmonary tuberculosis. Diagn Cytopathol. 2014;42(5):391-5.
232. Kutzner H, Argenyi ZB, Requena L, Rutten A, Hugel H. A new application of BCG antibody for rapid screening of various tissue microorganisms. J Am Acad Dermatol. 1998;38(1):56-60.
233. Sumi MG, Mathai A, Reuben S, Sarada C, Radhakrishnan VV. Immunocytochemical method for early laboratory diagnosis of tuberculous meningitis. Clin Diagn Lab Immunol. 2002;9(2):344-7.
234. Harboe M, Wiker HG. The 38-kDa protein of Mycobacterium tuberculosis: a review. J Infect Dis. 1992;166(4):874-84.
235. Thangaraj HS, Bull TJ, De Smet KA, Hill MK, Rouse DA, Moreno C, et al. Duplication of genes encoding the immunodominant 38 kDa antigen in Mycobacterium intracellulare. FEMS Microbiol Lett. 1996;144(2-3):235-40.
236. Freeman R, Magee J, Barratt A, Wheeler J, Steward M, Lee M, et al. Rapid immunochromatographic assay for diagnosis of tuberculosis: antibodies detected may not be specific. J Clin Microbiol. 1999;37(6):2111-2.
237. Damiani G, Biano A, Beltrame A, Vismara D, Mezzopreti MF, Colizzi V, et al. Generation and characterization of monoclonal antibodies to 28-, 35-, and 65-kilodalton proteins of Mycobacterium tuberculosis. Infect Immun. 1988;56(5):1281-7.
238. Nagai S, Wiker HG, Harboe M, Kinomoto M. Isolation and partial characterization of major protein antigens in the culture fluid of Mycobacterium tuberculosis. Infect Immun. 1991;59(1):372-82.
239. Oettinger T, Andersen AB. Cloning and B-cell-epitope mapping of MPT64 from Mycobacterium tuberculosis H37Rv. Infect Immun. 1994;62(5):2058-64.
240. Andersen AB, Ljungqvist L, Haslov K, Bentzon MW. MPB 64 possesses 'tuberculosis-complex'-specific B- and T-cell epitopes. Scand J Immunol. 1991;34(3):365-72.
241. Wiker HG, Harboe M, Nagai S. A localization index for distinction between extracellular and intracellular antigens of Mycobacterium tuberculosis. J Gen Microbiol. 1991;137(4):875-84.
242. Harboe M, Nagai S, Patarroyo ME, Torres ML, Ramirez C, Cruz N. Properties of proteins MPB64, MPB70, and MPB80 of Mycobacterium bovis BCG. Infect Immun. 1986;52(1):293-302.
243. Li H, Ulstrup JC, Jonassen TO, Melby K, Nagai S, Harboe M. Evidence for absence of the MPB64 gene in some substrains of Mycobacterium bovis BCG. Infect Immun. 1993;61(5):1730-4.
244. Mahairas GG, Sabo PJ, Hickey MJ, Singh DC, Stover CK. Molecular analysis of genetic differences between Mycobacterium bovis BCG and virulent M. bovis. J Bacteriol. 1996;178(5):1274-82.
245. Elhay MJ, Oettinger T, Andersen P. Delayed-type hypersensitivity responses to ESAT-6 and MPT64 from Mycobacterium tuberculosis in the guinea pig. Infect Immun. 1998;66(7):3454-6.
246. Mustafa T, Wiker HG, Mfinanga SG, Morkve O, Sviland L. Immunohistochemistry using a Mycobacterium tuberculosis complex specific antibody for improved diagnosis of tuberculous lymphadenitis. Mod Pathol. 2006;19(12):1606-14.

247. Purohit MR, Sviland L, Wiker H, Mustafa T. Rapid and Specific Diagnosis of Extrapulmonary Tuberculosis by Immunostaining of Tissues and Aspirates With Anti-MPT64. Appl Immunohistochem Mol Morphol. 2017;25(4):282-8.
248. Steingart KR, Henry M, Laal S, Hopewell PC, Ramsay A, Menzies D, et al. A systematic review of commercial serological antibody detection tests for the diagnosis of extrapulmonary tuberculosis. Postgrad Med J. 2007;83(985):705-12.
249. World Health Organization. Commercial serodiagnostic tests for diagnosis of tuberculosis, Policy Statement. WHO, Geneva Switzerland. 2011;WHO/HTM/TB/2011.5.
250. Steingart KR, Ramsay A, Dowdy DW, Pai M. Serological tests for the diagnosis of active tuberculosis: relevance for India. Indian J Med Res. 2012;135(5):695-702.
251. Steingart KR, Flores LL, Dendukuri N, Schiller I, Laal S, Ramsay A, et al. Commercial serological tests for the diagnosis of active pulmonary and extrapulmonary tuberculosis: an updated systematic review and meta-analysis. PLoS Med. 2011;8(8):e1001062.
252. Grenier J, Pinto L, Nair D, Steingart K, Dowdy D, Ramsay A, et al. Widespread use of serological tests for tuberculosis: data from 22 high-burden countries. Eur Respir J. 2012;39(2):502-5.
253. Pai M, Sotgiu G. Diagnostics for latent TB infection: incremental, not transformative progress. Eur Respir J. 2016;47(3):704-6.
254. Metcalfe JZ, Everett CK, Steingart KR, Cattamanchi A, Huang L, Hopewell PC, et al. Interferon-gamma release assays for active pulmonary tuberculosis diagnosis in adults in low- and middle-income countries: systematic review and meta-analysis. J Infect Dis. 2011;204 Suppl 4:S1120-9.
255. Rangaka MX, Wilkinson KA, Glynn JR, Ling D, Menzies D, Mwansa-Kambafwile J, et al. Predictive value of interferon-gamma release assays for incident active tuberculosis: a systematic review and meta-analysis. Lancet Infect Dis. 2012;12(1):45-55.
256. Menzies D, Gardiner G, Farhat M, Greenaway C, Pai M. Thinking in three dimensions: a web-based algorithm to aid the interpretation of tuberculin skin test results. Int J Tuberc Lung Dis. 2008;12(5):498-505.
257. Andersen P, Munk ME, Pollock JM, Doherty TM. Specific immune-based diagnosis of tuberculosis. Lancet. 2000;356(9235):1099-104.
258. Farhat M, Greenaway C, Pai M, Menzies D. False-positive tuberculin skin tests: what is the absolute effect of BCG and non-tuberculous mycobacteria? Int J Tuberc Lung Dis. 2006;10(11):1192-204.
259. Nash DR, Douglass JE. Anergy in active pulmonary tuberculosis. A comparison between positive and negative reactors and an evaluation of 5 TU and 250 TU skin test doses. Chest. 1980;77(1):32-7.
260. Redelman-Sidi G, Sepkowitz KA. IFN-gamma release assays in the diagnosis of latent tuberculosis infection among immunocompromised adults. Am J Respir Crit Care Med. 2013;188(4):422-31.
261. Berthet FX, Rasmussen PB, Rosenkrands I, Andersen P, Gicquel B. A Mycobacterium tuberculosis operon encoding ESAT-6 and a novel low-molecular-mass culture filtrate protein (CFP-10). Microbiology. 1998;144 (Pt 11):3195-203.
262. Harboe M, Oettinger T, Wiker HG, Rosenkrands I, Andersen P. Evidence for occurrence of the ESAT-6 protein in Mycobacterium tuberculosis and virulent Mycobacterium bovis and for its absence in Mycobacterium bovis BCG. Infect Immun. 1996;64(1):16-22.
263. Pai M, Menzies D. Interferon-gamma release assays: what is their role in the diagnosis of active tuberculosis? Clin Infect Dis. 2007;44(1):74-7.
264. Fan L, Chen Z, Hao XH, Hu ZY, Xiao HP. Interferon-gamma release assays for the diagnosis of extrapulmonary tuberculosis: a systematic review and meta-analysis. FEMS Immunol Med Microbiol. 2012;65(3):456-66.
265. Aggarwal AN, Agarwal R, Gupta D, Dhooria S, Behera D. Correction for Aggarwal et al., Interferon Gamma Release Assays for Diagnosis of Pleural Tuberculosis: a Systematic Review and Meta-Analysis. J Clin Microbiol. 2016;54(2):508.
266. Yu J, Wang ZJ, Chen LH, Li HH. Diagnostic accuracy of interferon-gamma release assays for tuberculous meningitis: a meta-analysis. Int J Tuberc Lung Dis. 2016;20(4):494-9.

267. Gakis C. Adenosine deaminase (ADA) isoenzymes ADA1 and ADA2: diagnostic and biological role. Eur Respir J. 1996;9(4):632-3.
268. Trajman A, Pai M, Dheda K, van Zyl Smit R, Zwerling AA, Joshi R, et al. Novel tests for diagnosing tuberculous pleural effusion: what works and what does not? Eur Respir J. 2008;31(5):1098-106.
269. Riquelme A, Calvo M, Salech F, Valderrama S, Pattillo A, Arellano M, et al. Value of adenosine deaminase (ADA) in ascitic fluid for the diagnosis of tuberculous peritonitis: a meta-analysis. J Clin Gastroenterol. 2006;40(8):705-10.
270. Gupta BK, Bharat A, Debapriya B, Baruah H. Adenosine Deaminase Levels in CSF of Tuberculous Meningitis Patients. J Clin Med Res. 2010;2(5):220-4.
271. Liang QL, Shi HZ, Wang K, Qin SM, Qin XJ. Diagnostic accuracy of adenosine deaminase in tuberculous pleurisy: a meta-analysis. Respir Med. 2008;102(5):744-54.
272. Vorster MJ, Allwood BW, Diacon AH, Koegelenberg CF. Tuberculous pleural effusions: advances and controversies. J Thorac Dis. 2015;7(6):981-91.
273. Lee SJ, Kim HS, Lee SH, Lee TW, Lee HR, Cho YJ, et al. Factors influencing pleural adenosine deaminase level in patients with tuberculous pleurisy. Am J Med Sci. 2014;348(5):362-5.
274. Tuon FF, Higashino HR, Lopes MI, Litvoc MN, Atomiya AN, Antonangelo L, et al. Adenosine deaminase and tuberculous meningitis--a systematic review with meta-analysis. Scand J Infect Dis. 2010;42(3):198-207.
275. Jimenez Castro D, Diaz Nuevo G, Perez-Rodriguez E, Light RW. Diagnostic value of adenosine deaminase in nontuberculous lymphocytic pleural effusions. Eur Respir J. 2003;21(2):220-4.
276. Burgess LJ, Maritz FJ, Le Roux I, Taljaard JJ. Combined use of pleural adenosine deaminase with lymphocyte/neutrophil ratio. Increased specificity for the diagnosis of tuberculous pleuritis. Chest. 1996;109(2):414-9.
277. Garcia-Zamalloa A, Taboada-Gomez J. Diagnostic accuracy of adenosine deaminase and lymphocyte proportion in pleural fluid for tuberculous pleurisy in different prevalence scenarios. PLoS One. 2012;7(6):e38729.
278. Marais S, Thwaites G, Schoeman JF, Torok ME, Misra UK, Prasad K, et al. Tuberculous meningitis: a uniform case definition for use in clinical research. Lancet Infect Dis. 2010;10(11):803-12.
279. Light RW, Macgregor MI, Luchsinger PC, Ball WC, Jr. Pleural effusions: the diagnostic separation of transudates and exudates. Ann Intern Med. 1972;77(4):507-13.
280. de Almeida SM, Nogueira MB, Raboni SM, Vidal LR. Laboratorial diagnosis of lymphocytic meningitis. Braz J Infect Dis. 2007;11(5):489-95.
281. Rahman NM, Chapman SJ, Davies RJ. Pleural effusion: a structured approach to care. Br Med Bull. 2004;72:31-47.
282. Seibert AF, Haynes J, Jr., Middleton R, Bass JB, Jr. Tuberculous pleural effusion. Twenty-year experience. Chest. 1991;99(4):883-6.
283. Chow KM, Chow VC, Hung LC, Wong SM, Szeto CC. Tuberculous peritonitis-associated mortality is high among patients waiting for the results of mycobacterial cultures of ascitic fluid samples. Clin Infect Dis. 2002;35(4):409-13.
284. Wright CA. Fine-needle aspiration biopsy of lymph nodes. CME. 2012;30(2):56-60.
285. Wright CA, van der Burg M, Geiger D, Noordzij JG, Burgess SM, Marais BJ. Diagnosing mycobacterial lymphadenitis in children using fine needle aspiration biopsy: cytomorphology, ZN staining and autofluorescence -- making more of less. Diagn Cytopathol. 2008;36(4):245-51.
286. Rammeh S, Romdhane E, Arfaoui Toumi A, Houcine Y, Lahiani R, Sassi A, et al. Efficacy of Fine-Needle Aspiration Cytology in the Diagnosis of Tuberculous Cervical Lymphadenitis. Acta Cytol. 2018;62(2):99-103.
287. Orell SR, Sterrett GF. Infectious diseases. Orell and Sterrett`s fine needle aspiration cytology. 5th ed: Churchill Livingstone, Elsevier Limited; 2012. p. 451-67.
288. Shah KK, Pritt BS, Alexander MP. Histopathologic review of granulomatous inflammation. Journal of Clinical Tuberculosis and Other Mycobacterial Diseases. 2017;7:1-12.

289. Lai KK, Stottmeier KD, Sherman IH, McCabe WR. Mycobacterial cervical lymphadenopathy. Relation of etiologic agents to age. JAMA. 1984;251(10):1286-8.
290. Wright CA, Hoek KG, Marais BJ, van Helden P, Warren RM. Combining fine-needle aspiration biopsy (FNAB) and high-resolution melt analysis to reduce diagnostic delay in Mycobacterial lymphadenitis. Diagn Cytopathol. 2010;38(7):482-8.
291. Senkoro M, Mfinanga S, Egwaga S, Mtandu R, Kamara DV, Basra D, et al. Prevalence of pulmonary tuberculosis in adult population of Tanzania: a national survey, 2012. Int J Tuberc Lung Dis. 2016;20(8):1014-21.
292. Nigeria FRo. Report FIRST National TB Prevalence Survey 2012, Nigeria. 2012.
293. World Health Organization. Global tuberculosis report 2018. WHO, Geneva Switzerland. 2018;WHO/CDS/TB/2018.20.
294. Bothamley GH, Ditiu L, Migliori GB, Lange C, contributors T. Active case finding of tuberculosis in Europe: a Tuberculosis Network European Trials Group (TBNET) survey. Eur Respir J. 2008;32(4):1023-30.
295. Lonnroth K, Corbett E, Golub J, Godfrey-Faussett P, Uplekar M, Weil D, et al. Systematic screening for active tuberculosis: rationale, definitions and key considerations. Int J Tuberc Lung Dis. 2013;17(3):289-98.
296. Fox GJ, Barry SE, Britton WJ, Marks GB. Contact investigation for tuberculosis: a systematic review and meta-analysis. Eur Respir J. 2013;41(1):140-56.
297. World Health Organization. Recommendations for the investigation of contacts of persons with infectious tuberculosis in low- and middle-income countries. WHO, Geneva Switzerland. 2012;WHO/HTM/TB/2012.9.
298. Purohit MR, Purohit R, Mustafa T. Patient Health Seeking and Diagnostic Delay in Extrapulmonary Tuberculosis: A Hospital Based Study from Central India. Tuberc Res Treat. 2019;2019:4840561.
299. Finnie RK, Khoza LB, van den Borne B, Mabunda T, Abotchie P, Mullen PD. Factors associated with patient and health care system delay in diagnosis and treatment for TB in sub-Saharan African countries with high burdens of TB and HIV. Trop Med Int Health. 2011;16(4):394-411.
300. Storla DG, Yimer S, Bjune GA. A systematic review of delay in the diagnosis and treatment of tuberculosis. BMC Public Health. 2008;8:15.
301. Meintjes G, Schoeman H, Morroni C, Wilson D, Maartens G. Patient and provider delay in tuberculosis suspects from communities with a high HIV prevalence in South Africa: a cross-sectional study. BMC Infect Dis. 2008;8:72.
302. Kamolratanakul P, Sawert H, Kongsin S, Lertmaharit S, Sriwongsa J, Na-Songkhla S, et al. Economic impact of tuberculosis at the household level. Int J Tuberc Lung Dis. 1999;3(7):596-602.
303. Mauch V, Woods N, Kirubi B, Kipruto H, Sitienei J, Klinkenberg E. Assessing access barriers to tuberculosis care with the tool to Estimate Patients' Costs: pilot results from two districts in Kenya. BMC Public Health. 2011;11:43.
304. Belay M, Bjune G, Ameni G, Abebe F. Diagnostic and treatment delay among Tuberculosis patients in Afar Region, Ethiopia: a cross-sectional study. BMC Public Health. 2012;12:369.
305. Saldana L, Abid M, McCarthy N, Hunter N, Inglis R, Anders K. Factors affecting delay in initiation of treatment of tuberculosis in the Thames Valley, UK. Public Health. 2013;127(2):171-7.
306. Basnet R, Hinderaker SG, Enarson D, Malla P, Morkve O. Delay in the diagnosis of tuberculosis in Nepal. BMC Public Health. 2009;9:236.
307. Gele AA, Bjune G, Abebe F. Pastoralism and delay in diagnosis of TB in Ethiopia. BMC Public Health. 2009;9:5.
308. Kalra A. Care seeking and treatment related delay among childhood tuberculosis patients in Delhi, India. Int J Tuberc Lung Dis. 2017;21(6):645-50.
309. Wandwalo ER, Morkve O. Delay in tuberculosis case-finding and treatment in Mwanza, Tanzania. Int J Tuberc Lung Dis. 2000;4(2):133-8.
310. Ngadaya ES, Mfinanga GS, Wandwalo ER, Morkve O. Delay in tuberculosis case detection in Pwani region, Tanzania. A cross sectional study. BMC Health Serv Res. 2009;9:196.

311. Hinderaker SG, Madland S, Ullenes M, Enarson DA, Rusen I, Kamara D. Treatment delay among tuberculosis patients in Tanzania: data from the FIDELIS initiative. BMC Public Health. 2011;11:306.
312. World Health Organization. Treatment of tuberculosis: guidelines - 4th edition. WHO, Geneva, Switzerland. 2009;WHO/HTM/TB/2009.420.
313. Nahid P, Dorman SE, Alipanah N, Barry PM, Brozek JL, Cattamanchi A, et al. Official American Thoracic Society/Centers for Disease Control and Prevention/Infectious Diseases Society of America Clinical Practice Guidelines: Treatment of Drug-Susceptible Tuberculosis. Clin Infect Dis. 2016;63(7):e147-e95.
314. Ministry of Health and Social Welfare, National Tuberculosis and Leprosy Programme. Manual for the Management of Tuberculosis and Leprosy. Ministry of Health and Social Welfare, National Tuberculosis and Leprosy Programme, Dar Es Salaam, The United Republic of Tanzania. 2013;6th edition.
315. World Health Organization. Guidance for national tuberculosis programmes on the management of tuberculosis in children, 2nd edition. 2014;WHO/HTM/TB/2014.03.
316. Thwaites GE. Advances in the diagnosis and treatment of tuberculous meningitis. Curr Opin Neurol. 2013;26(3):295-300.
317. Horsburgh CR, Jr., Barry CE, 3rd, Lange C. Treatment of Tuberculosis. N Engl J Med. 2015;373(22):2149-60.
318. Monedero I, Caminero JA. Evidence for promoting fixed-dose combination drugs in tuberculosis treatment and control: a review. Int J Tuberc Lung Dis. 2011;15(4):433-9.
319. Geri G, Passeron A, Heym B, Arlet JB, Pouchot J, Capron L, et al. Paradoxical reactions during treatment of tuberculosis with extrapulmonary manifestations in HIV-negative patients. Infection. 2013;41(2):537-43.
320. Cho OH, Park KH, Kim T, Song EH, Jang EY, Lee EJ, et al. Paradoxical responses in non-HIV-infected patients with peripheral lymph node tuberculosis. J Infect. 2009;59(1):56-61.
321. Meintjes G, Lawn SD, Scano F, Maartens G, French MA, Worodria W, et al. Tuberculosis-associated immune reconstitution inflammatory syndrome: case definitions for use in resource-limited settings. Lancet Infect Dis. 2008;8(8):516-23.
322. Bell LC, Breen R, Miller RF, Noursadeghi M, Lipman M. Paradoxical reactions and immune reconstitution inflammatory syndrome in tuberculosis. Int J Infect Dis. 2015;32:39-45.
323. Breen RA, Smith CJ, Bettinson H, Dart S, Bannister B, Johnson MA, et al. Paradoxical reactions during tuberculosis treatment in patients with and without HIV co-infection. Thorax. 2004;59(8):704-7.
324. Muller M, Wandel S, Colebunders R, Attia S, Furrer H, Egger M, et al. Immune reconstitution inflammatory syndrome in patients starting antiretroviral therapy for HIV infection: a systematic review and meta-analysis. Lancet Infect Dis. 2010;10(4):251-61.
325. Lawn SD, Meintjes G, McIlleron H, Harries AD, Wood R. Management of HIV-associated tuberculosis in resource-limited settings: a state-of-the-art review. BMC Med. 2013;11:253.
326. Hopewell PC, Fair EL, Uplekar M. Updating the International Standards for Tuberculosis Care. Entering the era of molecular diagnostics. Ann Am Thorac Soc. 2014;11(3):277-85.
327. Sharma V, Mandavdhare HS, Lamoria S, Singh H, Kumar A. Serial C-reactive protein measurements in patients treated for suspected abdominal tuberculosis. Dig Liver Dis. 2018;50(6):559-62.
328. Gaikwad UN, Gaikwad NR. Modalities to monitor the treatment response in tuberculosis. Indian J Tuberc. 2018;65(2):109-17.
329. Goletti D, Lindestam Arlehamn CS, Scriba TJ, Anthony R, Cirillo DM, Alonzi T, et al. Can we predict tuberculosis cure? What tools are available? Eur Respir J. 2018;52(5).
330. Wilson D, Nachega J, Morroni C, Chaisson R, Maartens G. Diagnosing smear-negative tuberculosis using case definitions and treatment response in HIV-infected adults. Int J Tuberc Lung Dis. 2006;10(1):31-8.
331. EuroQol G. EuroQol--a new facility for the measurement of health-related quality of life. Health Policy. 1990;16(3):199-208.

332. Karnofsky DA, Abelmann WH, Craver LF, Burdhenal JH. The use of the nitrogen mustards in the palliative treatment of carcinoma: With Particular reference to bronchogenic carcinoma. Cancer. 1948(November):634-56.
333. Franchi D, Wenzel RP. Measuring health-related quality of life among patients infected with human immunodeficiency virus. Clin Infect Dis. 1998;26(1):20-6.
334. Peus D, Newcomb N, Hofer S. Appraisal of the Karnofsky Performance Status and proposal of a simple algorithmic system for its evaluation. BMC Med Inform Decis Mak. 2013;13:72.
335. Bernabe-Ortiz A, Carcamo CP, Sanchez JF, Rios J. Weight variation over time and its association with tuberculosis treatment outcome: a longitudinal analysis. PLoS One. 2011;6(4):e18474.
336. Hoa NB, Lauritsen JM, Rieder HL. Changes in body weight and tuberculosis treatment outcome in Viet Nam. Int J Tuberc Lung Dis. 2013;17(1):61-6.
337. Krapp F, Veliz JC, Cornejo E, Gotuzzo E, Seas C. Bodyweight gain to predict treatment outcome in patients with pulmonary tuberculosis in Peru. Int J Tuberc Lung Dis. 2008;12(10):1153-9.
338. Khan A, Sterling TR, Reves R, Vernon A, Horsburgh CR. Lack of weight gain and relapse risk in a large tuberculosis treatment trial. Am J Respir Crit Care Med. 2006;174(3):344-8.
339. Pai M, Minion J, Steingart K, Ramsay A. New and improved tuberculosis diagnostics: evidence, policy, practice, and impact. Curr Opin Pulm Med. 2010;16(3):271-84.
340. Mann G, Squire SB, Bissell K, Eliseev P, Du Toit E, Hesseling A, et al. Beyond accuracy: creating a comprehensive evidence base for TB diagnostic tools. Int J Tuberc Lung Dis. 2010;14(12):1518-24.
341. Bruns DE. The STARD initiative and the reporting of studies of diagnostic accuracy. Clin Chem. 2003;49(1):19-20.
342. Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, et al. Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. Clin Chem Lab Med. 2003;41(1):68-73.
343. Whiting PF, Rutjes AW, Westwood ME, Mallett S, Deeks JJ, Reitsma JB, et al. QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies. Ann Intern Med. 2011;155(8):529-36.
344. Schunemann HJ, Oxman AD, Brozek J, Glasziou P, Jaeschke R, Vist GE, et al. Grading quality of evidence and strength of recommendations for diagnostic tests and strategies. BMJ. 2008;336(7653):1106-10.
345. Mustafa T, Leversen NA, Sviland L, Wiker HG. Differential in vivo expression of mycobacterial antigens in Mycobacterium tuberculosis infected lungs and lymph node tissues. BMC Infect Dis. 2014;14:535.
346. Baba K, Dyrhol-Riise AM, Sviland L, Langeland N, Hoosen AA, 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.
347. Tadele A, Beyene D, Hussein J, Gemechu T, Birhanu A, Mustafa T, et al. Immunocytochemical detection of Mycobacterium Tuberculosis complex specific antigen, MPT64, improves diagnosis of tuberculous lymphadenitis and tuberculous pleuritis. BMC Infect Dis. 2014;14:585.
348. National Bureau of Statistics Ministry of Finance Dar es Salaam, Office of Chief Government Statistician President's Office Finance Economy and Development Planning Zanzibar. Population distributon by age and sex. The United Republic of Tanzania. 2013 [Available from: https://ihi.eprints.org/2169/1/Age_Sex_Distribution.pdf.
349. Kessy F, Omar M. Status and progress in human developement in Zanzibar. The Economic and Social Research Foundation, Dar es Salaam, Tanzania. 2016.
350. Tanzania Commission for AIDS (TACAIDS), Zanzibar AIDS Commission (ZAC), National Bureau of Statistics (NBS), Office of the Chief Government Statistician (OCGS), ICF International. Tanzania HIV/AIDS and Malaria Indicator Survey 2011-12. 2013 [Available from: https://dhsprogram.com/pubs/pdf/AIS11/AIS11.pdf.

351. Ministry of Health Zanzibar. Zanzibar Integrated HIV, TB and Leprosy Programme Annual Report 2015. 2016.
352. Zanzibar MoH, Zanzibar RGo. Zanzibar Health Sector Strategic Plan III 2013/14-2018/19 Zanzibar2013 [Available from: http://tanzania.um.dk/en/danida-en/health/.
353. Ministry of Health RGoZ. 2013/14 Performance Report. Zanzibar; 2014.
354. Valenstein PN. Evaluating diagnostic tests with imperfect standards. Am J Clin Pathol. 1990;93(2):252-8.
355. Alonzo TA, Pepe MS. Using a combination of reference tests to assess the accuracy of a new diagnostic test. Stat Med. 1999;18(22):2987-3003.
356. Naaktgeboren CA, Bertens LC, van Smeden M, de Groot JA, Moons KG, Reitsma JB. Value of composite reference standards in diagnostic research. BMJ. 2013;347:f5605.
357. Bialocerkowski A, Bragge P. Measurement error and reliability testing: Application to rehabilitation. International Journal of Therapy and Rehabilitation. 2008;15(10).
358. Salazar MK. Interviewer bias. How it affects survey research. AAOHN J. 1990;38(12):567-72.
359. Althubaiti A. Information bias in health research: definition, pitfalls, and adjustment methods. J Multidiscip Healthc. 2016;9:211-7.
360. Ministry of Health and Social Welfare RGoZ, Organization WH. Zanzibar traditional and alternative medicine policy, 2008. Zanzibar2008 [cited 2019 20th of September]. Available from: https://www.afro.who.int/sites/default/files/2017-05/Zanzibar-traditional-and-alternative-medicine-policy-2008_0.pdf.
361. Meier zu Biesen C, Dilger H, Nienstedt T. Bridging gaps in health care and healing: Traditional medicine and the biomedical health care sector in Zanzibar 2012 [cited 2019 20th of September]. Available from: https://www.polsoz.fu-berlin.de/ethnologie/forschung/arbeitsstellen/medical_anthropology/personen/mitglieder/dilger/Meier_zu_Biesen_et_al_Bridging_Gaps_in_Health_Care_and_Healing_FINAL_REPORT_2012.pdf.
362. Sreeramareddy CT, Ramakrishnareddy N, Shah RK, Baniya R, Swain PK. Clinico-epidemiological profile and diagnostic procedures of pediatric tuberculosis in a tertiary care hospital of western Nepal-a case-series analysis. BMC Pediatr. 2010;10:57.
363. Cruz AT, Starke JR. Clinical manifestations of tuberculosis in children. Paediatr Respir Rev. 2007;8(2):107-17.
364. Shingadia D, Novelli V. Diagnosis and treatment of tuberculosis in children. Lancet Infect Dis. 2003;3(10):624-32.
365. Marais BJ, Wright CA, Schaaf HS, Gie RP, Hesseling AC, Enarson DA, et al. Tuberculous lymphadenitis as a cause of persistent cervical lymphadenopathy in children from a tuberculosis-endemic area. Pediatr Infect Dis J. 2006;25(2):142-6.
366. Wright CA, Warren RM, Marais BJ. Fine needle aspiration biopsy: an undervalued diagnostic modality in paediatric mycobacterial disease. Int J Tuberc Lung Dis. 2009;13(12):1467-75.
367. Fanny ML, Beyam N, Gody JC, Zandanga G, Yango F, Manirakiza A, et al. Fine-needle aspiration for diagnosis of tuberculous lymphadenitis in children in Bangui, Central African Republic. BMC Pediatr. 2012;12:191.
368. Wright CA, Hesseling AC, Bamford C, Burgess SM, Warren R, Marais BJ. Fine-needle aspiration biopsy: a first-line diagnostic procedure in paediatric tuberculosis suspects with peripheral lymphadenopathy? Int J Tuberc Lung Dis. 2009;13(11):1373-9.
369. Derese Y, Hailu E, Assefa T, Bekele Y, Mihret A, Aseffa A, et al. Comparison of PCR with standard culture of fine needle aspiration samples in the diagnosis of tuberculosis lymphadenitis. J Infect Dev Ctries. 2012;6(1):53-7.
370. Arora B, Arora DR. Fine needle aspiration cytology in diagnosis of tuberculous lymphadenitis. Indian J Med Res. 1990;91:189-92.
371. Gupta AK, Nayar M, Chandra M. Critical appraisal of fine needle aspiration cytology in tuberculous lymphadenitis. Acta Cytol. 1992;36(3):391-4.
372. Nayak S, Mani R, Kavatkar AN, Puranik SC, Holla VV. Fine-needle aspiration cytology in lymphadenopathy of HIV-positive patients. Diagn Cytopathol. 2003;29(3):146-8.
373. Wright CA, Pienaar JP, Marais BJ. Fine needle aspiration biopsy: diagnostic utility in resource-limited settings. Ann Trop Paediatr. 2008;28(1):65-70.

374. Thomas JO, Adeyi D, Amanguno H. Fine-needle aspiration in the management of peripheral lymphadenopathy in a developing country. Diagn Cytopathol. 1999;21(3):159-62.
375. Seddon JA, Jenkins HE, Liu L, Cohen T, Black RE, Vos T, et al. Counting children with tuberculosis: why numbers matter. Int J Tuberc Lung Dis. 2015;19 Suppl 1:9-16.
376. Jenkins HE. Global Burden of Childhood Tuberculosis. Pneumonia (Nathan). 2016;8.
377. Tattevin P, Che D, Fraisse P, Gatey C, Guichard C, Antoine D, et al. Factors associated with patient and health care system delay in the diagnosis of tuberculosis in France. Int J Tuberc Lung Dis. 2012;16(4):510-5.
378. Mfinanga SG, Morkve O, Sviland L, Kazwala RR, Chande H, Nilsen R. Patient knowledge, practices and challenges to health care system in early diagnosis of mycobacterial adenitis. East Afr Med J. 2005;82(4):173-80.
379. Leutscher P, Madsen G, Erlandsen M, Veirum J, Ladefoged K, Thomsen V, et al. Demographic and clinical characteristics in relation to patient and health system delays in a tuberculosis low-incidence country. Scand J Infect Dis. 2012;44(1):29-36.
380. Yimer SA, Bjune GA, Holm-Hansen C. Time to first consultation, diagnosis and treatment of TB among patients attending a referral hospital in Northwest, Ethiopia. BMC Infect Dis. 2014;14:19.
381. Osei E, Akweongo P, Binka F. Factors associated with DELAY in diagnosis among tuberculosis patients in Hohoe Municipality, Ghana. BMC Public Health. 2015;15:721.
382. Hoa NP, Thorson AE, Long NH, Diwan VK. Knowledge of tuberculosis and associated health-seeking behaviour among rural Vietnamese adults with a cough for at least three weeks. Scand J Public Health Suppl. 2003;62:59-65.
383. Rajeswari R, Chandrasekaran V, Suhadev M, Sivasubramaniam S, Sudha G, Renu G. Factors associated with patient and health system delays in the diagnosis of tuberculosis in South India. Int J Tuberc Lung Dis. 2002;6(9):789-95.
384. Guo N, Marra F, Marra CA. Measuring health-related quality of life in tuberculosis: a systematic review. Health Qual Life Outcomes. 2009;7:14.
385. Brown J, Capocci S, Smith C, Morris S, Abubakar I, Lipman M. Health status and quality of life in tuberculosis. Int J Infect Dis. 2015;32:68-75.
386. Tanvejsilp P, Loeb M, Dushoff J, Xie F. Out-of-Pocket Expenditures, Indirect Costs and Health-Related Quality of Life of Patients with Pulmonary Tuberculosis in Thailand. Pharmacoecon Open. 2018;2(3):281-96.
387. Sun Y, Yang Z, Wan C, Xu C, Chen L, Xu L, et al. Development and validation of the pulmonary tuberculosis scale of the system of Quality of Life Instruments for Chronic Diseases (QLICD-PT). Health Qual Life Outcomes. 2018;16(1):137.
388. Abdulelah J, Sulaiman SAS, Hassali MA, Blebil AQ, Awaisu A, Bredle JM. Development and Psychometric Properties of a Tuberculosis-Specific Multidimensional Health-Related Quality-of-Life Measure for Patients with Pulmonary Tuberculosis. Value Health Reg Issues. 2015;6:53-9.
389. Dhingra VK, Rajpal S. Health related quality of life (HRQL) scoring (DR-12 score) in tuberculosis--additional evaluative tool under DOTS. J Commun Dis. 2005;37(4):261-8.
390. EuroQol Research Foundation. EQ-5D-3L User Guide, 2018. 2018 [cited 2019 2nd of October]. Available from: https://euroqol.org/publications/user-guides.
391. Saleem S, A AM, Ghulam A, Ahmed J, Hussain H. Health-related quality of life among pulmonary tuberculosis patients in Pakistan. Qual Life Res. 2018;27(12):3137-43.
392. Kittikraisak W, Kingkaew P, Teerawattananon Y, Yothasamut J, Natesuwan S, Manosuthi W, et al. Health related quality of life among patients with tuberculosis and HIV in Thailand. PLoS One. 2012;7(1):e29775.
393. Kruijshaar ME, Lipman M, Essink-Bot ML, Lozewicz S, Creer D, Dart S, et al. Health status of UK patients with active tuberculosis. Int J Tuberc Lung Dis. 2010;14(3):296-302.
394. Kind P, Dolan P, Gudex C, Williams A. Variations in population health status: results from a United Kingdom national questionnaire survey. BMJ. 1998;316(7133):736-41.
395. Rockwood N, du Bruyn E, Morris T, Wilkinson RJ. Assessment of treatment response in tuberculosis. Expert Rev Respir Med. 2016;10(6):643-54.

396. Singh SK, Tiwari KK. Tuberculous lymphadenopathy: Experience from the referral center of Northern India. Niger Med J. 2016;57(2):134-8.
397. British Thoracic Society Research Committee. Short course chemotherapy for tuberculosis of lymph nodes: a controlled trial. . Br Med J (Clin Res Ed). 1985;290(6475):1106-8.
398. Jindal SK, Aggarwal AN, Gupta D, Ahmed Z, Gupta KB, Janmeja AK, et al. Tuberculous lymphadenopathy: a multicentre operational study of 6-month thrice weekly directly observed treatment. Int J Tuberc Lung Dis. 2013;17(2):234-9.

9. PAPERS

I

PLOS ONE

RESEARCH ARTICLE

MPT64 antigen detection test improves routine diagnosis of extrapulmonary tuberculosis in a low-resource setting: A study from the tertiary care hospital in Zanzibar

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Abstract

Background

Extrapulmonary tuberculosis (EPTB) is a diagnostic challenge. An immunochemistry-based MPT64 antigen detection test (MPT64 test) has reported higher sensitivity in the diagnosis of EPTB compared with conventional methods. The objective of this study was to implement and evaluate the MPT64 test in routine diagnostics in a low-resource setting.

Methods

Patients with presumptive EPTB were prospectively enrolled at Mnazi Mmoja Hospital, Zanzibar, and followed to the end of treatment. Specimens collected were subjected to routine diagnostics, GeneXpert® MTB/RIF assay and the MPT64 test. The performance of the MPT64 test was assessed using a composite reference standard, defining the patients as tuberculosis (TB) cases or non-TB cases.

Results

Patients (n = 132) were classified as confirmed TB (n = 12), probable TB (n = 34), possible TB (n = 18), non-TB (n = 62) and uncategorized (n = 6) cases. Overall, in comparison to the composite reference standard for diagnosis, the sensitivity, specificity, positive predictive value, negative predictive value and accuracy of the MPT64 test was 69%, 95%, 94%, 75% and 82%, respectively. The MPT64 test performance was best in TB lymphadenitis cases (n = 67, sensitivity 79%, specificity 97%) and in paediatric TB (n = 41, sensitivity 100%, specificity 96%).

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Competing interests: The authors have declared that no competing interests exist.

Conclusions

We show that the MPT64 test can be implemented in routine diagnostics in a low-resource setting and improves the diagnosis of EPTB, especially in TB lymphadenitis and in children.

Introduction

Despite efforts to develop new diagnostic tools for tuberculosis (TB), the diagnosis of extrapulmonary TB (EPTB) remains a challenge. The various clinical presentations of EPTB are non-specific, and the disease is often paucibacillary leading to low sensitivities of routine diagnostic methods such as; acid-fast bacilli (AFB) microscopy [1–3] and culture [1, 4, 5]. Furthermore, mycobacterial culture has a long turnaround time, and its technical and logistic demands limits its use in resource-limited settings. Histology can be used in the diagnosis of EPTB, but lacks specificity as several other conditions may present similar histological features [6]. Most nucleic acid amplification tests show better sensitivity, but are complex, expensive, technically demanding and prone to contamination, limiting their use in low-resource diagnostic settings [7–10]. The development of the GeneXpert® MTB/RIF (Xpert) assay is a landmark in TB diagnostics, but reported sensitivities of the assay for EPTB samples are highly heterogeneous and vary widely across different sample types [11–14]. Due to lack of a low-cost, robust, rapid and accurate diagnostic method, EPTB is either over- or underdiagnosed, leading to increased morbidity and mortality. Thus, there is a need for better diagnostic tools, which are implementable and sustainable in resource-limited settings.

MPT64 is a protein secreted by the *Mycobacterium tuberculosis* (Mtb) complex species, not detected in non-tuberculous mycobacteria (NTM) [15, 16] and bacillus Calmette-Guérin strains with RD2 deletion [17]. Earlier studies have investigated the diagnostic potential of an immunochemistry-based MPT64 antigen detection test (MPT64 test) showing sensitivity and specificity comparable to nested polymerase chain reaction (PCR) [4, 5, 18, 19].

Zanzibar is a semi-autonomous region of the United Republic of Tanzania and comprises the main islands Unguja and Pemba. The region has 1.3 million inhabitants [20], a prevalence of bacteriologically confirmed pulmonary TB of 124 per 100 000 [21], and a low adult human immunodeficiency virus (HIV) prevalence of 1% [22]. In 2013, 30% of the new TB patients were registered as EPTB cases [23]. The aim of the present study was to implement and evaluate the performance of the MPT64 test in routine diagnostics at the tertiary care hospital in Zanzibar, a low-resource setting with a high TB burden.

Materials and methods

Study participants

The study was conducted at Mnazi Mmoja Hospital (MMH), Unguja, Zanzibar. MMH is the only tertiary referral hospital in Zanzibar, and provides in addition primary and secondary health care for some districts. Patients of all ages presenting with symptoms suggestive of EPTB were prospectively enrolled from hospital wards and out-patients departments between 1st August 2014 and 31st August 2015. Patients who consented and where a representative sample was collected were included in the study. Those who had received anti-TB treatment (ATT) during the previous year were excluded. All patients were interviewed using a pretested structured questionnaire, and a physical examination was performed. Diagnostic imaging was done if required and possible. Response to ATT was assessed at 2–3 months and at the end of treatment by using criteria based on improvement in signs and symptoms, weight gain and objective measures such as

repeated chest radiographs, abdominal ultrasound and reduction of lymph node swellings. Patients not starting ATT were followed until recovery or until a diagnosis other than TB was established.

Study questionnaire

The study questionnaire was developed in English, translated to Swahili, then translated back to English. The translations were performed by two separate individuals fluent in both languages. The original English version and the back-translated version were compared to assess the validity. Prior to testing of the questionnaire among patients, two bilingual individuals at Zanzibar evaluated both the English (S1 and S2 Texts) and Swahili versions (S3 and S4 Texts) to assess the meaning of the questions according to the local setting. The questionnaire was tested among three adult inpatients at the medical ward at MMH to identify unclear or ambiguous questions and the questionnaire was adjusted accordingly.

Sample collection and processing

Fine-needle aspiration cytology (FNAC) from peripheral lymph nodes was performed by the hospital pathologist (MM) using a 23-g needle. Four smears were prepared from each aspirate; one each for cytology and AFB microscopy, and two for immunocytochemical (ICC) staining. The slides for ICC staining were fixed in 95% alcohol before being transported to the laboratory. The needle was rinsed with 2 ml of sterile 0.9% saline solution and distributed equally for the Xpert assay and Mtb culture. All fluids were aspirated aseptically, and subjected to routine diagnostic investigations, in addition to the Xpert assay. The specimens were centrifuged at 3000g for 10 minutes and smears were made from the 20μl of the sediment for cytology, AFB microscopy and ICC staining. The biopsies were divided equally and one half transported in 0.9% saline for Mtb culture and the other half fixed in 4% phosphate buffered formaldehyde for conventional paraffin embedding. From the formalin-fixed, paraffin-embedded biopsies, five-μm-thick tissue sections were prepared for histology, AFB microscopy and immunohistochemical (IHC) staining.

Diagnostic procedure

AFB microscopy was performed using Ziehl-Neelsen (ZN) staining. Culture was done at the Public Health Laboratory–Ivo de Carneri (PHL-IdC) located at Pemba island, on Lowenstein-Jensen medium according to the standard protocol. Positive cultures were confirmed by smear microscopy and sent to the Central Tuberculosis and Leprosy Reference Laboratory at Tanzania mainland for species identification and drug sensitivity testing. The Xpert assay was performed according to the standard protocol recommended by WHO [24].The specimens were stored at 4˚C for a maximum of 7 days if it was not analyzed on the same day as the sampling. The Xpert assay was not performed on biopsies. The slides for cytological and histological examination were stained with Papanicolaou stain and haematoxylin-eosin, respectively. Two laboratory technologists working at MMH were trained to perform the ICC/IHC staining (immunostaining) procedures and the pathologist at MMH (MM) received training in evaluation of the immunostaining. The immunostaining was performed as described earlier [5, 18] with some modifications, by using an in-house polyclonal anti-MPT64 primary antibody at 1/250 dilution and Dako kit (Dako Envision® + System-HRP, K4009, Dako, Glostrup, Denmark), to demonstrate the presence of MPT64 antigens. Briefly, for ICC staining, the slides were hydrated through decreasing grades of alcohol, washed in distilled water for 10 minutes and incubated with hydrogen peroxide for 15 minutes to inhibit the endogenous peroxidase activity. Thereafter, the primary antibody was applied and incubated for 60 minutes. Anti-rabbit dextran polymer conjugated to

horseradish peroxidase was then applied to the slides for 45 minutes. To visualize the bound antibody, the slides were incubated for 10 minutes with 3-amino-9-ethylcarbazol and hydrogen peroxide-containing substrate, and the background counterstained with Mayer's hematoxylin. The slides were mounted in Immu-Mount (Thermo Fisher Scientific). Between the incubation steps the slides were washed with wash buffer (Dako Wash buffer 10x, S3006, Dako, Glostrup, Denmark). For IHC staining, tissue sections were deparaffinized with xylene, hydrated and after microwave antigen retrieval using citrate buffer, pH 6.2, subsequently incubated with hydrogen peroxide for 10 minutes. Additional steps were as in the ICC staining procedure.

Evaluation of immunostaining

The stained slides were evaluated at 20x magnification using a light microscope, and possible positive signals were further assessed at 40x magnification. The pathologist (MM) evaluating the slides was blinded for the ZN staining and the Xpert assay results. Signals were regarded as positive if seen as reddish granular intracytoplasmic staining or extracellular staining in necrotic areas. The sample was evaluated as weakly positive if 1–2 strong positive or 3 weakly positive spots were seen, as positive if > 2 strong positive spots or > 3 weakly positive spots, negative if no positive signal and as inconclusive if ≤ 2 weakly positive spots or only uncertain spots were seen.

Patient categories and morphological criteria

The patients were categorized by using a composite reference standard (CRS) combining the various diagnostic criteria into 5 separate groups as described in Table 1. The MPT64 test results were not available during the categorization of patients. Briefly, the morphological criteria taken to be consistent with TB were the presence of granuloma with or without necrosis, poorly formed granulomas with necrosis or necrosis without granulomas in the biopsy specimens. In FNAC smears from lymph nodes these were granulomatous inflammation with or without necrosis or necrotic material without granulomas, and in cytological smears from effusion/cerebrospinal fluid (CSF) the predominance of lymphocytes was taken to be suggestive of tuberculosis.

Statistical analysis

Data was analyzed using Statistical Package for the Social Sciences (SPSS) for Windows version 24.0. Chi-square test was used to compare differences in categorical variables. The performance of the different diagnostic procedures was calculated using the CRS as a reference. Cross-tabulation was used to calculate sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy. P value < 0.05 was considered statistically significant.

Ethical considerations

Ethical clearance was obtained from the Regional Committee for Medical and Health Research Ethics, Western-Norway (REK Vest) and the Zanzibar Medical Research and Ethics Committee (ZAMREC). All study participants provided informed written consent. For children, consent was provided by the parent/guardian, in addition, children between 7–18 years had to sign the consent form as well. The biological specimens were collected on clinical demand and not based on participation in the study.

Table 1. Criteria for categorization of patients into various categories of the composite reference standard.

Confirmed TB case	Positive mycobacterial culture and/or *M. tuberculosis* detected by the Xpert assay
Probable TB case	Clinical presumptive EPTB and a good response to ATT at 2–3 months and/or end of treatment **or** clinical presumptive EPTB and bacteriologically confirmed concomitant pulmonary TB **and one of the following** • AFB seen on ZN staining of extrapulmonary material • Radiological findings suggestive of EPTB • Effusions/CSF: lymphocytosis on fluid cytology and protein level > 3 g/dl (> 1 g/l for CSF) • FNAC/biopsy–morphological features consistent with TB
Possible TB case	a) Patient started ATT based on clinical presumptive EPTB[a] **and one of the following** • AFB seen on ZN staining of extrapulmonary material • Radiological findings suggestive of EPTB • Effusions/CSF: lymphocytosis on fluid cytology and protein level > 3 g/dl (> 1 g/l for CSF) • FNAC/biopsy–morphological features consistent with TB b) Clinical presumptive EPTB and a good response to ATT at 2–3 months and/or end of treatment
Non-TB case[b] (control subject)	Negative mycobacterial culture **and/or** *M. tuberculosis* not detected by the Xpert assay **and one of the following** • Improvement without ATT and/or response to specific non-tuberculous therapy • Cytology/histology examination concluded other diagnosis than TB • Alternative diagnosis concluded by the clinician • Patient started on ATT based on clinical presumptive EPTB, but did not respond to treatment
Uncategorized patient	not possible to categorize the patient

NOTE. TB, tuberculosis; EPTB, extrapulmonary tuberculosis; ATT, antituberculous treatment; AFB, acid fast bacilli; ZN, Ziehl-Neelsen; CSF, cerebrospinal fluid; FNAC, fine-needle aspiration cytology.

[a] Patient died before observation time to assess response to treatment or was lost to follow-up.

[b] Culture was missing in 4 cases and the Xpert assay was missing in 28 cases. Among these, 3 patients had neither culture or Xpert assay results.

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Results

A total of 146 patients were approached and 132 patients were enrolled in the study. The total number of collected biological specimens were 152 from the 132 study participants. Fig 1 provides an overview of patients included and specimens collected in the study. According to the CRS, 12 (9%) were categorized as confirmed TB cases; 34 (26%) as probable TB cases; 18 (14%) as possible TB cases, 62 (47%) as non-TB cases and 6 (5%) patients were uncategorized. The uncategorized patients and the specimens collected in these patients were excluded from further analyses. Thus, 126 patients and the laboratory results from 145 specimens were included in the data analysis. In most patients, one specimen from the presumptive site of infection was collected and examined with the various diagnostic procedures. Two different specimens were collected from the same site in 19 patients (FNAC and biopsy, n = 17; ascites and biopsy, n = 1; pericardial effusion and biopsy, n = 1). All specimens were examined with the MPT64 test, whereas the routine methods were missed in some specimens; 143 (99%), 125 (86%) and 72 (50%) of the specimens were examined with ZN staining, culture and the Xpert assay, respectively.

Clinical characteristics

The demographic and baseline characteristics, as well as the distribution of presumptive sites of infection among the study participants, are described in Table 2. The age distribution

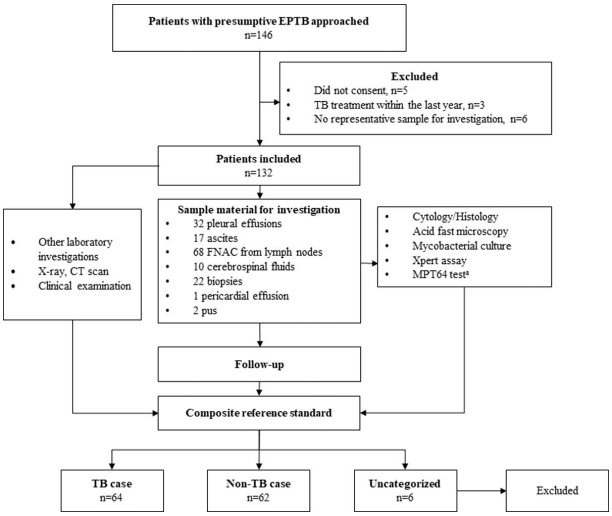

Fig 1. Flow-chart showing the study design and patient flow in the study. NOTE. EPTB, extrapulmonary tuberculosis; TB, tuberculosis; CT, computed tomography; FNAC, fine-needle aspiration cytology. [a] Not included in the composite reference standard.

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differed significantly between the TB and non-TB cases. The majority of the TB patients were between 15–44 years (59%), whereas the non-TB patients were predominantly either children (40%) or above 44 years (29%). HIV status was known in 94 patients, and 21% of these were HIV positive. In the HIV positive patients, 14/20 (70%) were categorized as TB cases; 3 as confirmed TB, 5 as probable TB and 6 as possible TB cases, respectively. Overall, there was a significant difference in the presumptive sites of EPTB between adults and children ($P = .047$). In children, there was a higher proportion of lymphadenitis 29/41 (71%), and lower proportions of pleuritis 7/41 (17%), peritonitis 2/41 (5%) and other sites 3/41 (7%), while the corresponding proportions among adults were 38/85 (45%), 24/85 (28%), 14/85 (17%) and 9/85 (11%), respectively. Among the paediatric TB cases (n = 16) the sites of infection were TB lymphadenitis (n = 11), pleural TB (n = 3), abdominal TB (n = 1) and TB pericarditis (n = 1).

Most patients (73%) presented with both local and systemic signs and symptoms, more so in the TB cases compared to non-TB cases, but the difference in proportions was not significant. The final diagnoses among the non-TB cases were malignant tumor (n = 18), benign tumor (n = 5), benign reactive lymphadenopathy (n = 13), heart failure (n = 5), liver disease (n = 6), meningitis/encephalitis (n = 6), pneumonia (n = 1), endometriosis (n = 1), hydatid cyst (n = 1), sialadenitis (n = 1) and sclerosing lymphocytic mastitis (n = 1). In 1 patient spontaneous resolution of ascites and pleural effusion was observed, 2 patients did not respond to anti-TB treatment and malignancy was suspected but not confirmed, and in 1 patient the treating physician did not suspect TB after throughout evaluation.

MPT64 test performance compared to routine laboratory diagnostic tests and the Xpert assay

The results of all diagnostic procedures among various categories of patients and from available specimens are presented in Table 3. The MPT64 test was positive in a higher proportion

Table 2. Demographic and baseline characteristics of the 126 categorized study participants, n(%).

Characteristics	TB cases[a] n = 64	Non-TB cases n = 62	*P* value[b]
Sex			.842
Male	35 (55)	35 (56)	
Female	29 (45)	27 (44)	
Age (years)			.014*
< 15	16 (25)	25 (40)	
15–29	17 (27)	8 (13)	
30–44	21 (33)	11 (18)	
≥45	10 (16)	18 (29)	
In/outpatient			.216
Inpatient	22 (34)	28 (45)	
Outpatient	42 (66)	34 (55)	
HIV status			.517[c]
Positive	14 (23)	6 (18)	
Negative	46 (77)	28 (82)	
Unknown	4 (-)	28 (-)	
Presumptive site of infection			.177
Lymphadenitis	34 (53)	33 (53)	
Pleuritis	20 (31)	11 (18)	
Peritonitis	6 (9)	10 (16)	
Other sites[d]	4 (6)	8 (13)	
Symptoms/signs at time of inclusion			.189
Local	14 (22)	20 (32)	
Local and systemic	50 (78)	42 (68)	

NOTE. TB, tuberculosis; HIV, human immunodeficiency virus.

[a] Confirmed, probable and possible TB cases.

[b] Comparing group differences between TB and non-TB cases.

[c] Only comparing patients with known HIV status.

[d] TB cases, meningitis (n = 2), spondylitis (n = 1), pericarditis (n = 1); Non-TB cases, meningitis (n = 6), osteomyelitis (n = 1), mastitis (n = 1).

* Statistically significant.

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of specimens as compared to the other tests. In total, 65% of the specimens in TB cases demonstrated a positive MPT64 test, compared to 12%, 13% and 16% demonstrating positive results with ZN staining, culture and the Xpert assay, respectively. In specimens examined with all diagnostic tests, the MPT64 test was positive in 81%, compared to 14%, 16% and 16% of the specimens showing a positive result with ZN staining, culture and the Xpert assay, respectively (Table 3). In confirmed TB cases 83% of the specimens had a positive MPT64 test as compared to 67% positivity for culture and the Xpert assay. All ZN and/or Xpert assay positive samples were positive with the MPT64 test. Among culture positive samples, 6/8 (75%) were positive with the MPT64 test. FNAC from lymph nodes was the specimen with the highest number of positive MPT64 results (76%) compared to pleural fluid, ascites and CSF. Further, all FNAC from lymph nodes that were positive by ZN staining, culture and/or the Xpert assay were also positive with the MPT64 test. In non-TB cases, the MPT64 test was negative in 73/76 (96%) of the specimens. Fig 2 shows the staining pattern at various sites of infection.

Table 3. Results of diagnostic procedures in effusions, CSF, aspirates and biopsies.

Final diagnosis	Total number of specimens	Number of specimens (%) positive by			
		ZN	LJ culture	Xpert assay	MPT64 test
TB cases–all tests performed[a]					
All samples	37	5/37 (14)	6/37 (16)	6/37 (16)	30/37 (81)
FNAC LN	21	4/21 (19)	4/21 (19)	6/21 (29)	19/21 (90)
Pleural effusion	8	0/8 (-)	0/8 (-)	0/8 (-)	5/8 (63)
TB cases[b]					
All samples	69	8/69 (12)	8/60 (13) [c]	6/38 (16) [d]	45/69 (65)
FNAC LN	34	6/34 (18)	5/30 (17)	6/22 (27)	26/34 (76)
Pleural effusion	20	0/20 (0)	1/19 (5)	0/8 (0)	10/20 (50)
Ascites	6	0/6 (0)	1/6 (17)	0/5 (0)	4/6 (67)
CSF	2	0/2 (0)	1/1 (100)	0/1 (0)	1/2 (50)
Biopsies	5	1/5 (20)	0/2 (0)	-	2/5 (40)
Pericardial effusion	1	0/1 (0)	0/1 (0)	0/1 (0)	1/1 (100)
Pus	1	1/1 (100)	0/1 (0)	0/1 (0)	1/1 (100)
Confirmed TB cases	12	3/12 (25)	8/12 (67)	6/9 (67)	10/12 (83)
Probable TB cases	39	5/39 (13)	0/31 (0)	0/18 (0)	20/39 (51)
Possible TB cases	18	0/18 (0)	0/17 (0)	0/11 (0)	15/18 (83)
Non-TB cases[e]					
All samples	76	0/74 (0) [f]	0/65 (0) [g]	0/34 (0) [h]	3/76 (4)
FNAC LN	32	0/31 (0)	0/31 (0)	0/20 (0)	0/32 (0)
Pleural effusion	11	0/11 (0)	0/10 (0)	0/5 (0)	1/11 (9)
Ascites	10	0/10 (0)	0/9 (0)	0/3 (0)	1/10 (10)
CSF	6	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)
Biopsies	16	0/15 (0)	0/8 (0)	-	1/16 (6)
Pericardial effusion	0	-	-	-	-
Pus	1	0/1 (0)	0/1 (0)	-	0/1 (0)

NOTE. CSF, cerebrospinal fluid; ZN, Ziehl-Neelsen staining; LJ, Lowenstein-Jensen; TB, tuberculosis; FNAC, fine-needle aspiration cytology; LN, lymph node.

[a] Only specimens analyzed with all methods (ZN, LJ culture, Xpert and MPT64 test).

[b] Five patients with two different specimens from the same site (FNAC and biopsy (n = 4), pericardial effusion and biopsy (n = 1)).

[c] Contaminated (n = 2) and specimens not sent for culture (n = 7) excluded.

[d] Invalid results (n = 1) and specimens not analyzed with the Xpert assay (n = 30) excluded.

[e] Fourteen patients with two different specimens from same site (FNAC and biopsy (n = 13), ascites and biopsy (n = 1)).

[f] Specimens not examined with ZN (n = 2) excluded.

[g] Contaminated (n = 1) and specimens not sent for culture (n = 10) excluded.

[h] Specimens not analyzed with the Xpert assay (n = 42) excluded.

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Diagnostic validation of the MPT64 test

The diagnostic validity of the MPT64 test and other methods in lymphadenitis, pleuritis and paediatric TB using the CRS as reference standard are shown in Table 4. The sensitivity, NPV and accuracy of the MPT64 test was better than the other diagnostic tests. The performance of the MPT64 test was best in TB lymphadenitis, were the sensitivity of the MPT64 test was significantly higher as compared to TB pleuritis ($P = .025$).

The performance of the MPT64 test was better in children (n = 41) as compared to adults (n = 85) with a sensitivity of 100% and 58% ($P = .002$) and a specificity of 96% and 95%, respectively. In the HIV positive patients (n = 20) the sensitivity of the MPT64 test was lower

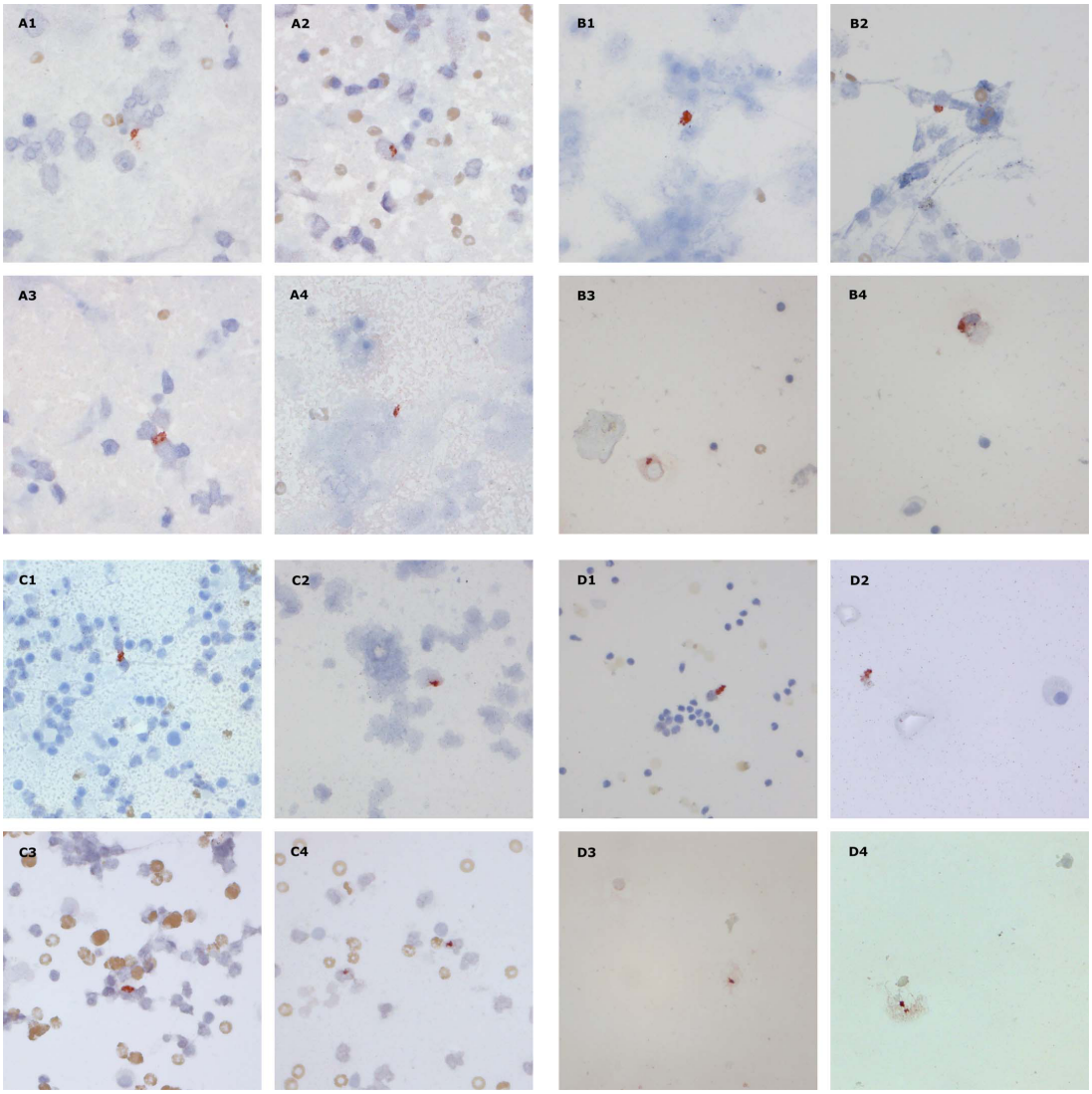

Fig 2. Patterns of immunostaining with anti-MPT64 antibody in various specimens. The signals are seen as granular, reddish staining. A, fine-needle aspirates from lymph nodes, signals were extracellular probably due to cell lysis (A1), mostly intracytoplasmic (A2-A3), and in necrotic areas (A4); B, pleural effusion, intracytoplasmic staining; C1-C2, pus/abscess, intracytoplasmic staining; C3-C4, pericardial effusion, intracytoplasmic staining, and non-specific staining mainly of red blood cells; D1-D2, ascites, intracytoplasmic staining (D1), extracellular probably due to cell lysis (D2); D3-D4, cerebrospinal fluid, extracellular probably due to cell lysis.

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compared to HIV negative cases (n = 74) (57% and 70%, respectively), but the difference was not significant.

Table 4. Diagnostic validation of various procedures among lymphadenitis, pleuritis and children using the CRS as reference standard.

	Number of patients	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV %	NPV %	Accuracy %
Lymphadenitis[a]	67					
MPT64 test	67	79 (62–91)	97 (84–100)	96	82	88
ZN	67	18 (7–35)	100 (89–100)	100	54	58
Culture	62	16 (5–34)	100 (89–100)	100	54	58
Xpert assay	42	27 (11–50)	100 (83–100)	100	56	62
Pleuritis	31					
MPT64 test	31	50 (27–73)	91 (59–100)	91	50	65
ZN	31	0 (0–17)	100 (72–100)	NA	35	35
Culture	29	5 (0–26)	100 (69–100)	100	36	38
Xpert assay	13	0 (0–37)	100 (48–100)	NA	38	38
Children	41					
MPT64 test	41	100 (79–100)	96 (80–100)	94	100	98
ZN	41	13 (2–38)	100 (86–100)	100	64	66
Culture	38	19 (4–46)	100 (85–100)	100	63	66
Xpert assay	22	10 (0–45)	100 (74–100)	100	57	59

NOTE. CRS, composite reference standard; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; ZN, Ziehl-Neelsen staining; NA, not applicable.

[a] Results from FNAC and biopsy (n = 17) are combined.

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Table 5. Relationship between various cytomorphological features in fine-needle aspirates from lymph nodes and results of diagnostic procedures.

	Number of specimens positive (%) by			
Cytomorphology	**ZN**	**Culture**	**Xpert assay**	**MPT64 test**
TB cases (n = 34)				
Gr. infl with necrosis (n = 3) *	0/3 (0)	0/2 (0)	0/1 (0)	1/3 (33)
Gr. infl without necrosis (n = 1) *	0/1 (0)	0/1 (0)	0/1 (0)	1/1 (100)
Supp. infl with necrosis (n = 6) *	1/6 (17)	2/5 (40)	2/4 (50)	6/6 (100)
Lymphoid cells and necrosis (n = 4) *	2/4 (50)	1/3 (33)	1/2 (50)	3/4 (75)
Abundant necrosis (n = 5) *	2/5 (40)	1/4 (25)	1/4 (25)	3/5 (60)
Reactive lymph node hyperplasia (n = 5) **	0/5 (0)	0/5 (0)	0/3 (0)	5/5 (100)
Acute supp. infl (n = 8) **	1/8 (13)	1/8 (13)	2/7 (29)	7/8 (88)
Inconclusive (n = 2) **	0/2 (0)	0/2 (0)	-	0/2 (0)
Non-TB cases (n = 32)				
Reactive lymph node hyperplasia (n = 13)	0/13 (0)	0/12 (0)	0/8 (0)	0/13 (0)
Acute supp. infl (n = 2)	0/2 (0)	0/2 (0)	0/1 (0)	0/2 (0)
Abundant necrosis (n = 1)	0/1 (0)	0/1 (0)	-	0/1 (0)
Benign tumor (n = 3)	0/3 (0)	0/3 (0)	0/1 (0)	0/3 (0)
Malign tumor (n = 10)	0/9 (0)	0/10)	0/9 (0)	0/10 (0)
Inconclusive (n = 3)	0/3 (0)	0/3(0)	0/1 (0)	0/3 (0)

NOTE. ZN, Ziehl-Neelsen staining; TB, tuberculosis; Gr. infl, granulomatous inflammation; Supp. infl, suppurative inflammation.

* Morphological features consistent with tuberculosis. 16% HIV positive.

** 31% HIV positive.

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Cytology/histology

In FNAC from lymph nodes, cytomorphological features consistent with TB were reported in only 19/34 of the cases, even though the majority of these patients (78%) were HIV negative. The proportion of HIV positives was slightly lower among the cases with cytology consistent with TB (16%) as compared to those without (31%), but the difference was not statistically significant. The sensitivity of cytology to detect TB was thus 56%. Table 5 shows the results of the various diagnostic procedures in relation to the cytomorphological features. The MPT64 test was positive in 14/19 (74%) of the cases showing cytomorphological patterns consistent with TB, while ZN staining, culture and the Xpert assay were positive in only 5/19 (26%), 4/15 (27%) and 4/12 (33%), respectively. Among the TB patients without cytomorphological features consistent with TB, the MPT64 test was positive in 12/15 (80%), ZN staining in 1/15 (7%), culture in 1/15 (7%) and the Xpert assay in 2/10 (20%) of the patients. In 4/34 TB lymphadenitis cases a lymph node biopsy was performed and the histomorphological picture showed granulomatous inflammation with necrosis (n = 3) and necrosis without granulomas (n = 1). In these biopsies, the MPT64 test was positive in 1/4 (25%). Biopsy of pericardium was performed in one TB patient showing necrosis infiltrated by inflammatory cells, the MPT64 test gave a positive result in this biopsy.

Discussion

This is the first study to show that the immunochemistry-based MPT64 test, applied on human specimens from patients with presumptive EPTB, can be implemented in a low-resource routine diagnostic setting leading to significant improvement in the diagnosis of EPTB. The results are comparable with previous clinical studies performed in more controlled settings, especially for TB lymphadenitis [5, 25]. The overall performance of the MPT64 test was better compared to the other diagnostic tests, with a sensitivity of 83% in the confirmed TB cases.

In FNAC specimens from lymph nodes, the MPT64 test was positive in 76% of the TB cases (confirmed, probable and possible TB cases) compared to none of the non-TB cases, demonstrating high sensitivity and excellent specificity. The superior performance of the MPT64 test for diagnosing TB lymphadenitis using FNAC specimens can have important clinical implications. FNAC is a simple, safe, cost-effective, minimally invasive procedure ideal for use in resource-limited settings [26, 27]. The procedure can be performed in out-patient settings, also in peripheral areas. Fixed slides can then be transported to a hospital with diagnostic facilities for performing cytological evaluation [26, 27]. Further, the possibility of FNAC to distinguish TB and malignant disease is very important [28], as empirical use of ATT in patients with peripheral lymphadenopathy may lead to undue delay of a malignant diagnosis. In the current study, cytological evaluation of FNAC reported suspected malignancy in 10/66 (15%) patients presenting with peripheral lymphadenopathy.

The cytomorphological features in patients with TB lymphadenitis varied greatly in our study, and only 56% of TB cases had morphological features consistent with TB infection, even if most patients were HIV negative, implying the limited use of cytology for an accurate diagnosis of TB. This emphasises the need of additional tests. AFB microscopy does not distinguish between the *M. tuberculosis* and NTM, and has low sensitivity in TB lymphadenitis [25, 29]. Even though culture remains the gold standard of diagnosis, the need for advanced laboratory facilities and the long turnaround time is a challenge in resource-limited settings. The MPT64 test could provide a rapid and confirmative diagnosis of TB lymphadenitis using FNAC specimens, where culture results are absent or takes weeks to be completed. In the current study, all culture positive FNAC from lymph nodes were positive with the MPT64 test.

The sensitivity of the MPT64 test was significantly higher in children than in adults. This could be biased by the higher proportion of TB lymphadenitis cases amongst the children. Still, the sensitivity of the MPT64 test in FNAC specimens from lymph nodes was better in children than in adults (100% vs. 65%). FNAC has been suggested as the diagnostic modality of choice also in children [27]. In the recent years childhood TB has received increased attention, and global estimates imply that the diagnosis of TB is often missed in children and only one third of children developing active TB are notified [30]. In endemic areas, peripheral lymphadenitis is the most common extra-thoracic site of TB in children [31]. The MPT64 test could therefore be very useful in the correct diagnosis of TB lymphadenitis among children.

In the present study, we have also evaluated the MPT64 test according to HIV status, and found no significant difference in sensitivity or specificity when comparing HIV negative to HIV positive patients, implying that the MPT64 test could have an important clinical impact also in this patient group. However, because of the low number of HIV positive cases (n = 20) in this study, the test needs to be evaluated using a larger sample size.

Developing new laboratory diagnostic tests for EPTB is demanding, because of the range of various specimens, challenges with obtaining adequate samples, defining optimal sample volumes, the diverse ways of sample processing and the problem of imperfect reference standards. Culture is still used as the gold standard, but is known to be of limited value in EPTB [12, 13], which makes it difficult to evaluate a new diagnostic test. Using a suboptimal reference standard may potentially misclassify patients as TB or non-TB cases and bias the results of the test under evaluation [32]. To overcome this challenge, we chose to compare the MPT64 test with a CRS and the patients were categorized according to this CRS (Table 1). Culture and Xpert assay results were available in 86% and 50% of the specimens included in the data analysis. Only 8 specimens were positive with culture and 6 specimens were positive with the Xpert assay. The CRS classified 64 patients as TB cases. Therefore, using only culture as a reference standard would have underestimated the true value of the MPT64 test. The low sensitivity of culture in this study could partly be explained by loss of viable bacilli during transport to PHL-IdC at Pemba, the paucibacillary nature of EPTB disease and the possibility of uneven distribution of bacilli in the specimens sent to analyses. Further, two patients had started ATT for 5 and 17 days before specimens were collected, influencing the bacterial viability.

The evaluation of the Xpert assay is challenging in our study, as only 50% of the specimens were examined with this method. A previous study described a sensitivity of 70.6% in lymph nodes when the Xpert assay was compared against culture [33]. In a review, a pooled sensitivity of the Xpert assay in lymph node samples was reported to be 83.1% when compared against culture and 81.2% when using a CRS as a reference standard [12]. In the current study only 5 lymph node samples were culture positive, of these 3/4 (75%) were positive with the Xpert assay. Even though the numbers are low, one could get an impression that the sensitivity of the Xpert assay compared to culture is comparable to other studies using culture as a reference standard. The reason for the low sensitivity of the Xpert assay compared to the CRS in the current study could be due to different criteria incorporated in the CRS in our study and other studies reporting a higher sensitivity of the Xpert assay assessed against a CRS.

There are some limitations of this study. The sample size is small which makes it difficult to do further subgroup analysis of the performance of the MPT64 test according to all presumptive sites of infection. Secondly, there is a heterogeneity in the number of tests performed in patients with different types of EPTB clinical presentation. This is due to the study design, where the new MPT64 test was evaluated for its performance in the routine, without interfering with other routine diagnostic procedures. All samples were not subjected to all routine diagnostic methods due to various reasons. This may have influenced the performance of the component tests and the new test under assessment. Thirdly, the CRS may have reduced

specificity, as defining a TB case based on clinical presumptive EPTB and response to ATT does not provide an accurate diagnosis of TB. It was therefore decided to subdivide the TB cases into "confirmed", "probable" and "possible" TB cases and present the results of the various diagnostic tests for the separate groups.

Conclusions

The MPT64 test is a robust, rapid, sensitive, and specific test for the etiological diagnosis of EPTB. It can differentiate between *Mycobacterium tuberculosis* complex species and NTM, and performs better than conventional methods and the Xpert assay. The test is particularly useful in correct diagnosis of TB lymphadenitis and in childhood TB, and performs equally well in HIV infected patients. Like any diagnostic test it should be interpreted together with the clinical history, examination and routine investigations. We show that the MPT64 test can be implemented in a routine laboratory in a low-resource setting, where improved diagnostics may have a valuable impact on patient management and outcome.

Supporting information

S1 File. Dataset.
(SAV)

S1 Text. Study questionnaire, English version (patients $\geq$ 18 years).
(PDF)

S2 Text. Study questionnaire, English version (patients $<$ 18 years).
(PDF)

S3 Text. Study questionnaire, Swahili version (patients $\geq$ 18 years).
(PDF)

S4 Text. Study questionnaire, Swahili version (patients $<$ 18 years).
(PDF)

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References

1. Chakravorty S, Sen MK, Tyagi JS. Diagnosis of extrapulmonary tuberculosis by smear, culture, and PCR using universal sample processing technology. J Clin Microbiol. 2005; 43(9):4357–62. https://doi.org/10.1128/JCM.43.9.4357-4362.2005 PMID: 16145077; PubMed Central PMCID: PMCPMC1234147.
2. Malbruny B, Le Marrec G, Courageux K, Leclercq R, Cattoir V. Rapid and efficient detection of Mycobacterium tuberculosis in respiratory and non-respiratory samples. Int J Tuberc Lung Dis. 2011; 15 (4):553–5. https://doi.org/10.5588/ijtld.10.0497 PMID: 21396219.
3. Hillemann D, Rusch-Gerdes S, Boehme C, Richter E. Rapid molecular detection of extrapulmonary tuberculosis by the automated GeneXpert MTB/RIF system. J Clin Microbiol. 2011; 49(4):1202–5. https://doi.org/10.1128/JCM.02268-10 PMID: 21270230; PubMed Central PMCID: PMCPMC3122824.
4. Purohit MR, Mustafa T, Wiker HG, Morkve O, Sviland L. Immunohistochemical diagnosis of abdominal and lymph node tuberculosis by detecting Mycobacterium tuberculosis complex specific antigen MPT64. Diagn Pathol. 2007; 2:36. https://doi.org/10.1186/1746-1596-2-36 PMID: 17894882; PubMed Central PMCID: PMCPMC2203973.
5. Purohit MR, Mustafa T, Wiker HG, Sviland L. Rapid diagnosis of tuberculosis in aspirate, effusions, and cerebrospinal fluid by immunocytochemical detection of Mycobacterium tuberculosis complex specific antigen MPT64. Diagn Cytopathol. 2012; 40(9):782–91. https://doi.org/10.1002/dc.21637 PMID: 21416644.
6. Kumar V, Abbas AK, Aster JC. Robbins Basic Pathology. 9th edition: Elsevier Saunders; 2013 p. 472–504.
7. Pai M, Ling DI. Rapid diagnosis of extrapulmonary tuberculosis using nucleic acid amplification tests: what is the evidence? Future Microbiol. 2008; 3(1):1–4. https://doi.org/10.2217/17460913.3.1.1 PMID: 18230027.
8. Pai M, Flores LL, Pai N, Hubbard A, Riley LW, Colford JM Jr. Diagnostic accuracy of nucleic acid amplification tests for tuberculous meningitis: a systematic review and meta-analysis. Lancet Infect Dis. 2003; 3(10):633–43. PMID: 14522262.
9. Pai M, Flores LL, Hubbard A, Riley LW, Colford JM Jr. Nucleic acid amplification tests in the diagnosis of tuberculous pleuritis: a systematic review and meta-analysis. BMC Infect Dis. 2004; 4:6. https://doi.org/10.1186/1471-2334-4-6 PMID: 15102325; PubMed Central PMCID: PMCPMC387423.
10. Daley P, Thomas S, Pai M. Nucleic acid amplification tests for the diagnosis of tuberculous lymphadenitis: a systematic review. Int J Tuberc Lung Dis. 2007; 11(11):1166–76. PMID: 17958977.
11. Lawn SD, Mwaba P, Bates M, Piatek A, Alexander H, Marais BJ, et al. Advances in tuberculosis diagnostics: the Xpert MTB/RIF assay and future prospects for a point-of-care test. Lancet Infect Dis. 2013; 13(4):349–61. https://doi.org/10.1016/S1473-3099(13)70008-2 PMID: 23531388; PubMed Central PMCID: PMCPMC4844338.
12. 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. https://doi.org/10.1183/09031936.00007814 PMID: 24696113
13. Vadwai V, Boehme C, Nabeta P, Shetty A, Alland D, Rodrigues C. Xpert MTB/RIF: a new pillar in diagnosis of extrapulmonary tuberculosis? J Clin Microbiol. 2011; 49(7):2540–5. https://doi.org/10.1128/JCM.02319-10 PMID: 21593262; PubMed Central PMCID: PMCPMC3147857.

14. Tortoli E, Russo C, Piersimoni C, Mazzola E, Dal Monte P, Pascarella M, et al. Clinical validation of Xpert MTB/RIF for the diagnosis of extrapulmonary tuberculosis. Eur Respir J. 2012; 40(2):442–7. https://doi.org/10.1183/09031936.00176311 PMID: 22241741.

15. Harboe M, Nagai S, Patarroyo ME, Torres ML, Ramirez C, Cruz N. Properties of proteins MPB64, MPB70, and MPB80 of Mycobacterium bovis BCG. Infect Immun. 1986; 52(1):293–302. PMID: 3514457; PubMed Central PMCID: PMCPMC262233.

16. Elhay MJ, Oettinger T, Andersen P. Delayed-type hypersensitivity responses to ESAT-6 and MPT64 from Mycobacterium tuberculosis in the guinea pig. Infect Immun. 1998; 66(7):3454–6. PMID: 9632623; PubMed Central PMCID: PMCPMC108370.

17. Mahairas GG, Sabo PJ, Hickey MJ, Singh DC, Stover CK. Molecular analysis of genetic differences between Mycobacterium bovis BCG and virulent M. bovis. J Bacteriol. 1996; 178(5):1274–82. PMID: 8631702; PubMed Central PMCID: PMCPMC177799.

18. Mustafa T, Wiker HG, Mfinanga SG, Morkve O, Sviland L. Immunohistochemistry using a Mycobacterium tuberculosis complex specific antibody for improved diagnosis of tuberculous lymphadenitis. Mod Pathol. 2006; 19(12):1606–14. https://doi.org/10.1038/modpathol.3800697 PMID: 16980944.

19. Baba K, Dyrhol-Riise AM, Sviland L, Langeland N, Hoosen AA, 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. https://doi.org/10.1097/PAI.0b013e31816c3f79 PMID: 18698260.

20. National Bureau of Statistics, Ministry of Finance, Dar es Salaam, Office of Chief Government Statistician, President's Office, Finance, Economy and Development Planning, Zanzibar. Population distributon by age and sex. The United Republic of Tanzania, 2013. Available from: https://ihi.eprints.org/2169/1/Age_Sex_Distribution.pdf.

21. Senkoro M, Mfinanga S, Egwaga S, Mtandu R, Kamara DV, Basra D, et al. Prevalence of pulmonary tuberculosis in adult population of Tanzania: a national survey, 2012. Int J Tuberc Lung Dis. 2016; 20 (8):1014–21. https://doi.org/10.5588/ijtld.15.0340 PMID: 27393533.

22. Tanzania Commission for AIDS (TACAIDS), Zanzibar AIDS Commission (ZAC), National Bureau of Statistics (NBS), Office of the Chief Government Statistician (OCGS), ICF International. Tanzania HIV/AIDS and Malaria Indicator Survey 2011–12. Dar es Salaam, The United Republic of Tanzania, 2013. Available from: https://dhsprogram.com/pubs/pdf/AIS11/AIS11.pdf.

23. Ministry of Health, Zanzibar, Zanzibar Intergrated HIV, Tuberculosis and Leprosy Programme. Annual Report 2013. Zanzibar, The United Republic of Tanzania, 2014.

24. World Health Organization. Xpert MTB/RIF Implementation Manual: Technical and Operational 'How-To'; Practical Considerations. Geneva, WHO, 2014. Available from: https://www.ncbi.nlm.nih.gov/pubmed/25473699.

25. Tadele A, Beyene D, Hussein J, Gemechu T, Birhanu A, Mustafa T, et al. Immunocytochemical detection of Mycobacterium Tuberculosis complex specific antigen, MPT64, improves diagnosis of tuberculous lymphadenitis and tuberculous pleuritis. BMC Infect Dis. 2014; 14:585. https://doi.org/10.1186/s12879-014-0585-1 PMID: 25421972; PubMed Central PMCID: PMCPMC4262190.

26. Wright CA, Pienaar JP, Marais BJ. Fine needle aspiration biopsy: diagnostic utility in resource-limited settings. Ann Trop Paediatr. 2008; 28(1):65–70. https://doi.org/10.1179/146532808X270707 PMID: 18318952.

27. Wright CA, Warren RM, Marais BJ. Fine needle aspiration biopsy: an undervalued diagnostic modality in paediatric mycobacterial disease. Int J Tuberc Lung Dis. 2009; 13(12):1467–75. PMID: 19919763.

28. Thomas JO, Adeyi D, Amanguno H. Fine-needle aspiration in the management of peripheral lymphadenopathy in a developing country. Diagn Cytopathol. 1999; 21(3):159–62. PMID: 10450098.

29. Aljafari AS, Khalil EA, Elsiddig KE, El Hag IA, Ibrahim ME, Elsafi ME, et al. Diagnosis of tuberculous lymphadenitis by FNAC, microbiological methods and PCR: a comparative study. Cytopathology. 2004; 15(1):44–8. PMID: 14748791.

30. Jenkins HE. Global Burden of Childhood Tuberculosis. Pneumonia (Nathan). 2016; 8. https://doi.org/10.1186/s41479-016-0018-6 PMID: 28003956; PubMed Central PMCID: PMCPMC5166554.

31. Marais BJ, Gie RP, Schaaf HS, Hesseling AC, Enarson DA, Beyers N. The spectrum of disease in children treated for tuberculosis in a highly endemic area. Int J Tuberc Lung Dis. 2006; 10(7):732–8. PMID: 16848333.

32. Alonzo TA, Pepe MS. Using a combination of reference tests to assess the accuracy of a new diagnostic test. Stat Med. 1999; 18(22):2987–3003. PMID: 10544302.

33. Moure R, Martin R, Alcaide F. Effectiveness of an integrated real-time PCR method for detection of the Mycobacterium tuberculosis complex in smear-negative extrapulmonary samples in an area of low tuberculosis prevalence. J Clin Microbiol. 2012; 50(2):513–5. https://doi.org/10.1128/JCM.06467-11 PMID: 22162564; PubMed Central PMCID: PMCPMC3264142.

II

PLOS ONE

RESEARCH ARTICLE

Diagnostic delay in extrapulmonary tuberculosis and impact on patient morbidity: A study from Zanzibar

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Abstract

Background

Early and proper treatment of tuberculosis could have an important impact on the morbidity, mortality and the economic situation of patients. There is insufficient knowledge on the extent of diagnostic delay and the associated factors in extrapulmonary tuberculosis (EPTB). The aims of this study were to assess the health care seeking behaviour, EPTB knowledge and diagnostic delay in presumptive EPTB patients at the main referral hospital in Zanzibar, factors associated with longer delay, and the impact of untreated EPTB on self-rated health.

Materials and methods

Prospective data collection using a semi-structured questionnaire in patients presenting with symptoms suggestive of EPTB. The time between the onset of symptoms and first visit to a health care provider (patient delay), and then to the initiation of treatment (health system delay) and total delay were analysed according to sociodemographic and clinical factors and health care seeking trajectories. The EQ-5D-3L was used among the adult EPTB patients to assess the impact of treatment on self-rated health.

Results

Of the 132 patients with median age of 27 years (interquartile range 8–41), 69 were categorized as TB cases and 63 as non-TB cases. The median patient, health system and total delays were 14, 34 and 62 days respectively, among the EPTB patients. A longer health system delay with repeated visits to the same health care level was reported. Significantly better self-rated health status was described after treatment. The knowledge regarding extrapulmonary disease was low.

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Competing interests: The authors have declared that no competing interests exist.

Conclusion

Many EPTB patients, presenting to the main referral hospital in Zanzibar, experience a long delay in the initiation of treatment, specially patients with TB lymphadenitis. The health system delay is the major contributor to the total delay. The improvement of self-rated health after treatment implies that timely treatment has the potential to reduce morbidity and the economic loss for the patient.

Introduction

Tuberculosis (TB) continues to be a major global public health problem. In 2015, the World Health Organization (WHO) estimated that there were 10.4 million incident cases of TB worldwide [1]. Of the notified new cases, extrapulmonary TB (EPTB) accounted for 15% of the cases [1]. The proportion of EPTB is higher in females [2–6], people with African or Asian origin [2–4], TB and human immunodeficiency virus (HIV) co-infected patients [2, 3, 7] and at younger ages [2–6].

Timely detection and proper treatment of TB are two of the key elements of an effective TB control programme [8]. In the context of the WHO's End TB strategy, which calls for early diagnosis and treatment of all TB cases [9], the detection, treatment and follow-up of EPTB cases should also be given priority. Diagnosing EPTB is challenging as it frequently has non-specific clinical presentation and may simulate other conditions, which may contribute towards delay in diagnosis. In addition, lack of rapid, simple and accurate diagnostic tools for diagnosing EPTB may prolong diagnostic delay [10]. Since EPTB is rarely infectious the aspect of transmission is not as important as in pulmonary TB (PTB) patients, but delay in diagnosis and treatment could lead to increased disease severity, more complications and economic costs for the patient and the families affected.

Most studies on the diagnostic delay in TB have focused on PTB and adults [11–13]. Fewer studies have included children and EPTB patients, and these studies have reported longer delays among EPTB patients as compared to PTB [14–17]. However, there is insufficient knowledge on the extent of the various delays and the associated factors among EPTB patients. It is important to identify factors contributing to the distinct types of delays in EPTB, which could provide information for evidence-based intervention to improve case-finding and prompt diagnosis and treatment.

The United Republic of Tanzania is among the 30 high TB burden countries in the world [1]. Zanzibar, a semi-autonomous part of the United Republic of Tanzania, comprising the two main islands Unguja and Pemba and some smaller islands, has 1.3 million inhabitants [18]. According to a national TB prevalence survey in 2012, the estimated prevalence of bacteriologically confirmed PTB in Zanzibar was 124/100 000 in the adult population [19]. Among the 814 notified incident TB cases in Zanzibar in 2015, 195 (24%) were EPTB cases [20]. Zanzibar has 100% directly observed treatment coverage, however, the notified TB cases are still below the estimated numbers of TB cases.

The aims of this study were to assess the health care seeking behavior, EPTB knowledge and diagnostic delay in presumptive EPTB patients presenting at the Mnazi Mmoja Hospital (MMH), Zanzibar, the factors associated with longer delay and the impact of untreated EPTB on self-rated health status of patients. The presumptive EPTB cases were categorized into TB and non-TB cases based on a composite reference standard allowing us to compare the TB and non-TB cases with similar clinical presentation.

Materials and methods

Study setting

The study was conducted at MMH, which is the main referral hospital in Zanzibar. The public health care system in Zanzibar is divided into three levels [21]. The primary level consists of primary health care units (PHCU), PHCU+, which are supposed to provide additional services of delivery, dental, dispensing and laboratory, and primary health care centres/cottage hospitals (PHCC) which serve as referral level for PHCU and PHCU+. PHCCs has both in- and outpatient services, average capacity of 30 beds, and provides additional services such as diagnostic imaging (ultrasound and x-ray). The secondary and tertiary level consist of district hospitals, special and referral hospitals. In 2013, there were 100 PHCUs, 34 PHCU+, 4 PHCCs, 3 district hospitals, 2 special hospitals and 1 main referral hospital (MMH) in Zanzibar [21]. MMH, situated in the capital at Unguja Island, provides primary and secondary health care for some districts, in addition to tertiary health care. There is a good distribution and access to primary health care services, with 95% of the population living within 5 km to the nearest public health facility [21]. However, there is a limited capacity to perform invasive procedures for diagnostic purposes at the primary level of health care. MMH is the only public hospital with the capacity to perform the diagnostic cytological/histological evaluation of fine-needle aspirates and biopsies. In 2013/14, there were 4 private hospitals, all situated at Unguja Island, 71 private clinics/dispensaries, 25 pharmacies and 335 over-the-counter drug shops in Zanzibar [22]. The private health facilities are predominantly located in the major towns. The TB preventive, diagnostic and treatment services are integrated into the public health service at all levels. Anti-TB treatment (ATT) is free of charge and all public health facilities and some private facilities are providing TB treatment services. TB/HIV collaborative activities are well-organized, and all TB patients are offered HIV testing and counselling.

Study design and population

The study participants were prospectively enrolled at MMH, from 1st of August 2014 until 31st of August 2015, from outpatient departments and the hospital wards. The study population was patients of all ages presenting with symptoms suggestive of EPTB. The current study was part of a larger study evaluating the implementation of a new diagnostic test for the diagnosis of EPTB at MMH [23], and eligible patients were included if a representative biological specimen was sampled for laboratory investigations from the presumptive site of EPTB infection. Those who did not give an informed written consent or had received ATT during the previous year were excluded. All study participants were interviewed at the time of inclusion using a pretested semi-structured questionnaire and closely followed until a diagnosis was concluded by the local clinicians. Additional visits to health care providers after inclusion in the study were documented. Patients starting ATT were further assessed at 2–3 months and at end of ATT to evaluate response to treatment, and patients not starting ATT were followed until recovery or until a diagnosis was established. At the end of the study, patients were categorized as TB cases or non-TB cases using a composite reference standard, including clinical signs and symptoms, radiological findings, results from various laboratory investigations, response to specific non-tuberculous therapy and response to ATT [23].

Data collection and outcomes

The main outcomes were patient, health system and total delays in days. Additionally, we assessed general TB knowledge and EPTB knowledge among all the study participants and self-rated health status in adult EPTB patients before ATT and after the completion of ATT.

The following definitions were used. The onset of symptoms referred to the time at which the first symptom appeared, either localized symptoms or constitutional symptoms which led to the care-seeking. The patient delay was defined as the time interval between the onset of symptoms and the patients'first visit to a health care provider because of those symptoms. The health system delay was defined as the time interval between the patients'first visit to a health care provider and the initiation of ATT. The total delay was defined as the sum of patient delay and health system delay. Health care providers were defined as modern health facilities such as private clinics/dispensaries, PHCU, PHCU+, PHCC or hospitals owned by the government or the private sectors. Registered pharmacies were also included, whereas, non-formal health providers, such as traditional healers and over-the-counter drug stores were not included in this definition.

A semi-structured questionnaire was developed in English and translated to the local language, Swahili. Further, the Swahili version was translated back to English by a person who had not seen the original version. The two English versions were compared to evaluate the validity of the questions. To adjust the language to the local setting, two local bilingual individuals assessed the questions prior to testing the questionnaire among inpatients ($n = 3$) at MMH to clarify confusing questions and estimating the required time for filling in the questionnaire. Two local medical officers, who underwent training, conducted all the interviews. Included in the questionnaire were questions related to sociodemographic characteristics, symptoms and their duration, estimation of the time interval from the onset of symptoms until the first contact with a health care provider, number of different health care providers contacted due to the current illness and other questions regarding medical history, health care seeking behavior and TB knowledge [23]. The date of starting ATT and medical history were counterchecked from patient medical records and TB treatment cards.

The study participants'TB knowledge was assessed by questions related to symptoms, site of infection, transmission and treatment of TB (S1 Table). A scoring system was designed based on these questions and the median score among all the respondents was used as a cut-off point to dichotomize the responses into two categories, lesser (below the median) and higher (equal or above the median) TB knowledge. The TB knowledge questions were answered by the parent/guardian for study participants $<$ 15 years of age.

In addition to the questionnaire, the adult patients starting ATT answered the EQ-5D 3 level version (EQ-5D-3L) [24], before and after ATT, to provide a simple descriptive health profile on self-rated health. The EQ-5D-3L comprises the EQ-5D descriptive system and the EQ visual analogue scale. The EQ-5D descriptive system consists of 5 dimensions (mobility, self-care, usual activities, pain/discomfort and anxiety/depression) with 3 levels (level 1, no problems; level 2, some problems; level 3, extreme problems). The EQ VAS comprises a vertical, visual analogue scale, with endpoints labelled "Best imaginable health state", marked 100, and "Worst imaginable health state", marked 0, and records the respondent's self-rated health at the time of completion. The EQ-5D-3L was provided in the local language (EuroQoL Group 1990).

The exposure variables included age, sex, educational level, housing, HIV status, site of TB disease, self-treatment before care-seeking, distance to the nearest health care provider, initial health care provider visited after the onset of symptoms, antibiotics given at first visit, number of visits to health care providers, and TB knowledge.

Statistical analysis

Descriptive statistics were presented, and group differences were compared using the Chi-square test for proportions. Differences in the delay variables were assessed by Mann-Whitney

or Kruskal-Wallis tests for differences between the groups as well as Wilcoxon signed rank test for repeated measures. The general significance level was set to 0.05. Due to the larger number of tests we had to consider multiple testing effects. We used the Bonferroni adjustment, leading to the marginal levels of 0.0083 (6 tests) for sociodemographic and clinical characteristics, 0.01 (5 tests) for first health care provider visited, 0.0042 (12 tests) for the delay, and 0.0125 (4 tests) for TB knowledge score. Data analysis was performed using SPSS 24 (Armonk, NY) and graphics was created using Matlab 9.0 (Natick, MA).

Ethical considerations

Ethical clearance was obtained from the Regional Committee for Medical and Health Research Ethics, Western-Norway (REK Vest) and the Zanzibar Medical Research and Ethics Committee (ZAMREC). All study participants signed an informed written consent. In patients < 18 years, the written consent was signed by the parent/guardian, in addition, patients ≥ 7 years had to sign the consent form.

Results

Sociodemographic and clinical characteristics

Of 146 eligible patients presenting with presumptive EPTB, 132 patients were enrolled in the study and 14 excluded (5 refused to participate, 3 on treatment within the last year, 6 with no representative sample for laboratory investigation). Sixty-nine participants were categorized as TB cases, and 63 as non-TB cases. The sociodemographic and clinical characteristics of the included study participants are described in Table 1. The TB and non-TB patients did not differ significantly with regard to sex, residence, educational level, HIV status or presumptive site of infection. The median age was 27 years (interquartile range (IQR), 8–41 years). Most of the non-TB patients were either children or ≥ 45 years, whereas the majority of the TB patients were between 15–44 years. HIV status was known in 99 (75%) patients, and 22 (22%) of these were HIV positive.

Health care seeking behaviour and care seeking pathways

Table 2 shows the distribution of health care seeking trajectories among the TB and non-TB patients. The majority of patients consulted public health care providers as their first contact with the health care system after the onset of symptoms. Patients who first visited a public health care provider were similar to those who initially went to a private health care provider with regard to sex, age groups, residence, HIV status and TB category (TB or non-TB cases) (all $P > .1$). Further, no differences were found regarding the same variables when comparing those who contacted MMH first with patients who initially visited any health care provider other than MMH. Overall, 39 patients (30%) sought care at MMH as their first contact, while 32 (24%) at PHCUs, 22 (17%) at private clinics/dispensaries, 18 (14%) at PHCC/district hospitals, 18 (14%) at private hospitals and 2 (2%) at pharmacies. The practice of self-medication before contacting a health care provider was common and reported by 46% of the patients. When excluding patients only consulting MMH, 45/99 (45%) patients, reported repeated visits at the same level in the health care system before referral to MMH. Repeated visits to MMH before the commence of ATT were reported by 56/69 (81%) of the TB cases. Nearly half of the TB patients had > 3 visits to a health care provider, and 26% had visited ≥ 3 different health care providers, either at the same level or different level of health care, before the initiation of ATT. The most common care-seeking pathways among TB patients were; 18 (26%) patients had only visited MMH, 14 (20%) private clinic/dispensary and MMH, 9 (13%) private hospital

Table 1. Sociodemographic and clinical characteristics of the study participants, n (%).

Characteristics		TB patients	non-TB patients	*P* value
		***n* = 69**	***n* = 63**	
Sex				.811
	Male	38 (55%)	36 (57%)	
	Female	31 (45%)	27 (43%)	
Age groups, years				.010
	0–14	18 (26%)	25 (40%)	
	15–44	40 (58%)	20 (32%)	
	≥45	11 (16%)	18 (29%)	
Residential status				.871
	Urban	48 (70%)	43 (68%)	
	Rural	21 (30%)	20 (32%)	
Highest educational level[a]				.592
	≤ primary school	26 (51%)	21 (57%)	
	> primary school	25 (49%)	16 (43%)	
Main household income[b]				
	Agriculture/fishing	19 (33%)		
	Governmental/private sector	15 (26%)		
	Self-employed	24 (41%)		
Housing[c]				
	Own house	37 (59%)		
	Renting/living with relatives	26 (41%)		
Number of people in household[d]				
	1–5	24 (38%)		
	6–10	25 (40%)		
	≥11	14 (22%)		
HIV status				.396[e]
	Negative	48 (75%)	29 (83%)	
	Positive	16 (25%)	6 (17%)	
	Unknown	5 (-)	28 (-)	
Presumptive site of infection[f]				.263
	Lymphadenitis	36 (52%)	33 (52%)	
	Pleuritis	20 (29%)	12 (19%)	
	Other sitesf	13 (18%)	18 (29%)	

Abbreviations: TB, tuberculosis; HIV, human immunodeficiency virus.

[a] Only adult patients. Patients with missing values excluded (n = 1).

[b, c, d] Patients with missing values excluded (n = [b]; 11, [c, d]; 6). Not recorded for non-TB patients.

[e] Only comparing patients with known HIV serostatus.

[f] TB patients; meningitis (n = 4), spondylitis (n = 1), pericarditis (n = 1), peritonitis (n = 7). Non-TB patients; meningitis (n = 6), osteomyelitis (n = 1), mastitis (n = 1), peritonitis (n = 10).

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and MMH, 9 (13%) PHCU and MMH, and 7 (10%) patients had been in contact with PHCC/district hospital and MMH. The patients were also asked to report if they had visited a traditional healer during the care-seeking interval. Only 7/69 (10%) of the TB patients reported visiting a traditional healer, 6 of these patients were females and all were ≥ 15 years. An illustration of health care seeking pathways among the TB cases is presented in Fig 1.

Table 2. Distribution of health care seeking trajectories and TB knowledge, n (%).

Variable		TB patients	non-TB patients	*P* value
		***n* = 69**	***n* = 63**	
Self-treatment before care seeking[a]				.959
	Yes	31 (46%)	29 (46%)	
	No	37 (54%)	34 (54%)	
Distance to nearest HCP (time)[b]				
	< 30 min	15 (23%)		
	30–60 min	32 (49%)		
	> 60 min	18 (28%)		
First HCP visited[c]				.281
	Public	44 (64%)	45 (73%)	
	Private	25 (36%)	17 (27%)	
First HCP visited[c]				.861
	MMH	21 (30%)	18 (29%)	
	HCP other than MMH	48 (70%)	44 (71%)	
Antibiotics given at first visit[d]				.869
	Yes	38 (58%)	36 (59%)	
	No	28 (42%)	25 (41%)	
Given a diagnosis prior to interview[e]				.672
	No	36 (59%)	38 (66%)	
	Yes, other diagnosis than tuberculosis	21 (34%)	18 (31%)	
	Yes. Tuberculosis	4 (7%)	2 (3%)	
Number of different HCP[f]				
	1	18 (26%)	14 (23%)	
	2	33 (48%)	28 (45%)	
	3	11 (16%)	13 (21%)	
	≥4	7 (10%)	7 (11%)	
Number of visits to HCP[g]				
	1	2 (3%)	5 (9%)	
	2	10 (14%)	14 (25%)	
	3	24 (35%)	19 (33%)	
	4	7 (10%)	8 (14%)	
	≥5	26 (38%)	11 (19%)	
TB knowledge[h]				.607
	Good (≥median)	38 (56%)	38 (60%)	
	Poor (<median)	30 (44%)	25 (40%)	
EPTB knowledge[h]				
	Yes	1 (1%)	0 (-)	
	No	67 (99%)	63 (100%)	

Abbreviations: TB, tuberculosis; HCP, health care provider; MMH, Mnazi Mmoja Hospital; EPTB, extrapulmonary tuberculosis.

[a] Patients with missing values excluded (n = 1)

[b] Patients with missing values excluded (n = 4). Not recorded for non-TB patients.

[c, d, e] Patients with missing values excluded (n = [c]; 1, [d]; 5, [e]; 13).

[f, g] Patients with missing values excluded (n = [f];1, [g]; 6). No comparison between TB patients and non-TB patient because the number of various health carer providers visited and the number of visits for TB patients were recorded until starting antituberculosis treatment, but for non-TB patients only to the day of the interview.

[h] Patients with missing values excluded (n = 1).

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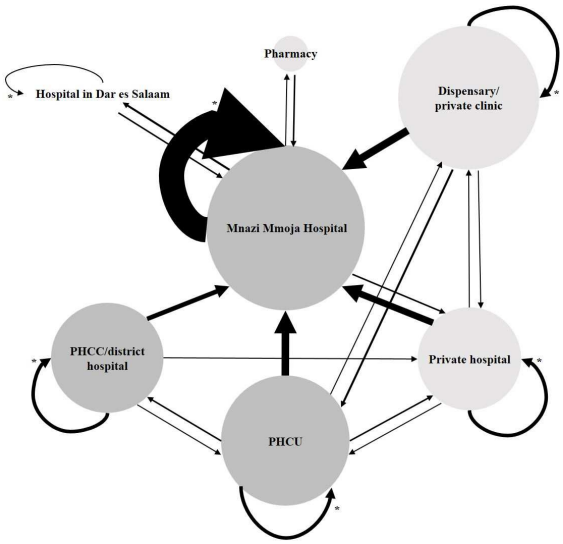

Fig 1. Health care seeking pathways in TB patients presenting at Mnazi Mmoja Hospital, Zanzibar. The size of the arrows shows the number of patients going in the direction of the arrow in the health care system. The size of the circles correlates to the number of patients visiting each respective site as the first place of contact (Mnazi Mmoja hospital, n = 21; private clinic/dispensary, n = 16; primary health care unit, n = 14; primary health care centre/district hospital, n = 9; private hospital, n = 8; pharmacy, n = 1). Colour—Dark grey, public health sector; light grey, private health sector. *Repeated visits at the same health care level, either same place or different health care provider at the same level. Abbreviations: PHCC, primary health care centre; PHCU, primary health care unit.

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Diagnostic delay and associated factors

The patient, health system and total delays observed in TB patients are described according to different variables in Table 3. The median patient delay was 14 days (TB cases IQR, 5–28 days; non-TB cases IQR, 6–28 days) with no significant difference between TB and non-TB cases ($P = .794$). Patients with TB lymphadenitis reported a significantly longer patient delay compared to patients with other sites of TB disease. Interestingly, the median patient delay for those who first consulted MMH was 28 days compared to 11 days for those who initially visited other health facilities, but the difference was not statistically significant.

The median health system delay among TB patients was 34 days (IQR, 19–76 days). Patients seeking care at MMH at first consultation reported a significantly shorter health system delay as compared to the patients visiting other health care providers first ($P = .001$). A longer health system delay was found in patients with > 3 visits to health care providers compared to those with ≤ 3 visits ($P < .001$). The health system delay tended to be longer among respondents with higher educational level, TB lymphadenitis and who were HIV negative, as compared to those with lower educational level, other sites of TB infection and who were HIV positive, respectively, but without reaching statistical significance. Among patients first consulting other health facilities than MMH, the health system delay before referral to MMH was significantly longer than the delay at MMH (median, 22 vs. 11 days, $P = .011$).

The median total delay among TB patients was 62 days (IQR, 31–126 days), 15% had much longer total delay exceeding 6 months and 8% had total delay exceeding one year. Among the patients delaying > 6 months, 90% were diagnosed with TB lymphadenitis. The health system

Table 3. Patient delay, health system delay and total delay among TB patients by sociodemographic and clinical variables and health care seeking trajectories[a].

Variable		Patient delay (days)			Health system delay (days)			Total delay (days)		
		n	median (IQR)	*P* value	*n*	median (IQR)	*P* value	*n*	median (IQR)	*P* value
Sex				.400			.235			.777
	Male	38	13 (5–28)		36	36 (20–106)		36	58 (29–151)	
	Female	30	18 (6–47)		29	28 (14–67)		29	69 (31–118)	
Age groups, years				.674			.585			.137
	0–14	18	10 (5–28)		17	28 (22–65)		17	36 (28–113)	
	15–44	40	18 (4–42)		39	41 (16–115)		39	72 (34–163)	
	≥45	10	11 (7–26)		9	23 (16–61)		9	34 (26–94)	
Educational level[b]				.831			.032*			.297
	≤primary school	25	17 (4–79)		23	23 (11–51)		23	61 (31–108)	
	>primary school	25	16 (6–28)		25	48 (22–118)		25	91 (33–171)	
Housing				.253			.318			.031*
	Own house	36	13 (4–21)		35	28 (21–51)		35	41 (28–96)	
	Renting/living with relatives	26	21 (5–47)		26	59 (16–118)		26	98 (54–167)	
HIV status				.734[c]			.055[c]			.727[c]
	Negative	48	15 (5–36)		46	42 (21–110)		46	64 (30–155)	
	Positive	15	17 (5–41)		14	21 (10–67)		14	67 (31–121)	
	Unknown	5	8 (5–16)		5	28 (21–43)		5	36 (31–53)	
Site of TB infection				< **.001****			.091			< **.001****
	Lymphadenitis	35	28 (14–76)		34	55 (22–118)		34	99 (62–189)	
	Pleuritis	20	6 (4–11)		19	28 (16–48)		19	34 (20–65)	
	Other sites	13	6 (3–17)		12	22 (16–42)		12	30 (26–60)	
Self-treatment before care seeking				.870			.962			.903
	Yes	30	18 (4–42)		28	39 (17–80)		28	61 (30–141)	
	No	37	11 (6–28)		36	27 (20–79)		36	68 (30–127)	
First HCP visited				.025*			**.001****			.445
	MMH	21	28 (8–111)		19	21 (7–34)		19	91 (31–124)	
	Other than MMH	47	11 (4–21)		46	46 (23–111)		46	61 (28–133)	
Antibiotics given at first visits							.141			
	Yes		-		35	41 (21–118)			-	
	No		-		27	24 (17–60)			-	
Distance to nearest HCP (time)				.553			.500			.993
	< 30 min	15	7 (6–38)		15	28 (16–86)		15	72 (21–179)	
	30–60 min	32	14 (4–28)		31	41 (23–86)		31	61 (30–129)	
	< 60 min	17	17 (6–67)		17	23 (14–56)		17	61 (30–124)	
Number of visits to HCP							< **.001****			
	≤3		-		34	21 (12–29)			-	
	>3		-		31	69 (41–138)			-	
TB knowledge				.930			.280			.531
	Good (≥median)	37	14 (5–28)		35	28 (16–70)		35	62 (29–112)	
	Poor (<median)	30	14 (5–47)		29	41 (21–113)		29	65 (31–184)	

Abbreviations: TB, tuberculosis; IQR, interquartile range; HIV, human immunodeficiency virus, HCP, health care provider; MMH, Mnazi Mmoja Hospital

[a] Patients with missing values excluded

[b] Only adult patients included.

[c] Only comparing patients with known HIV serostatus

* *P* value < 0.05

** Statistically significant, *P* value < 0.0042.

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delay was significantly longer as compared to the patient delay ($P < .001$) and the greatest contributor to the total delay. In 80% of the patients the health system delay was longer than the patient delay. A longer total delay was noted for patients renting a house/living with relatives compared to patients owning their house, but the difference was not statistically significant. The only significant difference in total delay was found when comparing delay according to the site of TB disease, where a longer total delay was experienced by patients with TB lymphadenitis ($P < .001$), where 26% of the TB lymphadenitis patients faced a total delay exceeding 6 months.

TB knowledge

Table 2 shows the level of TB knowledge among the TB and non-TB patients and S1 Table presents the distribution of answers to the various TB knowledge questions according to TB category, sex, HIV status and educational level. The knowledge about extrapulmonary site of TB was extremely poor. Fifty-five percent knew that the lungs could be affected by TB, as compared to only one patient who mentioned an extrapulmonary site. Most patients (93%) had heard of TB disease before being included in the study, and 73% answered respiratory and/or constitutional symptoms when asked about symptoms of TB disease. None of the respondents mentioned any other local symptoms than respiratory symptoms. Most of the respondents knew that TB is a curable disease (69%) and that TB can spread from person to person (79%). The median TB knowledge score was 5 (IQR, 3–6). The respondents with education above primary level had a higher TB knowledge score compared to patients with lower educational level ($P = .007$).

Morbidity due to TB and improvement in self-rated health status after treatment

In the adult EPTB patients, 19/47 (40%) reported that they had completely stopped working and 20/47 (43%) reported reduced working capacity due to the current illness. Among respondents with TB lymphadenitis (n = 23), 70% reported reduced working capacity/stopped working, while this was noted in 96% of the respondents with other sites of TB infection (n = 24). The TB lymphadenitis patients reported a median of 90 days (IQR, 60–120 days) of reduced working capacity/stopped working, and patients with other sites of TB infection a median of 30 days (IQR, 14–60 days). Adult EPTB patients (n = 31) reported significantly higher self-rated health in the EQ VAS after compared to before ATT. The median EQ VAS score before and after ATT was 60% (IQR 48–80%) and 96% (IQR 90–100%) ($P < .001$), respectively. Respondents with TB lymphadenitis (n = 17) had a relatively better self-rated health before treatment, (EQ VAS; median, 79%, IQR, 50–91%) as compared to patients with other sites of TB infection (pleuritis, n = 10; peritonitis, n = 3; spondylitis, n = 1) (EQ VAS; median, 50%, IQR, 38.5–60%) ($P = .015$). Both groups reported significantly higher self-rated health after treatment as compared to before treatment (TB lymphadenitis, $P = .002$; other sites of TB infection, $P = 0.001$) (Fig 2). A similar pattern was observed when the health status was assessed with the EQ-5D descriptive system. Before treatment, patients with other sites of TB infection described more problems in all dimensions as compared to patients with TB lymphadenitis. Still, both groups reported lesser problems at the end of treatment than before treatment (Fig 3).

Discussion

This study found that many EPTB patients, presenting to the main referral hospital in Zanzibar, experienced significant delay from the onset of symptoms until the start of TB treatment.

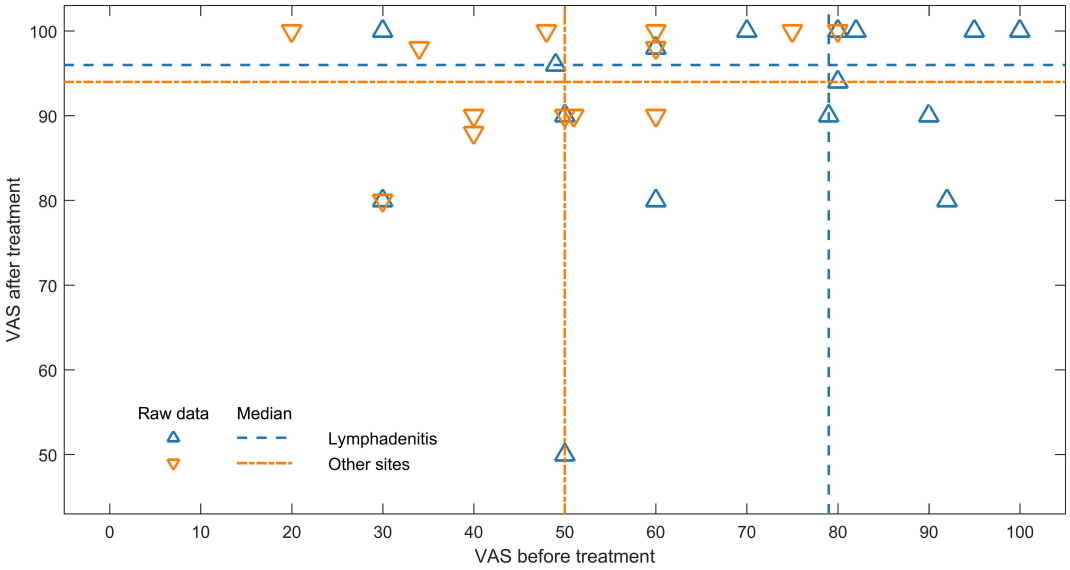

Fig 2. Scatterplot showing the EQ visual analogue scale scores before and after TB treatment. The scores are divided according to the site of TB infection, where the TB lymphadenitis patients are indicated with blue colour and other sites of TB infection with orange colour. The median scores in both groups before and after treatment are shown. Both groups reported significantly higher self-rated health after treatment.

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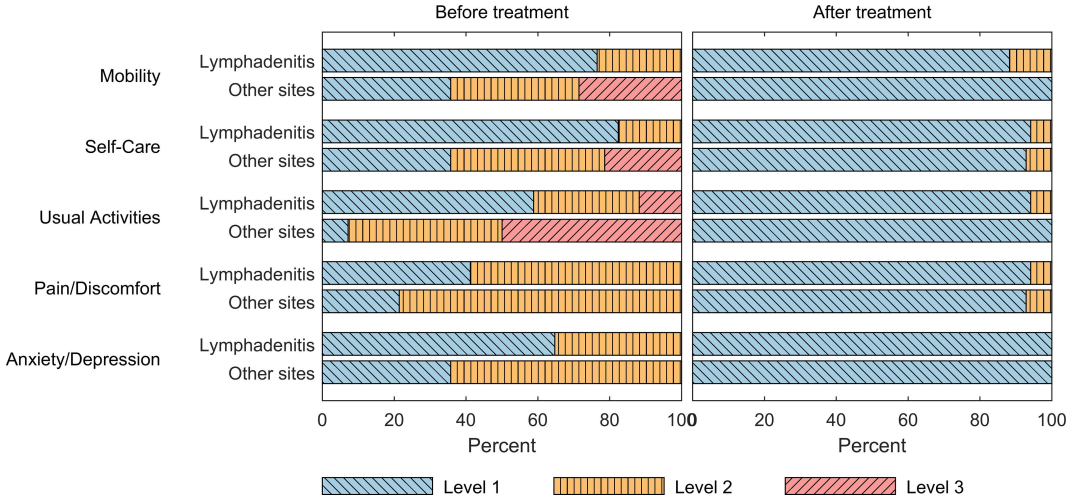

Fig 3. Stacked bar plot describing the patient statements in each of the 5 dimensions of the EQ-5D descriptive system before and after TB treatment. Level 1, no problems; level 2, some problems; level 3, extreme problems. Both groups reported lesser problems after treatment.

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Even though EPTB have been reported as a predictor of longer delays [14–16, 25–27], most earlier studies have been focusing on delay and associated factors in PTB, particularly smear-positive PTB. Cases of EPTB are seldom infectious and thus have less consequences for the spread of the TB disease, and probably therefore have not been studied specifically. However, for the patients, delay in the start of treatment may results in increased morbidity and mortality. A study from South-Africa [28], reported increased mortality when provider delay was $\geq$ 30 days. Further, TB poses an economic burden on affected patients and their household [29, 30]. In our study, 83% of the adult EPTB patients reported that they had completely stopped working or had reduced working capacity due to the current illness, a finding similar to a study from Kenya [30], where 85% of the respondents reported a decrease in the number of hours worked per week as a result of TB illness. Further, the adult EPTB patients in our study reported significantly higher self-rated health using the EQ VAS and described lesser problems in the EQ-5D descriptive system after TB treatment. Thus, this indicates that reducing the delay in the diagnosis and treatment of EPTB could decrease patient morbidity and have a positive impact on the economic situation for the patients and their families.

To the best of our knowledge there is no defined consensus on what constitutes acceptable and unacceptable delay in the diagnosis and initiation of treatment in EPTB patients. Some studies also including EPTB patients have used $>$ 30 days and $>$ 14 days as unacceptable patient and health system delay, respectively [17, 31]. Using these cut-offs, 76% of the EPTB patients in the present study reported to a health care provider within the acceptable patient delay, whereas only 17% had acceptable health system delay.

The total delay with a median of 62 days was found to be in agreement with previous reports including EPTB patients from resource-constrained settings [17, 25, 32], while other studies have found longer total delays [33]. Long total delays among EPTB patients have also been reported in industrialized countries [16, 27]. Previous studies have demonstrated that there is no consistent pattern of patient and health system delays between various settings. In the current study, health system delay was the major contributing factor to the total time to diagnosis and treatment, consistent with studies from settings as diverse as India [34], South Africa [28] and France [35]. Other studies from Nepal [32], Ethiopia [26] and Denmark [27] have reported patient delay as a major contributor towards total delay. These studies indicate that factors contributing to delay may vary depending on the setting and the study population.

The median patient delay of 14 days in our study was short compared to the patient delay reported in studies from Rwanda [25], Angola [36], Ethiopia [26] and mainland Tanzania [37], but longer than a study among children in Delhi, India [34]. These differences may be explained by the different distribution of urban and rural populations in the various studies, and in the availability of medical care. Unlike the findings reported from the United Kingdom [38] and Norway [14] we found no difference in patient delay according to age groups. In addition, no difference in patient delay was noted between men and women, a finding similar to Ethiopia [26], but different from South Africa [28], where a longer patient delay was reported among males. Self-treatment before consulting a health care provider was common in our study, but were not associated with longer patient delay, a finding different from a study from Ethiopia [15] reporting self-treatment as a predictor of patient delay. In our study few (10%) patients reported visiting a traditional healer, which may also be an explanation for the relative short patient delay, as consulting traditional healers is consistently reported to be associated with longer patient delay [12].

The median health system delay of 34 days was longer than acceptable and a significant contributor towards total delay in our study. This is comparable with delay reported in previous studies also including EPTB patients from Ethiopia [15], South Africa [28] and Rwanda [25], but longer than reported in another study from Ethiopia [26]. The shorter delay in the

Ethiopian study could be explained by patients presenting with more advanced symptoms of disease since the median patient delay was 60 days in this study. Again, other studies from Norway [14] and United Kingdom [16] reported both longer patient and health system delay among EPTB patients than the present study, which could be because of a lower index of suspicion of TB in low-endemic countries. A long health system delay may partly be explained by the wide range of clinical presentation of EPTB disease, the low sensitivity of routine diagnostic methods such as acid-fast bacilli microscopy [39, 40] and mycobacterial culture [39, 41], lack of trained personnel and facilities for performing invasive procedures, thus increasing the difficulty in obtaining adequate biological samples for laboratory investigations, and the limited capacity for diagnostic imaging in low-resource settings. In addition, being dependent on patients returning for follow-up visits to receive results from various investigations or patients delaying consultation at a higher health care level even though a referral has been advised, could be contributing factors to the health system delay. A longer health system delay was found in patients with >3 visits to a health care provider, which is in agreement with a study from India reporting that number of providers consulted until TB diagnosis was associated with health system delay [34]. As concluded in a review of delay in the diagnosis and treatment of tuberculosis, "The core problem in delay of diagnosis and treatment seemed to be a vicious cycle of repeated visits at the same healthcare level, resulting in nonspecific antibiotic treatment and failure to access specialized TB services" [11]. In our study, 48% of the TB patients reported >3 visits and 38% ≥ 5 visits to a health care provider due to the current illness. Further, 81% reported repeated visits to MMH before diagnosis and initiation of treatment. In TB patients with initial visit to other health care providers than MMH, the health system delay prior to referral to MMH was longer as compared to the delay at MMH, and 47% reported repeated visits at the same level before referral to MMH. Advancing disease and the availability of diagnostic tools as imaging and laboratory investigations could have contributed to the shorter delay at MMH. At MMH, 37% of these patients were not started on ATT within 2 weeks of their first visit to the hospital. A high threshold for initiation of treatment, awaiting diagnostic proof, could also cause delay in the management of EPTB patient. On the other hand, starting treatment only based on clinical presumptive EPTB, leads to overtreatment. In our study, ATT was given to 19% of the non-TB cases, highlighting the importance of laboratory confirmation of EPTB and close follow-up of EPTB patients during treatment.

Patients with higher educational level tended to have longer health system delay, which is somewhat surprising. The explanation for this is uncertain, but there was a higher percentages of TB lymphadenitis cases among those with higher educational level compared to patients with lower educational level (56% vs 35%), which could explain the tendency for a longer health system delay in these patients.

In our study, patients with TB lymphadenitis experienced the longest median total delay of 99 days as compared to TB at other sites. A study from Tanzania also reported a long median diagnostic delay of 119 days (17 weeks) among TB lymphadenitis patients [36]. The indolent course of disease, lesser local symptoms, and fewer or no constitutional symptoms in lymphadenitis may be the reason for longer delay. Frequent involvement of cervical lymph nodes in different infections and lack of confirmatory TB tests may also have contributed towards the longer health system delay in lymphadenitis. Despite the indolent course, it is important to reduce the delay as these patients experienced significant improvement in self-rated health after treatment.

The level of TB knowledge in our study may be a reasonable indicator of the knowledge regarding TB in the general population in Zanzibar, since TB knowledge was assessed in both TB and non-TB patients and the TB patients were interviewed preferably before starting ATT, and by that they had not yet received the information following initiation of treatment. Our

study indicates that while patients had relatively good knowledge of PTB, the knowledge regarding EPTB was very low, identifying a potential area of intervention.

Our study has some limitations. The samples size is small, limiting the possibility of performing further statistical analysis on factors associated with longer delays. The patients were enrolled only from the main referral hospital in Zanzibar, and patients initiated on treatment at the primary or secondary care level are not represented in the study. Thus, the results may not necessarily be generalisable to all patients presenting with presumptive EPTB to the health care system in Zanzibar. The information regarding symptoms, onset of symptoms and health care seeking behaviour are mainly self-reported and implies the possibility of recall bias. We did not record the patients visit to over-the-counter drug shops, and there is a possibility that some of the patient first sought care at these sites, further reducing the actual patient delay. Further, the study participants were part of a study implementing and validating a new diagnostic test for EPTB at MMH, and being part of this study may potentially have influenced health system delay in either direction. Finally, use of a composite reference standard for categorization of TB patients is not perfect and some patients with other chronic bacterial infections could have responded to the ATT and wrongly categorized as TB patients. Similarly, inadequate response due to poor compliance or drug resistance could have led to wrong categorization of patients as non-TB cases. However, subanalysis of the bacteriologically or clinically confirmed TB groups did not show difference in various outcomes (data not presented due to small sample size), and unlikely to have an impact on the conclusions drawn in this study.

Conclusion

Many EPTB patients presenting to the main referral hospital in Zanzibar had delay in the diagnosis and treatment exceeding two months, and the greatest proportion of this delay occurred due to the health system delay. The self-rated health among adult EPTB patients was significantly higher after treatment, implying that appropriate and timely treatment of EPTB disease have the potential to reduce morbidity and the economic loss for the patient and their families. A reduction of diagnostic delay could be achieved through strengthening the knowledge and awareness of EPTB in the general population by incorporating information on EPTB in public health educational campaigns, continuous training of all health care providers, at all levels, in early recognition of symptoms suggestive of EPTB and the diagnostic possibilities, proper information and a scheduled follow-up of patients receiving a trial of antibiotics and strengthening the collaboration between the national TB programme and private and public health facilities such as outpatient clinics and hospital wards performing invasive diagnostic procedures. Further, since most peripheral health units do not have diagnostic facilities for diagnosing EPTB it could be feasible to establish algorithms of timely referral of presumptive EPTB patients to health facilities with diagnostic capacity and higher medical expertise to shorten the care-seeking pathway. Finally, support and strengthening of the laboratories performing TB diagnostics services is of utmost importance.

Supporting information

S1 Table. Distribution of answers to TB knowledge questions according to TB category, sex, HIV status and educational level.
(DOCX)

S1 File. Data set.
(SAV)

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References

1. World Health Organization. Global tuberculosis report 2016. 2016.
2. Gonzalez OY, Adams G, Teeter LD, Bui TT, Musser JM, Graviss EA. Extra-pulmonary manifestations in a large metropolitan area with a low incidence of tuberculosis. Int J Tuberc Lung Dis. 2003; 7 (12):1178–85. PMID: 14677893.
3. Yang Z, Kong Y, Wilson F, Foxman B, Fowler AH, Marrs CF, et al. Identification of risk factors for extra-pulmonary tuberculosis. Clin Infect Dis. 2004; 38(2):199–205. https://doi.org/10.1086/380644 PMID: 14699451.
4. Rieder HL, Snider DE Jr., Cauthen GM. Extrapulmonary tuberculosis in the United States. Am Rev Respir Dis. 1990; 141(2):347–51. https://doi.org/10.1164/ajrccm/141.2.347 PMID: 2301852.
5. Memish ZA, Bamgboye EA, Abuljadayel N, Smadi H, Abouzeid MS, Al Hakeem RF. Incidence of and risk factors associated with pulmonary and extra-pulmonary tuberculosis in Saudi Arabia (2010–2011). PLoS One. 2014; 9(5):e95654. https://doi.org/10.1371/journal.pone.0095654 PMID: 24824783; PubMed Central PMCID: PMCPMC4019475.
6. Sreeramareddy CT, Panduru KV, Verma SC, Joshi HS, Bates MN. Comparison of pulmonary and extra-pulmonary tuberculosis in Nepal- a hospital-based retrospective study. BMC Infect Dis. 2008; 8:8. https://doi.org/10.1186/1471-2334-8-8 PMID: 18218115; PubMed Central PMCID: PMCPMC2245948.
7. Jones BE, Young SM, Antoniskis D, Davidson PT, Kramer F, Barnes PF. Relationship of the manifestations of tuberculosis to CD4 cell counts in patients with human immunodeficiency virus infection. Am Rev Respir Dis. 1993; 148(5):1292–7. https://doi.org/10.1164/ajrccm/148.5.1292 PMID: 7902049.
8. World Health Organization. Early detection of tuberculosis: an overview of approches, guidelines and tools. WHO Document: WHO/HTM/STB/PSI/201121 Geneva, Switzerland, WHO, 2011. 2011.
9. World Health Organization. The End TB Strategy. WHO/HTM/TB/201519 Geneva, Switzerland, WHO, 2015. 2015.
10. Purohit M, Mustafa T. Laboratory Diagnosis of Extra-pulmonary Tuberculosis (EPTB) in Resource-constrained Setting: State of the Art, Challenges and the Need. J Clin Diagn Res. 2015; 9(4):EE01–6.

https://doi.org/10.7860/JCDR/2015/12422.5792 PMID: 26023563; PubMed Central PMCID: PMCPMC4437077.

11. Storla DG, Yimer S, Bjune GA. A systematic review of delay in the diagnosis and treatment of tuberculosis. BMC Public Health. 2008; 8:15. https://doi.org/10.1186/1471-2458-8-15 PMID: 18194573; PubMed Central PMCID: PMCPMC2265684.
12. Finnie RK, Khoza LB, van den Borne B, Mabunda T, Abotchie P, Mullen PD. Factors associated with patient and health care system delay in diagnosis and treatment for TB in sub-Saharan African countries with high burdens of TB and HIV. Trop Med Int Health. 2011; 16(4):394–411. https://doi.org/10.1111/j.1365-3156.2010.02718.x PMID: 21320240.
13. Cai J, Wang X, Ma A, Wang Q, Han X, Li Y. Factors associated with patient and provider delays for tuberculosis diagnosis and treatment in Asia: a systematic review and meta-analysis. PLoS One. 2015; 10(3):e0120088. https://doi.org/10.1371/journal.pone.0120088 PMID: 25807385; PubMed Central PMCID: PMCPMC4373856.
14. Farah MG, Rygh JH, Steen TW, Selmer R, Heldal E, Bjune G. Patient and health care system delays in the start of tuberculosis treatment in Norway. BMC Infect Dis. 2006; 6:33. https://doi.org/10.1186/1471-2334-6-33 PMID: 16504113; PubMed Central PMCID: PMCPMC1435913.
15. Belay M, Bjune G, Ameni G, Abebe F. Diagnostic and treatment delay among Tuberculosis patients in Afar Region, Ethiopia: a cross-sectional study. BMC Public Health. 2012; 12:369. https://doi.org/10.1186/1471-2458-12-369 PMID: 22621312; PubMed Central PMCID: PMCPMC3444375.
16. Saldana L, Abid M, McCarthy N, Hunter N, Inglis R, Anders K. Factors affecting delay in initiation of treatment of tuberculosis in the Thames Valley, UK. Public Health. 2013; 127(2):171–7. https://doi.org/10.1016/j.puhe.2012.11.010 PMID: 23313162.
17. Yimer SA, Bjune GA, Holm-Hansen C. Time to first consultation, diagnosis and treatment of TB among patients attending a referral hospital in Northwest, Ethiopia. BMC Infect Dis. 2014; 14:19. https://doi.org/10.1186/1471-2334-14-19 PMID: 24410927; PubMed Central PMCID: PMCPMC3898386.
18. National Bureau of Statistics, Ministry of Finance, Dar es Salaam, Office of Chief Government Statistician, President's Office, Finance, Economy and Development Planning, Zanzibar. Population distributon by age and sex The United Republic of Tanzania2013 [28 August 2017]. Available from: https://ihi.eprints.org/2169/1/Age_Sex_Distribution.pdf.
19. Senkoro M, Mfinanga S, Egwaga S, Mtandu R, Kamara DV, Basra D, et al. Prevalence of pulmonary tuberculosis in adult population of Tanzania: a national survey, 2012. Int J Tuberc Lung Dis. 2016; 20(8):1014–21. https://doi.org/10.5588/ijtld.15.0340 PMID: 27393533.
20. Ministry of Health Zanzibar. Zanzibar Integrated HIV, TB and Leprosy Programme Annual Report 2015. 2016.
21. Ministry of Health, Revolutionary Government of Zanzibar. Zanzibar Health Sector Strategic Plan III 2013/14-2018/19 Zanzibar2013 [cited 2017 30. October]. Available from: http://tanzania.um.dk/en/danida-en/health/.
22. Ministry of Health, Revolutionary Government of Zanzibar. 2013/14 Performance Report. Zanzibar, 2014.
23. Jorstad MD, 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; 13(5):e0196723. https://doi.org/10.1371/journal.pone.0196723 PMID: 29742144.
24. EuroQol G. EuroQol—a new facility for the measurement of health-related quality of life. Health Policy. 1990; 16(3):199–208. PMID: 10109801.
25. Lorent N, Mugwaneza P, Mugabekazi J, Gasana M, Van Bastelaere S, Clerinx J, et al. Risk factors for delay in the diagnosis and treatment of tuberculosis at a referral hospital in Rwanda. Int J Tuberc Lung Dis. 2008; 12(4):392–6. PMID: 18371264.
26. Gele AA, Bjune G, Abebe F. Pastoralism and delay in diagnosis of TB in Ethiopia. BMC Public Health. 2009; 9:5. https://doi.org/10.1186/1471-2458-9-5 PMID: 19128498; PubMed Central PMCID: PMCPMC2628652.
27. Leutscher P, Madsen G, Erlandsen M, Veirum J, Ladefoged K, Thomsen V, et al. Demographic and clinical characteristics in relation to patient and health system delays in a tuberculosis low-incidence country. Scand J Infect Dis. 2012; 44(1):29–36. https://doi.org/10.3109/00365548.2011.608081 PMID: 21923629.
28. Meintjes G, Schoeman H, Morroni C, Wilson D, Maartens G. Patient and provider delay in tuberculosis suspects from communities with a high HIV prevalence in South Africa: a cross-sectional study. BMC Infect Dis. 2008; 8:72. https://doi.org/10.1186/1471-2334-8-72 PMID: 18501019; PubMed Central PMCID: PMCPMC2413241.

29. Kamolratanakul P, Sawert H, Kongsin S, Lertmaharit S, Sriwongsa J, Na-Songkhla S, et al. Economic impact of tuberculosis at the household level. Int J Tuberc Lung Dis. 1999; 3(7):596–602. PMID: 10423222.

30. Mauch V, Woods N, Kirubi B, Kipruto H, Sitienei J, Klinkenberg E. Assessing access barriers to tuberculosis care with the tool to Estimate Patients' Costs: pilot results from two districts in Kenya. BMC Public Health. 2011; 11:43. https://doi.org/10.1186/1471-2458-11-43 PMID: 21244656; PubMed Central PMCID: PMCPMC3033813.

31. Osei E, Akweongo P, Binka F. Factors associated with DELAY in diagnosis among tuberculosis patients in Hohoe Municipality, Ghana. BMC Public Health. 2015; 15:721. https://doi.org/10.1186/s12889-015-1922-z PMID: 26220804; PubMed Central PMCID: PMCPMC4517499.

32. Basnet R, Hinderaker SG, Enarson D, Malla P, Morkve O. Delay in the diagnosis of tuberculosis in Nepal. BMC Public Health. 2009; 9:236. https://doi.org/10.1186/1471-2458-9-236 PMID: 19602255; PubMed Central PMCID: PMCPMC2716339.

33. Mesfin MM, Tasew TW, Tareke IG, Kifle YT, Karen WH, Richard MJ. Delays and care seeking behavior among tuberculosis patients in Tigray of northern Ethiopia. Ethiopian Journal of Health Development. 2005; 19:7–12.

34. Kalra A. Care seeking and treatment related delay among childhood tuberculosis patients in Delhi, India. Int J Tuberc Lung Dis. 2017; 21(6):645–50. https://doi.org/10.5588/ijtld.16.0563 PMID: 28482958.

35. Tattevin P, Che D, Fraisse P, Gatey C, Guichard C, Antoine D, et al. Factors associated with patient and health care system delay in the diagnosis of tuberculosis in France. Int J Tuberc Lung Dis. 2012; 16 (4):510–5. https://doi.org/10.5588/ijtld.11.0420 PMID: 22325560.

36. Segagni Lusignani L, Quaglio G, Atzori A, Nsuka J, Grainger R, Palma Mda C, et al. Factors associated with patient and health care system delay in diagnosis for tuberculosis in the province of Luanda, Angola. BMC Infect Dis. 2013; 13:168. https://doi.org/10.1186/1471-2334-13-168 PMID: 23566166; PubMed Central PMCID: PMCPMC3637285.

37. Mfinanga SG, Morkve O, Sviland L, Kazwala RR, Chande H, Nilsen R. Patient knowledge, practices and challenges to health care system in early diagnosis of mycobacterial adenitis. East Afr Med J. 2005; 82(4):173–80. PMID: 16122084.

38. Sherman LF, Fujiwara PI, Cook SV, Bazerman LB, Frieden TR. Patient and health care system delays in the diagnosis and treatment of tuberculosis. Int J Tuberc Lung Dis. 1999; 3(12):1088–95. PMID: 10599012.

39. Chakravorty S, Sen MK, Tyagi JS. Diagnosis of extrapulmonary tuberculosis by smear, culture, and PCR using universal sample processing technology. J Clin Microbiol. 2005; 43(9):4357–62. https://doi.org/10.1128/JCM.43.9.4357-4362.2005 PMID: 16145077; PubMed Central PMCID: PMCPMC1234147.

40. Malbruny B, Le Marrec G, Courageux K, Leclercq R, Cattoir V. Rapid and efficient detection of Mycobacterium tuberculosis in respiratory and non-respiratory samples. Int J Tuberc Lung Dis. 2011; 15 (4):553–5. https://doi.org/10.5588/ijtld.10.0497 PMID: 21396219.

41. Purohit MR, Mustafa T, Wiker HG, Sviland L. Rapid diagnosis of tuberculosis in aspirate, effusions, and cerebrospinal fluid by immunocytochemical detection of Mycobacterium tuberculosis complex specific antigen MPT64. Diagn Cytopathol. 2012; 40(9):782–91. https://doi.org/10.1002/dc.21637 PMID: 21416644.

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Evaluation of treatment response in extrapulmonary tuberculosis in a low-resource setting

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Abstract

Background: Diagnosing extrapulmonary tuberculosis (EPTB) is challenging and many patients are initiated on empirical anti-TB treatment without a laboratory confirmed diagnosis. Monitoring treatment response is thus important to ensure correct diagnosis and proper disease management. The definition of satisfactory response to treatment in EPTB remains unclear. The objectives of this study were to describe the clinical presentation of EPTB and the effect of treatment on clinical parameters. Further, to assess if simple clinical parameters, without laboratory data, could evaluate treatment response.

Methods: Prospective cohort study of presumptive EPTB patients at Mnazi Mmoja Hospital, Zanzibar. By using a composite reference standard, patients were categorized as TB or non-TB cases. The TB patients were followed during anti-TB treatment.

Results: There were 64 TB and 62 non-TB cases. The frequency of symptoms at baseline were comparable in TB and non-TB patients, with lymphadenitis and pleuritis as the most common manifestations. Among TB cases, there was a trend towards regression of lymphadenopathy after 2 months, and at treatment completion 24/28 (86%) cases showed full regression. Weight gain ≥5% was reported in 36/49 (73%) of the TB patients at 2 months and in 38/46 (83%) at treatment completion. After 2 months of treatment, a combination of clinical parameters; improvement of symptoms (50/50), ≥5% weight gain (36/49) and regression of physical signs (45/49) correlated with the treatment response.

Conclusions: An algorithm including only simple clinical parameters could be used as an easy tool to assess treatment responses in low-resource settings. However, this needs to be tested on a larger sample size.

Keywords: Treatment outcome, Clinical parameters, Weight gain, Tuberculous lymphadenitis, Tuberculous pleuritis

Background

Globally, extrapulmonary tuberculosis (EPTB) accounts for 15% of the notified incident tuberculosis (TB) cases [1]. Laboratory confirmation of EPTB is challenging due to the paucibacillary nature of disease and limited resources in high-TB endemic settings. The diagnosis is therefore often made only on medical history and clinical signs and many patients are started on empirical anti-TB treatment. It is thus important to monitor response to treatment to ensure diagnosis and appropriate disease management. Monitoring response to treatment in EPTB is challenging and clinical assessment is generally recommended [2]. However, the definition of satisfactory clinical response to treatment among the various forms of EPTB remains unclear. Earlier studies have suggested a combination of clinical and laboratory criteria for evaluating response to treatment [3, 4]. Repeated invasive procedures or measurement of inflammatory markers are often incorporated in the definition of treatment response [4]. In low-resource settings with limited capacity for laboratory investigations and

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invasive procedures, it would be useful to assess response to treatment in EPTB by using simple clinical criteria.

In Zanzibar the estimated prevalence of bacteriologically confirmed pulmonary TB (PTB) among the adult population is 124/100000 [5]. In 2015, EPTB accounted for 24% of the notified TB cases [6]. In contrast to PTB, the EPTB patients are not followed systematically by the TB programme during anti-TB treatment. Symptoms and signs are often not documented in the TB records and the only information available is "treatment completed", which does not always correspond to successful remission.

The objectives of this study were to describe the clinical presentation of EPTB among patients presenting at Mnazi Mmoja Hospital (MMH), Zanzibar, and to describe the effect of anti-TB treatment on clinical parameters. Further, to assess if simple clinical parameters, without laboratory support, could be used to reliably evaluate treatment response.

Methods

Study setting and population

This prospective cohort study was nested in a larger project at MMH [7], the main referral hospital in Zanzibar. Children and adults presenting with symptoms suggestive of EPTB were enrolled from in- and outpatient departments for a period of thirteen months starting 1st of August 2014. Patients were excluded if a specimen from the presumptive site of infection was not sent for laboratory investigations, if they did not give consent or had received anti-TB treatment during the previous year.

Data collection

A medical history was obtained from the patients using their local language, Swahili, and a physical examination was performed at the time of inclusion and at follow-up visits after the intensive phase of anti-TB treatment at 2 months (median 71 days, 5–95 percentile 55–109 days) and at the end of treatment (median 175 days, 5–95 percentile 145–244 days). At inclusion, a pretested semi-structured questionnaire was used [7]. At the follow-up visits, clinical assessment was done without a standard questionnaire. Improvement of symptoms; cough, fever, appetite, fatigue, local symptoms and general condition reported by the patient and assessed by clinician, and results from the physical examination were registered in the patient's study folder. Patients who were not started on anti-TB therapy were followed until a diagnosis was established or until recovery.

Clinical investigation

Enlarged lymph nodes were measured by the size of the long axis of the largest swelling using a measuring tape, if not available, an eye estimate was used. Any change during anti-TB treatment in the size of nodes, appearance of new nodes, formation of fistulae and/or abscesses were recorded. Size of the lymph nodes was designated to one of four categories; not palpable (palpable lymph nodes < 1 cm was also included in this group), ≤2 cm, > 2–4 cm or > 4 cm. Residual lymphadenopathy after treatment was defined as palpable lymph nodes > 1 cm. Pleural or peritoneal effusions were evaluated clinically and by X-ray and ultrasound when available. All patients were weighed, and weight change was recorded in kilograms (kg) and as percentage change in weight at two intervals; 1) between treatment onset and 2 months of treatment, and 2) between treatment onset and end of treatment.

Laboratory investigations

Acid-fast bacilli (AFB) microscopy, *Mycobacterium tuberculosis* (Mtb) culture, GeneXpert® MTB/RIF (Xpert) assay, and cytological/histological evaluation were performed on extrapulmonary specimens. In addition, other laboratory investigations such as bacteriological culture and Gram staining, and protein quantification and white cell count in effusions, were performed if available. CD4 cell counts and human immunodeficiency virus (HIV) ribonucleic acid levels were not available for the HIV positive patients.

Composite reference standard for categorization of patients

A composite reference standard (CRS) was used to categorize the study participants as "confirmed TB cases" (positive Mtb culture and/or Mtb detected by the Xpert assay), "probable TB cases" (clinical presumptive EPTB and either response to anti-TB treatment or bacteriologically confirmed PTB, and one of the following; AFB on Ziehl-Neelsen stain of extrapulmonary specimen or cytology/histology consistent with TB or lymphocytosis on fluid cytology or radiological findings suggestive of EPTB), "possible TB cases" (clinical presumptive EPTB and one of the following; AFB on Ziehl-Neelsen stain of extrapulmonary specimen or cytology/histology consistent with TB or lymphocytosis on fluid cytology or radiological findings suggestive of EPTB or response to anti-TB treatment) and "non-TB cases" (negative Mtb culture and/or Mtb not detected by the Xpert assay and one of the following; improvement without anti-TB treatment or cytology/histology concluded other diagnosis than TB or alternative diagnosis concluded by the local clinician or non-response to anti-TB treatment) [7]. Patients unable to be categorized by the CRS were termed "uncategorized" and excluded from further analyses [7].

Statistical analysis

Chi-square test was used in the analysis of categorical variables and Mann-Whitney U test and Kruskal-Wallis test for group-wise comparison of continuous variables. The general significance level was set to 0.05. Bonferroni correction was used to adjust for multiple testing effects leading to a marginal level of 0.0071 for symptoms (7 tests), 0.01 for presumptive TB lymphadenitis (5 tests) and 0.017 for weight gain (3 tests). Data were analysed using IBM SPSS Statistics for Windows, version 24 (Armonk, NY, USA), and graphics were created using Matlab 9.0 (Natick, MA, USA).

Results

Study participants

A total of 132 patients were enrolled and based on the CRS categorized as TB (confirmed, $n = 12$; probable, $n = 34$; possible, $n = 18$) and non-TB ($n = 62$) cases, while 6 patients were uncategorized and excluded from further analyses. Baseline sociodemographic and clinical characteristics are presented in Table 1.

Among the 64 TB cases, 10 (16%) died before first follow-up, and 2 (3%) were lost-to-follow. Thus, 52 TB patients were followed during treatment. Forty patients received standard first-line anti-TB regimen (isoniazid (H), rifampicin (R), pyrazinamide (Z) and ethambutol (E), 2HRZE/4HR), 2 patients an extended standard first-line regimen, 7 children received a regimen without ethambutol (2HRZ/4HR), whereas 3 patients received a retreatment regimen containing first-line drugs including streptomycin (S) (2HRZES/1HRZE/5HRE). An overall clinical improvement was noted in all 52 patients after 2 months of treatment (one patient only assessed after 5 months), but a transient paradoxical reaction with increase in lymphadenopathy was observed in 2 HIV positive patients. Empirical anti-TB treatment was initiated in 12/62 (19%) non-TB patients. Among these, 5/12 patients received a malignant diagnosis, 5/12 cases showed no response to treatment and were further investigated for an alternative diagnosis, 1/12 patients showed clinical improvement before starting anti-TB treatment and 1/12 cases (presumptive TB meningitis) only received anti-TB treatment for 2 weeks due to side-effects and improved without anti-TB therapy.

TB and non-TB patients present with similar symptoms

Table 2 presents the distribution of symptoms among TB and non-TB cases according to site of infection, age and HIV status. There was no significant difference in the frequency or type of constitutional symptoms when comparing TB with non-TB patients. All patients reported local symptoms from the site of infection. Among the TB cases, patients with lymphadenitis reported less frequently constitutional symptoms as compared to patients with pleuritis and other sites of infection. Paediatric patients described less constitutional symptoms compared to adults. Weight loss, reduced appetite or fatigue were reported more often in adults. Constitutional symptoms were more frequent in HIV positive patients.

Table 1 Characteristics of the study participants

	Total	Lymphadenitis		Pleuritis		Other sites of infection[a]	
	$n = 126$	$n = 67$		$n = 31$		$n = 28$	
		TB	Non-TB	TB	Non-TB	TB	Non-TB
Characteristics		$n = 34$	$n = 33$	$n = 20$	$n = 11$	$n = 10$	$n = 18$
Female, *n* (%)	56 (44%)	17 (50%)	14 (42%)	7 (35%)	5 (45%)	5 (50%)	8 (44%)
Age, years, median [IQR]	27 [8–41]	27 [7–38]	13 [7–49]	32 [23–50]	20 [6–41]	27 [19–34]	40 [26–50]
Children (< 15 years), *n* (%)	41 (33%)	11 (32%)	18 (55%)	3 (15%)	4 (36%)	2 (20%)	3 (17%)
HIV status, *n* (%)							
HIV positive	20 (16%)	7 (21%)	2 (6%)	5 (25%)	0 (–)	2 (20%)	4 (22%)
HIV negative	74 (59%)	25 (74%)	10 (30%)	15 (75%)	6 (55%)	6 (60%)	12 (67%)
Unknown	32 (25%)	2 (6%)	21 (64%)	0 (–)	5 (45%)	2 (20%)	2 (11%)
Comorbidities, *n* (%)							
Diabetes	4 (3%)	0 (–)	1 (3%)	1 (5%)	1 (9%)	1 (10%)	0 (–)
Heart disease/HT	12 (10%)	2 (6%)	3 (9%)	0 (–)	2 (18%)	1 (10%)	4 (24%)
COPD/Asthma	7 (6%)	3 (9%)	2 (6%)	0 (–)	1 (9%)	1 (10%)	0 (–)
Outpatient, *n* (%)	76 (60%)	32 (94%)	32 (97%)	7 (35%)	0 (–)	3 (30%)	2 (11%)

Abbreviations: *TB* tuberculosis, *IQR* interquartile range, *HIV* human immunodeficiency virus, *HT* hypertension, *COPD* chronic obstructive pulmonary disease
[a] TB cases: peritonitis ($n = 6$), meningitis ($n = 2$), pericarditis ($n = 1$), spondylitis ($n = 1$); non-TB cases: peritonitis ($n = 10$), meningitis ($n = 6$), mastitis ($n = 1$), osteomyelitis ($n = 1$)

Table 2 Distribution of constitutional symptoms reported at the time of inclusion, *n* (%)[a]

	Total *n* = 126	Symptoms						
		Constitutional[b]		Fever	Weight loss	Night sweats	Fatigue	Loss of appetite
		≥1 symptoms	≥3 symptoms					
Diagnosis								
TB	64	50 (78%)	23 (36%)	33 (52%)	36 (57%)	21 (33%)	16 (26%)	20 (32%)
Non-TB	62	42 (68%)	22 (35%)	26 (42%)	25 (42%)	18 (29%)	24 (39%)	18 (30%)
P value[c]		.189	.958	.279	.086	.604	.109	.741
TB cases								
Confirmed TB	12	9 (75%)	3 (25%)	6 (50%)	6 (50%)	3 (25%)	3 (27%)	4 (33%)
Probable TB	34	28 (82%)	16 (47%)	21 (62%)	23 (70%)	12 (35%)	8 (24%)	13 (38%)
Possible TB	18	13 (72%)	4 (22%)	6 (33%)	7 (39%)	6 (35%)	5 (29%)	3 (19%)
Site								
Lymphadenitis								
TB	34	22 (65%)	7 (21%)	14 (41%)	15 (45%)	7 (21%)	2 (6%)	5 (15%)
Non-TB	33	15 (45%)	6 (18%)	9 (27%)	8 (25%)	4 (12%)	5 (16%)	7 (21%)
Pleuritis								
TB	20	20 (100%)	12 (60%)	13 (65%)	16 (80%)	11 (55%)	9 (47%)	12 (63%)
Non-TB	11	11 (100%)	6 (55%)	7 (64%)	6 (55%)	7 (64%)	8 (73%)	4 (40%)
Other sites								
TB	10	8 (80%)	4 (40%)	6 (60%)	5 (50%)	3 (33%)	5 (50%)	3 (30%)
Non-TB	18	16 (89%)	10 (56%)	10 (56%)	11 (65%)	7 (39%)	11 (61%)	7 (39%)
Age								
Children								
TB	16	12 (75%)	2 (13%)	8 (50%)	5 (31%)	6 (38%)	1 (6%)	0 (–)
Non-TB	25	14 (56%)	6 (24%)	8 (32%)	6 (25%)	6 (24%)	7 (28%)	6 (25%)
Adults								
TB	48	38 (79%)	21 (44%)	25 (52%)	31 (66%)	15 (32%)	15 (33%)	20 (43%)
Non-TB	37	28 (76%)	16 (43%)	18 (49%)	19 (53%)	12 (32%)	17 (47%)	12 (32%)
HIV status[d]								
HIV positive								
TB	14	13 (93%)	6 (43%)	8 (57%)	8 (62%)	5 (38%)	6 (46%)	6 (43%)
Non-TB	6	6 (100%)	4 (67%)	6 (100%)	3 (60%)	1 (17%)	4 (67%)	3 (50%)
HIV negative								
TB	46	33 (72%)	16 (35%)	21 (46%)	27 (59%)	14 (30%)	9 (20%)	14 (32%)
Non-TB	28	20 (71%)	10 (36%)	11 (39%)	15 (54%)	8 (29%)	11 (39%)	8 (29%)

Abbreviations: *TB* tuberculosis, *HIV* human immunodeficiency virus
[a] Patients with missing values excluded
[b] The following were included as constitutional symptoms; fever, weight loss, night sweats, fatigue, loss of appetite. ≥1 and ≥ 3 refers to number of patients reporting one or more or three or more symptoms respectively
[c] *P* value < 0. 0071 (0.05/7) considered statistically significant
[d] Patients with unknown HIV status excluded

Findings in presumptive TB lymphadenitis

There was no statistically significant difference in the site or symmetry of lymph node involvement between TB and non-TB patients (Table 3). Still, a higher proportion of larger lymph nodes > 4 cm, matted or painful nodes and discharge/fistulas were seen in TB patients compared to non-TB patients.

There was a significant regression in lymph node size with anti-TB treatment, as shown in Fig. 1. During treatment enlargement of nodes, new fistulae or fresh abscesses were reported in 5 (2 HIV positive) patients. However, overall reduction in lymph node size was seen as an indicator for treatment response among TB patients.

Table 3 Findings in presumptive TB lymphadenitis at the time of inclusion, *n* (%)

		Diagnosis		
	Total	TB	Non-TB	*P* value[a]
Characteristics	*n* = 67	*n* = 34	*n* = 33	
Localization				.549[b]
Unilateral	40 (60%)	22 (65%)	18 (55%)	
Bilateral/generalized	27 (40%)	12 (35%)	15 (45%)	
Only cervical	50 (75%)			
Unilateral		17 (50%)	14 (42%)	
Bilateral		9 (26%)	10 (30%)	
Only axillary	6 (9%)			
Unilateral		3 (9%)	3 (9%)	
Bilateral		0 (–)	0 (–)	
Only inguinal	2 (3%)			
Unilateral		0 (–)	1 (3%)	
Bilateral		0 (–)	1 (3%)	
Cervical and axillary	6 (9%)			
Unilateral		2 (6%)	0 (–)	
Bilateral		1 (3%)	2 (6%)	
Cervical bilateral and axilla unilateral		1 (3%)	0 (–)	
Generalized	3 (4%)	1 (3%)	2 (6%)	
Size of largest lymph node[c]				.031
≤ 2 cm	9 (13%)	1 (3%)	8 (24%)	
> 2–4 cm	25 (37%)	13 (38%)	12 (36%)	
> 4 cm	33 (49%)	20 (59%)	13 (39%)	
Matted nodes	38 (57%)	24 (71%)	14 (42%)	.020
Painful	9 (13%)	7 (21%)	2 (6%)	.081
Discharge/fistula	7 (10%)	6 (18%)	1 (3%)	.051

Abbreviation: *TB* tuberculosis
[a] *P* value < 0. 01 (0.05/5) considered statistically significant
[b] Test for different risk for unilateral vs. bilateral/generalized localization in TB and Non-TB group
[c] Long axis of largest lymph node swelling

Regression of pleural and peritoneal effusion during anti-TB treatment

Among the 20 TB pleuritis cases, any regression of effusion was assessed by chest x-ray in 15 patients after 2 months and in 13 patients at treatment completion. Regression was noted in 14 (93%) and 13 (100%), respectively. Only 3/6 (50%) with abdominal TB were evaluated, and partial regression of peritoneal effusion was seen at 2 months and full regression at the end of treatment, as assessed by ultrasound.

Weight gain as indicator for treatment efficacy

Weight change during anti-TB treatment was recorded according to age, site of infection and HIV status (Fig. 2). After 2 months of treatment, 36/49 (73%) of the patients had ≥5% weight gain and 18/49 (37%) had ≥10% weight gain. At treatment completion, 38/46 (83%) of the cases had ≥5% and 26/46 (57%) ≥10% weight gain. Overall, the median weight gain at 2 months of treatment was 2.9 kg (interquartile range (IQR), 1.4–5.1 kg) and the median percentage weight gain 6.7% (IQR, 4.6–13.4%). After completion of treatment, the overall median weight gain was 4.0 kg (IQR, 2.2–7.5 kg) and 12.9% (IQR, 7.2–19.7%). The paediatric cases had a higher median percentage weight gain compared to the adult patients both at 2 months (11.1% vs 6.2%) and end of treatment (17.9% vs 9.5%), but the differences were not statistically significant. A weight gain ≥15% was described in 71% of the paediatric TB cases at treatment completion. In all HIV positive cases ($n = 5$) ≥5% weight gain was seen after treatment completion, and 4/5 patients had a weight gain ≥15%. There was no statistically significant difference in weight gain according to site of disease.

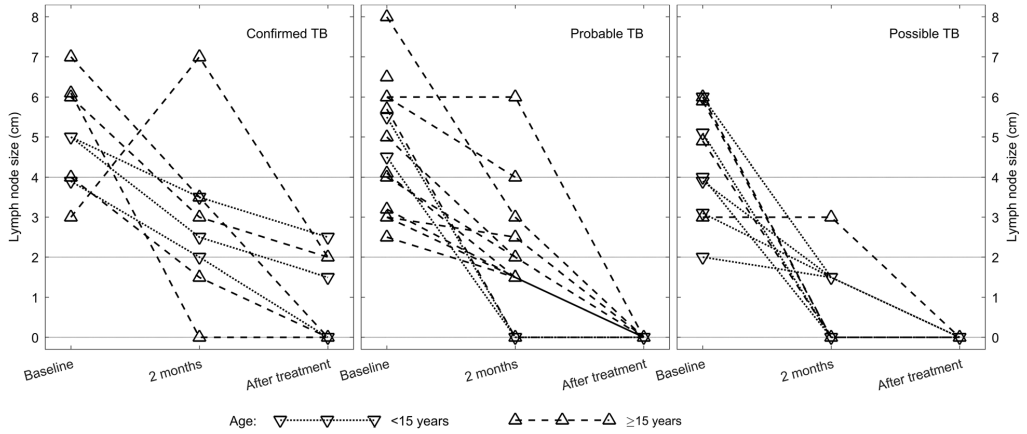

Fig. 1 Time series plot illustrating the regression of lymphadenopathy during anti-TB treatment. The lymph node size (long axis of largest swelling) is presented at baseline, after 2 months of treatment and at treatment completion according to TB category (confirmed, probable and possible TB cases) and age. Each patient is represented by an individual line. At baseline, all, except one case, had lymph node enlargement > 2 cm. At 2 months, there was a trend in regression of lymph node swellings, and at treatment completion, 4 (14%) cases had residual lymphadenopathy. The patients with residual lymphadenopathy were all confirmed TB cases. Abbreviation: TB, tuberculosis

Combination of clinical parameters for assessment of treatment outcome

Among the patients starting anti-TB treatment, a combination of the following three clinical parameters were assessed retrospectively; 1) Improvement of reported symptoms; 2) Weight gain (any weight gain or ≥ 5% gain); 3) Regression of lymph node swelling or pleural or peritoneal effusion or other local findings, during and after treatment compared to baseline. If a weight gain ≥5% was used, all TB cases, except one, had ≥2 parameters after 2 months of treatment, while non-TB cases fulfilled only 0 or 1 parameter (Fig. 3).

Discussion

In this study, we describe the clinical presentation of various forms of EPTB and changes in clinical parameters during the course of anti-TB treatment. Our results show that presenting signs and symptoms alone could not differentiate TB and non-TB cases among presumptive EPTB cases. There was regression in clinical signs in TB cases during treatment, and we suggest that a combination of 2–3 clinical parameters could predict good response early (2 months) in the treatment. These findings imply that in low-resource settings where anti-TB treatment is often started on clinical assumption,

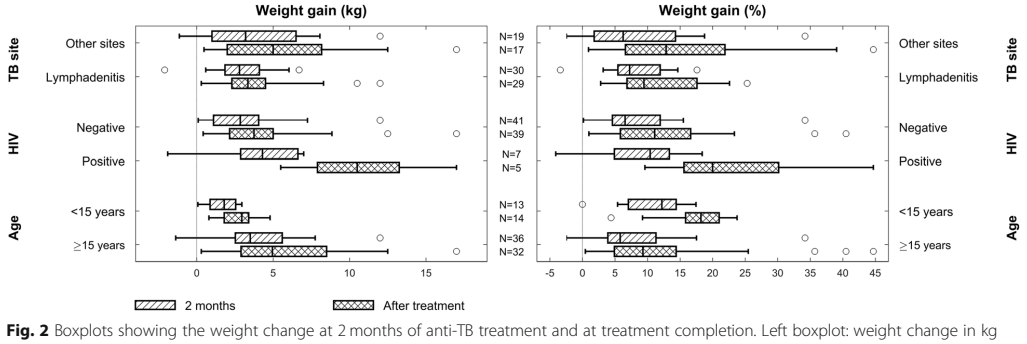

Fig. 2 Boxplots showing the weight change at 2 months of anti-TB treatment and at treatment completion. Left boxplot: weight change in kg between treatment onset and 2 months of treatment and between treatment onset and end of treatment according to site of TB infection, HIV status and age. Right boxplot: weight change as percentage change in weight between treatment onset and 2 months of treatment and between treatment onset and end of treatment according to site of TB infection, HIV status and age. Abbreviations: TB, tuberculosis; HIV, human immunodeficiency virus

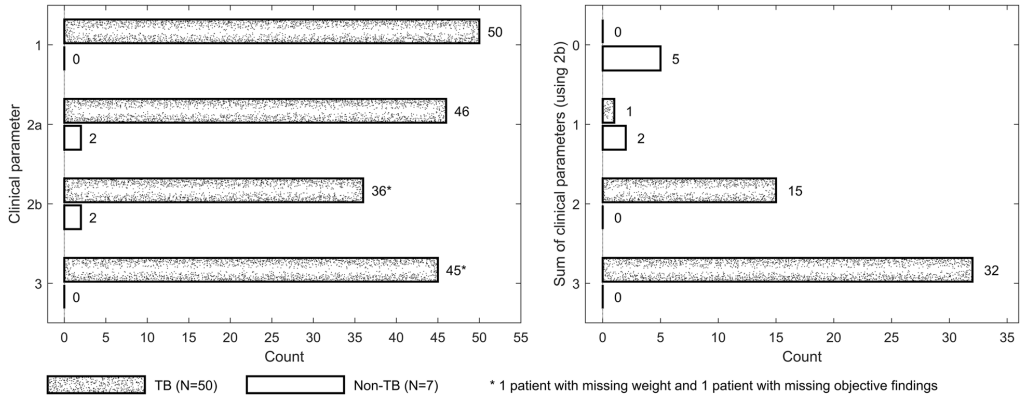

Fig. 3 Clinical parameters at 2 months of treatment for assessment of treatment outcome. Left figure: Shows the number of patients having each clinical parameter. 1 – improvement of reported symptoms such as coughing, fever, appetite, fatigue and general condition. 2a – any weight gain compared to baseline; 2b - ≥5% weight gain compared to baseline; 3 - regression of objective findings. Right figure: Shows the number of patients having the sum of clinical parameters. Abbreviation: TB, tuberculosis

unnecessary over-treatment can be reduced by systematic clinical monitoring of responses early during treatment.

Improvement in symptoms can be assessed by a symptoms count ratio [3], or self-rated health using the EQ-5D 3 level version [8]. Overall clinical improvement or performance status can be assessed by the health care worker using standardised systems, like the Karnofsky performance score [3, 9]. However, clinically relevant cut-off points for symptoms count ratio, improvement of self-rated health, performance scores and other clinical parameters such as weight gain need to be established in EPTB patients.

Weight gain is an easy, inexpensive parameter for assessing treatment response. Earlier studies, predominantly among PTB patients, have suggested an association with weight gain and successful treatment outcome [10], whereas lesser weight gain or weight loss were associated with poor outcome [10–12] or relapse [13]. Only a few studies have evaluated weight gain as a marker of treatment response in EPTB patients [3, 10, 12]. In our cohort of TB cases with successful treatment outcome, ≥5% weight gain was recorded in 73 and 83% of the cases at 2 months and at treatment completion.

Regression of lymphadenopathy could also be a reliable parameter for assessment of response to treatment, but appearance of newly enlarged nodes, enlargement of existing nodes, formation of new fistulae and abscesses during and after treatment [14, 15] can complicate the evaluation. Paradoxical reactions during anti-TB treatment [16], are described to occur quite frequently in TB/HIV co-infected patients [17, 18]. In a meta-analysis, the frequency was estimated to be 15.7% among patients treated for active TB and newly commencing antiretroviral therapy [16, 19]. Further, paradoxical reactions are reported to be relatively common in HIV negative EPTB patients [20–25]. Therefore, a single clinical parameter may not be enough, and a combination of parameters will be better to differentiate responders from non-responders.

The failure to treatment response could indicate not just alternative diagnoses but also poor compliance, paradoxical reactions or drug resistance in TB cases. These can be difficult to differentiate using only simple clinical parameters. Still, such criteria, incorporated systematically in the follow-up of TB patients, such as in TB registers or treatment cards, can be valuable and assist the health care provider in detecting patients who need clinical reassessment.

Current practice in the TB programme (in Zanzibar) does not detect EPTB patients with poor clinical response. The TB register and treatment cards document treatment completion as the only outcome for EPTB which is based only on the intake of drugs without concurrent record of clinical parameters. The combination of clinical parameters described in this study could be developed into an assessment tool, which can be attached to the patients TB treatment cards. However, the study sample is small, and the findings need to be validated on a larger number of patients. This simple tool has the potential to detect non-responders early at 2 months and standardize follow-ups. Early detection of non-responders would save costs, reduce morbidity due to side-effects, and further minimise the undue delay of alternative diagnoses.

The study has some limitations. Single centre study and the small sample size limits the generalisability of the results and sub-group analysis. There are few patients included with presumptive neurological or bone TB, and none with presumptive genital TB, urinary tract TB or other forms of abdominal TB besides peritoneal TB. Therefore, the results may not apply to all patients presenting with presumptive EPTB in Zanzibar. The possible TB cases were defined by clinically suspected EPTB and overall response to treatment. This does not provide an accurate diagnosis, as some of the patients may have other infections responding to anti-TB treatment. There was also a limited capacity to perform investigations to diagnose other infections and for the HIV patients CD4 counts and viremia were not known. The lack of standardized questionnaire and clinical registration during follow-up and standardized measurements of lymph node size may have influenced the results.

Conclusions

Clinical presentation alone cannot reliably diagnose EPTB, and empirical anti-TB treatment leads to over-treatment. A combination of simple clinical parameters could be used as an easy tool to assess treatment responses and thus improve patient management in low-resource settings. More and larger studies are needed for further evaluation and validation of these simple clinical parameters.

Abbreviations

AFB: Acid-fast bacilli; COPD: Chronic obstructive pulmonary disease; CRS: Composite reference standard; E: Ethambutol; EPTB: Extrapulmonary tuberculosis; H: Isoniazid; HIV: Human immunodeficiency virus; HT: Hypertension; IQR: Interquartile range; kg: Kilogram; MMH: Mnazi Mmoja Hospital; Mtb: *Mycobacterium tuberculosis*; PTB: Pulmonary tuberculosis; R: Rifampicin; S: Streptomycin;; TB: Tuberculosis; Xpert: GeneXpert® MTB/RIF; Z: Pyrazinamide

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Concept and study design: TM, LS, MDJ. Acquisition of funds for the study: TM. Acquisition of data: MDJ, MM. Overseeing data collection: TM. Analysis and interpretation of data: MDJ, TM, AMDR, JA. Drafting and revising the manuscript: MDJ, TM, LS, AMDR, JA, MM. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethical clearance was obtained from the Regional Committee for Medical and Health Research Ethics, Western-Norway (2014/46/REK vest) and the Zanzibar Medical Research and Ethics Committee (ZAMREC/0001/MAY/014). All study participants signed an informed written consent. In patients < 18 years, the written consent was signed by the parent/guardian, in addition, patients ≥7 years had to sign the consent form.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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References

1. World Health Organization. Global tuberculosis report 2016. WHO/HTM/TB/201613. Geneva: WHO; 2016.
2. Hopewell PC, Fair EL, Uplekar M. Updating the international standards for tuberculosis care. Entering the era of molecular diagnostics. Ann Am Thorac Soc. 2014;11(3):277–85.
3. Wilson D, Nachega J, Morroni C, Chaisson R, Maartens G. Diagnosing smear-negative tuberculosis using case definitions and treatment response in HIV-infected adults. Int J Tuberc Lung Dis. 2006;10(1):31–8.
4. Sharma V, Singh H, Mandavdhare HS. Defining 'satisfactory response' to therapy in abdominal tuberculosis : a work in progress. Infect Disord Drug Targets. 2018.
5. Senkoro M, Mfinanga S, Egwaga S, Mtandu R, Kamara DV, Basra D, et al. Prevalence of pulmonary tuberculosis in adult population of Tanzania: a national survey, 2012. Int J Tuberc Lung Dis. 2016;20(8):1014–21.
6. Ministry of Health Zanzibar. Zanzibar integrated HIV, TB and leprosy Programme annual report 2015. 2016.
7. Jorstad MD, 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;13(5):e0196723.
8. EuroQol G. EuroQol--a new facility for the measurement of health-related quality of life. Health Policy. 1990;16(3):199–208.
9. Karnofsky DA, Abelmann WH, Craver LF, Burdhenal JH. The use of the nitrogen mustards in the palliative treatment of carcinoma: with particular reference to bronchogenic carcinoma. Cancer. 1948;(November):634–56.
10. Bernabe-Ortiz A, Carcamo CP, Sanchez JF, Rios J. Weight variation over time and its association with tuberculosis treatment outcome: a longitudinal analysis. PLoS One. 2011;6(4):e18474.

11. Krapp F, Veliz JC, Cornejo E, Gotuzzo E, Seas C. Bodyweight gain to predict treatment outcome in patients with pulmonary tuberculosis in Peru. Int J Tuberc Lung Dis. 2008;12(10):1153–9.
12. Hoa NB, Lauritsen JM, Rieder HL. Changes in body weight and tuberculosis treatment outcome in Viet Nam. Int J Tuberc Lung Dis. 2013;17(1):61–6.
13. Khan A, Sterling TR, Reves R, Vernon A, Horsburgh CR. Lack of weight gain and relapse risk in a large tuberculosis treatment trial. Am J Respir Crit Care Med. 2006;174(3):344–8.
14. Short course chemotherapy for tuberculosis of lymph nodes: a controlled trial. British Thoracic Society Research Committee. Br Med J (Clin Res Ed). 1985;290(6475):1106–8.
15. Jawahar MS, Sivasubramanian S, Vijayan VK, Ramakrishnan CV, Paramasivan CN, Selvakumar V, et al. Short course chemotherapy for tuberculous lymphadenitis in children. BMJ. 1990;301(6748):359–62.
16. Bell LC, Breen R, Miller RF, Noursadeghi M, Lipman M. Paradoxical reactions and immune reconstitution inflammatory syndrome in tuberculosis. Int J Infect Dis. 2015;32:39–45.
17. Narita M, Ashkin D, Hollender ES, Pitchenik AE. Paradoxical worsening of tuberculosis following antiretroviral therapy in patients with AIDS. Am J Respir Crit Care Med. 1998;158(1):157–61.
18. Breen RA, Smith CJ, Bettinson H, Dart S, Bannister B, Johnson MA, et al. Paradoxical reactions during tuberculosis treatment in patients with and without HIV co-infection. Thorax. 2004;59(8):704–7.
19. Muller M, Wandel S, Colebunders R, Attia S, Furrer H, Egger M, et al. Immune reconstitution inflammatory syndrome in patients starting antiretroviral therapy for HIV infection: a systematic review and meta-analysis. Lancet Infect Dis. 2010;10(4):251–61.
20. Geri G, Passeron A, Heym B, Arlet JB, Pouchot J, Capron L, et al. Paradoxical reactions during treatment of tuberculosis with extrapulmonary manifestations in HIV-negative patients. Infection. 2013;41(2):537–43.
21. Cheng VC, Yam WC, Woo PC, Lau SK, Hung IF, Wong SP, et al. Risk factors for development of paradoxical response during antituberculosis therapy in HIV-negative patients. Eur J Clin Microbiol Infect Dis. 2003;22(10):597–602.
22. Cheng VC, Ho PL, Lee RA, Chan KS, Chan KK, Woo PC, et al. Clinical spectrum of paradoxical deterioration during antituberculosis therapy in non-HIV-infected patients. Eur J Clin Microbiol Infect Dis. 2002;21(11):803–9.
23. Cho OH, Park KH, Kim T, Song EH, Jang EY, Lee EJ, et al. Paradoxical responses in non-HIV-infected patients with peripheral lymph node tuberculosis. J Inf Secur. 2009;59(1):56–61.
24. Hawkey CR, Yap T, Pereira J, Moore DA, Davidson RN, Pasvol G, et al. Characterization and management of paradoxical upgrading reactions in HIV-uninfected patients with lymph node tuberculosis. Clin Infect Dis. 2005; 40(9):1368–71.
25. Jung JW, Shin JW, Kim JY, Park IW, Choi BW, Seo JS, et al. Risk factors for development of paradoxical response during anti-tuberculosis treatment in HIV-negative patients with pleural tuberculosis. Tohoku J Exp Med. 2011; 223(3):199–204.

10. APPENDICES

10.1 Appendix A – Study questionnaire and study information and consent form

QUESTIONNAIRE – patients ≥ 18 years

Date:

Consultant:

Hospital:

Department: □ OPD □ IPD

□ **Written informed consent** □ **Oral informed consent**

PATIENT IDENTIFICATION

Study Number: _____

Age_____Years

Gender: □ Male □ Female

Respondent: □ Patient □ Parent □ Spouse □ Child □ Other, relative/friend

Address: Region_____ Village_____

1. Marital status:

□ Single □ Married □ Widow/widower □ Separated □ Divorced

□ Cohabited □ Other, please specify_____

2. Level of education:

□ No formal education □ Not completed primary school

□ Completed primary school □Form I-IV

□ Form IV-VI □Above secondary education

□Adult education □Others (Please mention)_____

3. Religion:

□Muslim □ Christian □ Other, please mention_____

PREVIOUS MEDICAL HISTORY

4. Tobacco use: □ Yes □ No ____weeks/months /years

5. Smoking: □ Yes □ No ____weeks/months /years

6. Alcohol: □ Yes □ No ____weeks/months /years

7. Associated Diseases

COPD: □ Yes □ No

Renal Disease: □ Yes □ No

Liver Diseases: □ Yes □ No

Diabetes Mellitus: □ Yes □ No

Hypertension: □ Yes □ No

Other: □ Yes □ No

Describe other: _____

_____

8. Medication: ***Please write the names of the medication***

_____

PREVIOUS HISTORY OF TUBERCULOSIS

9. Have you been in contact with a person with known tuberculosis?

□ Yes □ No

10. Have you previously been diagnosed with pulmonary tuberculosis?

□ Yes □ No

11. Have you previously been diagnosed with extrapulmonary tuberculosis:

□ Yes □ No

If yes, please specify: _____

12. Have you previously been treated for tuberculosis?

□ Yes □ No

13. If you have been treated, what was the treatment outcome?

□ Cured

□ Treatment Completed

□ Treatment Interrupted

14. If treated, when was the last time that you completed any TB treatment?

_____

HEALTH-SEEKING BEHAVIOUR AND DIAGNOSTIC DELAY

Health seeking behavior for TB patients

Please remind the patient that this survey is confidential.

15. Please ask if the patient has experienced any of the following symptoms

General Symptoms

Fever: □ Yes □ No ____weeks/months

What kind of fever do you have?

□ High-grade □ Low-grade

When do you have fever?

□ Morning □ Day-time □ Evening □ Night □ all day

Loss of weight: □ Yes □ No ____weeks/months

Loss of appetite: □ Yes □ No ____weeks/months

Night Sweat: □ Yes □ No ____weeks/months

Fatigue: □ Yes □ No ____weeks/months

Amenorrhea/female: □ Yes □ No ____weeks/months

Body weakness: □ Yes □ No ____weeks/months

Frequent cold: □ Yes □ No ____weeks/months

Neck mass: □ Yes □ No ____weeks/months

Other: □ Yes □ No ____weeks/months

Respiratory Symptoms

Cough: □ Yes □ No ___weeks/months

Sputum: □ Yes □ No ___weeks/months

Cough with Sputum: □ Yes □ No ___weeks/months

Cough with blood: □ Yes □ No ___weeks/months

Chest pain: □ Yes □ No ___weeks/months

Difficult in breathing: □ Yes □ No ___weeks/months

Abdominal Symptoms

Swelling of/in stomach: □ Yes □ No ___weeks/months

Fullness of the stomach: □ Yes □ No ___weeks/months

Vomiting: □ Yes □ No ___weeks/months

Diarrhea: □ Yes □ No ___weeks/months

Other: □ Yes □ No ___weeks/months

Describe other: _____

_____

\

Neurological Symptoms

Headache: □ Yes □ No ___weeks/months

Photophobia: □ Yes □ No ___weeks/months

Vomiting: □ Yes □ No ___weeks/months

Dizziness: □ Yes □ No ___weeks/months

Vertigo: □ Yes □ No ___weeks/months

Weakness/Numbness of extremity:

□ Yes □ No ___weeks/months

Visual disturbance: □ Yes □ No ___weeks/months

Other: □ Yes □ No ___weeks/months

Describe other: _____

_____

_____

16. What were the major symptoms that first made you seek care?

□ Prolong Cough □ coughing blood □ Breathlessness

□ Chest pain □ Fever □ Weight loss

□ Fatigue\Weakness □ Loss of appetite □ Night sweats

□ Bone pain □ Lymph node swelling □ Diarrhoea

□ abdominal pain □ others (specify) _____

17. When did you first notice the symptoms?

_____

18. Did you practice any self-medication before you sought care?

□ Yes □ No

19. When did you first seek medical advice for this illness after noticing the symptoms? (Ask the respondent to specify how many days/weeks after noticing the symptoms)

□ Today □ 1-6 days □ 1-4 weeks □ 5-8weeks □ over 8 weeks

20. How many different places did you go to seek help for the current symptoms? (Ask the respondent to specify the various health care providers/health facilities)

_____places

21. How many times have you visited health facilities with the same symptoms before?

□ First visit □ Second visit □ Third visit

□ > 3 visits □ don't remember

22. Which place did you first seek care for your symptoms?

□ Regional Hospital □ District hospital/PHCC □ Health center/PHCU

□ Dispensary/private clinic □ Private Hospital □ Traditional healer

□ Pharmacy □ other, please specify_____

23. Did you get any medicine from there?

□ Yes □ No

24. If yes, what kind of medicine?

□ Antibiotics □ Anti-TB □ Herbs □ others, which_____

25. Were the symptoms relieved after taking medicines?

□ Yes □ No

26. What kind of diagnosis did you receive for your illness?

_____

27. Were any tests done at the first medical service?

□ Yes □ No

28. What type of tests?

□ Blood test □ Urine test □ Sputum □ X-ray

□Others, please specify_____

29. Did you take the results back to the doctor?

□ Yes □ No

30. Could you estimate the total cost for the previous visits/investigations related to your current illness?

Admission _____TZS

Consultations _____ TZS

Medication _____TZS

Laboratory tests/X-ray/CT_____TZS

Transportation _____TZS

31. Who referred you here to this health facility?

□ myself □ Traditional healers □ Religious leaders

□ Pharmacy/drug shop □ Village health worker □ Government dispensary

□ Government health center □ Government hospital □ Private dispensary/hospital

□ Charitable/NGO □ Member of the family □ Other_____

32. Have you ever been tested for HIV?

□ Yes □ No

33. Self-reported HIV results

□ HIV positive □ HIV negative □ don't know □ don't agree to disclose HIV status

34. Before today, had you heard of the illness tuberculosis?

□ Yes □ No

35. Do you know any symptoms of tuberculosis?

□ Chronic cough □ Spitting blood □ Shortness of breath

□ Chest pain □ Fever □Weight loss

□ Tiredness □ Loss of appetite

□ Others Please specify_____ ***(Do not probe but ask for more symptoms)***

36. Do you know which parts of the body that can be affected by tuberculosis?

_____

37. Can tuberculosis spread from person to person?

□ Yes □ No □ Uncertain

38. Do you drink unboiled milk?

□ Yes □ No

39. Do you eat raw meat?

□ Yes □ No

40. Did you know that consumption of raw animal products, like uncooked dairy products can lead to gastrointestinal tuberculosis as a result of transfer of the disease from animals to humans?

□ Yes □ No

41. Can tuberculosis be cured with medicines?

□ Yes □ No □ Uncertain

42. Do you know how long it takes to treat tuberculosis?

□ Yes □ No

If yes, do you know the approximate duration of treatment?

_____

43. Do people in your community associate tuberculosis with HIV?

□ Yes □ No □ Uncertain

If yes, why do they associate it with HIV? _____

44. Is there anything that would make it easier for people with tuberculosis to get treatment, not just in this clinic, but in other health facilities?

□ Yes □ No □ Uncertain

If yes, what could be done? _____

45. The moment you realized you may have contracted tuberculosis, did you have any problems deciding to seek care? If so, what types of problems?

_____

_____

_____

46. What fears do others have about TB that prevents them from seeking medical advice?

_____

47. If you consulted a traditional healer before seeking care at a modern health facility, what were the reasons which led you to first use the traditional healer?

PATIENT AND HOUSEHOLD COSTS, estimate of the patient income level

48. How long does it take you to go to the nearest health facility?

□ Less than 30 minutes □ between 30 minutes and one hour □ More than one hour

49. How far is this hospital to your home (in Kilometers) _____

50. How long (on average) does it take you to this health facility, waiting for your consultation and finally returning to your home\workplace?

_____Hours

51. How did you get to this health facility?

□ Walked □ Bicycle □Motorcycle □ Private car □ Dala Dala

52. If you have to take a Dala Dala, how much (on average) does it cost you to come to the clinic? _____TZS.

53. Do you usually have to make some special arrangements at home before coming to the clinic?

□ Yes □ No □ Uncertain

If yes, what arrangements?_____

54. What is your main occupation (past twelve months)?

□ Employed by government

□ Employed private for profit sector

□ Employed by NGO

□ Self-employed, business with employees

□ Self-employed, business no employee

□ Self-employed (merchant), farmer/ fishing

□ Unemployed

□ Retired

□ Pupil/ student

□ Disabled/ sick

□ Housewife

□ Other_____

55. What is the main income of your house hold?

□ Crop production □ Livestock □ Fishing

□ Hunting/ bee-keeping □ Poultry □ Farm wage

□ Other agricultural activity □ Wages (government) □ Wages (private)

□ Monetary savings (interest) □ Pensions

□ Property (rentals) □Self-employed payments (merchant)

□Other Specify_____

56. In the past 12 months, in what types of activities were you and any members of your household engaged? (Only income-generating activities)?

_____

_____

_____

57. How much did (NAME) earn (money) for the activities stated on average in the past 12 months? This should include not only salary or cash income: but also the value of goods produced or traded for other goods and services.

_____

58. Do you have reduced working capacity due to your current illness?

□ Yes, completely stopped working □ Yes, working but with reduced capacity

□ Working as normal

59. If you have reduced working capacity, how would you describe your reduced working capacity?

_____

60. When did you stop working or working with reduced capacity?

_____

61. Have any member of your household stopped working or reduced their work capacity because of your illness?

□ Yes □ No

If yes, for how long? _____days

If yes, how much reduced working capacity? _____

62. Have you/or any member of your household lost any wages or income because of your illness?

□ Yes □ No □ Uncertain

If yes, how much_____

63. Do you own a house?

□ Yes □ renting a house □ living with relatives /friends □ Homeless

64. How many people live in your household: _____ (number of people)

How many: Men: _____Women:_____Elderly:_____

Children (between 0-10):_____ Children (between 11-18):_____

65. What is the main source of drinking water for members of your household?

□ Piped water 1=Piped into dwelling 2= Piped into yard/plot 3=Public tap 4=Neighbors' tap

□ Water from open well

□ Water from covered well or borehole

□ Running water 1=spring; 2=river/stream; 3=pond/Lake; 4=Dam

□ Rain water

□ Tanker truck

□ Water vendor

□ Bottled water

□ Others Specify _____

66. What kind of toilet facilities does your household have?

□ Flush toilet □ Pit toilet/latrine 1=traditional pit latrine 2=ventilated pit latrine (VIP)

□ No facility/bush/field □ other, please specify _____

67. Do you share these facilities with other households?

□ Yes □ No

68. Does your household have?

□ Electricity □ Paraffin lamp □ Radio

□ Television □ Telephone/mobile □ Iron (either charcoal or electricity)

□ Refrigerator

69. What is the main source of energy for lighting in your household?

□ Main electricity □ Solar □ Gas

□ Paraffin-hurricane lamp □ Paraffin-Wick lamp □ Firewood

□ Candles □ other, please specify _____

70. What is the main material for the walls of your house or house you are living?

□ Grass □ Poles and mud □ Cement bricks

□ Backed bricks □ Timber □Stones

□ Others Specify_____

71. What is the roofing material of your house or house you are living?

□ Grass/leaves/mud □ Iron sheets □ Tiles □ Concrete □ Asbestos

□ Others Specify_____

72. Does any member of your household own

□ A bicycle □ A motorcycle or motor scooter □ A car □ A bank account

73. How many acres of land for farming/grazing are owned by the household?

□ Arable land_____acres □ Land for grazing_____acres

74. How many meals does your household usually have per day?

Meals_____

Request for participation in the study:

Improved diagnosis of tuberculosis by antigen detection from sputum and extrapulmonary samples using immunochemistry-based assays

This is a request for you to participate in a study on improving the diagnosis of tuberculosis. The study will include patients from both Zanzibar and Norway, which would give us the opportunity to compare findings and results from the two settings

Tuberculosis most often affects the lungs. However disease may spread anywhere in the body which makes the diagnosis very difficult.

Routinely used tests are not able to detect disease in many cases and therefore the disease remains undetected leading to the worsening of illness and ultimately death. If tuberculosis is diagnosed it is curable.

At the University of Bergen, Norway and Haukeland University Hospital, we have developed a new and better test for the detection of tuberculosis. In research settings, this test has been shown to be significantly better than the routine tests. We would like to ask you to participate in this study, because we want to examine if this new method could improve the routine diagnostics of tuberculosis.

This is a laboratory-based test and will be performed on the material collected for the routine tests like various fluids, tissue samples, and sputum. All the sample collection and invasive procedures will be performed solely for the purpose of diagnosis, and no extra invasive procedure will be performed only for the sake of this study. No extra money will be charged for the test.

For this research, it is necessary to:
• ask you questions based on questionnaires
• obtain information relevant for your disease from your medical record
• perform our new diagnostic method in your sample material already collected

The objectives of this study are:
- to implement this test in the routine diagnostic of tuberculosis at Mnazi Mmoja Hospital
- to study whether the test improves the diagnosis of tuberculosis
- to study whether it is feasible to implement the test by evaluation of the costs and the advantages
- to compare research findings from Zanzibar and Norway.
- to further develop new, similar methods for sputum and other samples.

What will happen to the samples and the information about you?
The samples and data that are registered about you will only be used in accordance with the purpose of the study as described above. All the data and samples will be processed without name or other directly recognisable type of information. It will not be possible to identify you in the results of the study when these are published.

Voluntary participation
Participation in the study is voluntary. If you wish to participate, sign the declaration of consent on the final page. You can withdraw your consent to participate in the study at any time and without stating any particular reason. This will not have any consequences for your further treatment. If you later on wish to withdraw your consent or have questions concerning the study, you may contact following person, Melissa Jorstad, phone number 0776 584 962, e-mail: Melissa.jorstad@gmail.com or Maryam Ali, phone number 0774 040 545

Biobank:
At Haukeland University Hospital we want to collect blood samples and other biological material to be stored in a general research biobank for further research studies. If you give your consent that your samples may be stored in a biobank, the samples may later be used for further research studies in order to increase the knowledge about tuberculosis and other infectious diseases. This information may be beneficial for you as well as for other patients.

Generally, the material and samples that are left after routine examinations or treatment will be stored in the biobank. In addition we request for extra blood samples (maximum 50 ml) during your follow-up visits. 50 ml corresponds to 1/10 the volume drawn at blood donation. You may refuse to give these extra blood samples and in that case only the left-over samples from routine examinations will be stored in the biobank.

If you agree to that your samples and de-identified data being released to Haukeland University Hospital, Bergen, Norway, you have to sign a separate declaration. The samples would then be stored in a biobank at Haukeland University Hospital.You may still participate in the study even if you do not consent to your samples and de-identified data being released to Haukeland University Hospital, your samples would then be destroyed after this study is completed.

If you give your consent, the samples will be frozen at Mnazi Mmoja Hospital and later transported, stored and kept indefinitely in a biobank at the Haukeland University Hospital, Norway.We follow the laws allowing us to store and perform research on biological material. The Biobank is approved by the Regional Committee for Research Ethics in Norway. The samples will be used in research only after the specific studies are approved by the Regional Committee for Research Ethics in Norway and Zanzibar.

The data will be treated confidentially and stored according to national guidelines for sensitive data. If future analyses give results that may have implications for your medical health, you will be informed.

Right to access and right to delete your data and samples
If you agree to participate in the study, you are entitled to have access to what information is registered about you. You are further entitled to correct any mistakes in the information we have registered. You may withdraw from the study at any time without giving any reason. You can also demand that your samples are destroyed and that data concerning you are eliminated, unless the data have already been incorporated in analyses or used in scientific publications.

The study and the biobank are funded by research grants from The Western Norway Regional Health Authority.

Information about the study
Your are entitled to receive information about the outcome/result of the study

Contact persons: Dr. Melissa Davidsen Jørstad, Haukeland University Hospital/Mnazi Mmoja Hospital
Dr. Msafiri Marijani, Mnazi Mmoja Hospital
Dr. Maryam Abdalla Ali, Mnazi Mmoja Hospital

HELSE BERGEN
Haukeland University Hospital

Consent for participation in the study
- Adults above 18 years

Project title:	Prosjektnummer
Improved diagnosis of tuberculosis by antigen detection from sputum and extrapulmonary samples using immunochemistry-based assays	310035

Name of project manager	Department
Tehmina Mustafa	Dep. of pulmonary medicine (Lungeavdelingen)

It is voluntary to participate in the study. If you would like to participate in the study you have to sign the consent form. If you agree to participate at this time, you may at any time and without giving a reason withdraw your consent without your treatment being affected. If you later on wish to withdraw your consent or have questions concerning the study, you may contact the following person, Melissa Jorstad, phone number …. or Maryam Ali, phone number ….

I am willing to participate in the study:

Block letters	Identity number

Date	Signature

I agree that my samples and de-identified data may be sent to Haukeland University Hospital, Norway, and that the biological material may be stored in a biobank and used for further research studies in order to increase knowledge about tuberculosis and other infectious diseases.

Block letters	Identity number

Date	Signature

To be completed by the representative of the research project

I confirm that I have provided information about the research project:

Date	Signature	

Comments:

N-5021 Bergen - Norway - Telephone + 47 55 97 50 00 - Fax + 47 55 97 20 05
E-mail: postmottak@helse-bergen.no
Internet: www.helse-bergen.no

10.2 Appendix B - Standard operating procedures for sample collection and processing and immunostaining

Fine needle aspiration cytology

1) Prepare slides for routine staining and immunocytochemical staining (superfrosted slides). Write sample ID on the frosted end of the slide.
2) Prepare two sterile tubes. Write sample ID on the tubes.
3) Aspirate material as per routine practice.
4) Slide preparation
    a. Prepare routine slides.
    b. Prepare 2-4 smears on superfrosted slides.
        i. Air-dry the smears in room temperature.
        ii. Fixate the smears in 96% alcohol for 20 minutes.

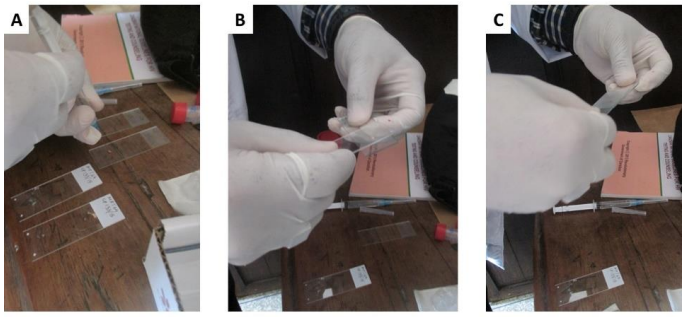

Figure: Preparation of smears from fine needle aspirates. Picture A: Using a syringe expel the material in the needle onto two slides (if enough material). Picture B and C: Place another slide on top of the slide with the expelled material, apply gentle pressure to spread the material, and then pull the slides apart.

1) Rinse the needle and syringe directly into two sterile tubes (equal volume in each tube) using 2 ml sterile normal saline.
2) Transport the slides to the laboratory.
3) Material in sterile tubes:
    a. Transport one tube to the TB laboratory at Mnazi Mmoja Hospital for GeneXpert® MTB/RIF.

b. Transport one tube to the TB clinic. The specimen will be sent to PHL-IdC on Pemba for mycobacterial culture.

Effusions and CSF

1) Divide the specimen equally in three (four) sterile tubes (if not already collected in three (four) tubes)
    a. Transport one tube to the TB clinic. The specimen will be sent to PHL-IdC on Pemba for mycobacterial culture.
    b. Transport one tube to the TB laboratory at Mnazi Mmoja Hospital for GeneXpert® MTB/RIF.
    c. One tube for cytology
    d. One tube for microbiological culture, Gram staining or other special stains.
2) Prepare slides for routine staining and immunocytochemical staining (superfrosted slides). Write sample ID on the frosted end of the slide.
3) Prepare 5 ml sterile tubes. Write sample ID and date on the tubes.
4) Centrifuge the sample at 3000 g for 10 minutes.
5) Transfer the supernatant to 5 ml sterile tubes.
    a. One for estimation of protein and glucose concentrations.
    b. Transfer the remaining supernatant to 5 ml sterile cryotubes for storage.
6) Use the pellet/sediment to make smears. Use 15-20 µl of the pellet/sediment for each smear.
    a. Place the material in the middle of the slide.
    b. Spread the sample material gentle with a pipette tip.
7) Prepare six slides per patient sample (If limited amount make slides for routine staining and reduce the number of slides for immunochemical staining)
    a. Slides for routine staining
        i. The slides for acid fast staining and Giemsa/PAP (or any other staining used for morphology) should be fixed according to the routine protocol.
    b. Four slides for immunochemical staining
        i. Air-dry the smear in room temperature.

Biopsies

1) In the operating room, divide the fresh surgical biopsy in two halves.
    a. Place one half of the specimen in a sterile container with normal saline.

i. Transport the container to the TB clinic. The specimen will be sent to PHL-IdC on Pemba for mycobacterial culture.

b. Place one half of the specimen in a container with formalin for fixation.

2) Perform conventional paraffin-embedding on the formalin-fixed specimen as per routine practice.
3) Prepare slides for routine staining and immunohistochemical staining (superfrosted slides). Write sample ID on the frosted end of the slide.
4) Prepare six consecutive 5 µm thick sections from the formalin-fixed paraffin-embedded biopsy.
    a. Slides for routine staining.
    b. Four slides for immunohistochemical staining.

Immunocytochemical staining of smears from FNAC, effusions and CSF

1) Preparation before the staining protocol.
    a. Prepare working solutions.
        i. Wash buffer: Dilute the concentrated wash buffer (Dako Wash buffer 10x, S3006, Dako, Glostrup, Denmark) 1:10 with distilled water.
        ii. Primary antibody: Dilute the primary antibody 1:250 in Antibody Diluent solution (Dako Antibody Diluent, S0809, Dako, Glostrup, Denmark).
    b. Rehydration step: Hydrate the alcohol-fixed slides in decreasing grades of alcohol.
        i. 80% alcohol, 4 minutes.
        ii. 70% alcohol, 4 minutes.
        iii. Distilled water, 10 minutes.
    c. Wash step: Using a Pasteur pipette rinse the slides with wash buffer 3 times, then apply wash buffer to cover the whole smear, after 1 minutes repeat the procedure (Repeat 3 times).
    d. Tap off excess water, and carefully wipe around the smear to remove remaining wash buffer. Encircle the region of the smear with the Dako Pen (Dako Pen, S2002, Dako, Glostrup, Denmark).
2) Staining protocol
    a. Step 1: Peroxidase block (EnVision® + System-HRP)

i. Apply peroxidase block to cover the whole specimen, incubate in humidity chamber for 15 minutes, to inhibit endogenous peroxidase activity.
ii. Wash step: Repeat step 1c.

b. Step 2: Primary antibody.
i. Apply diluted primary antibody to cover the whole specimen, incubate in humidity chamber for 60 minutes.
ii. Wash step: Repeat step 1c.

c. Step 3: Peroxidase labelled polymer (EnVision® + System-HRP).
i. Apply labelled polymer (horseradish peroxidase labelled polymer conjugated with secondary anti-rabbit antibodies) to cover the whole specimen, incubate in humidity chamber for 45 minutes.
ii. Wash step: Repeat step 1c.

d. Step 4: Substrate-chromogen (EnVision® + System-HRP).
i. Apply substrate-chromogen solution to cover the whole specimen, incubate in humidity chamber for 10 minutes.
ii. Wash step: Rinse the slides gently with wash buffer using a Pasteur pipette, then place in distilled water bath for 10 minutes.

e. Step 5: Mayer`s hematoxylin counterstain.

f. Step 6: Mounting. Mount the slides in Immu-Mount (Thermo Fischer Scientific).

Immunohistochemical staining of formalin-fixed, paraffin-embedded tissue sections

1) Preparation before the staining protocol.
a. Prepare working solutions.
i. Wash buffer: Dilute the concentrated wash buffer 1:10 with distilled water.
ii. Primary antibody: Dilute the primary antibody 1:250 in Antibody Diluent solution.
iii. Target retrieval solution (Citrate buffer): Dilute Target retrieval solution (Dako Target Retrieval Solution (10x), S1699, Dako, Glostrup, Denmark) 1:10 in distilled water. Final pH 6.00-6.20.

b. Deparaffinization and rehydration steps.

i. Xylene (to remove the paraffin), 25 minutes.
ii. 100% alcohol, 4 minutes x 2.
iii. 95% alcohol, 4 minutes.
iv. 80% alcohol, 4 minutes.
v. 70% alcohol, 4 minutes.
vi. Distilled water, 10 minutes.

c. Microwave antigen retrieval.
    i. Place the slides in Citrate buffer bath inside the microwave oven. First at 800W until the boiling point, then boil at 400-600W for 15 minutes.
    ii. Leave for 20 minutes in room temperature.
    iii. Wash in running distilled water to make the slides cool to room temperature.
d. Wash step: Using a Pasteur pipette rinse the slides with wash buffer 3 times, then apply wash buffer to cover the whole smear, after 1 minutes repeat the procedure (Repeat 3 times).
e. Tap off excess water, and carefully wipe around the smear to remove remaining wash buffer. Encircle the region of the smear with the Dako Pen.

2) Staining protocol
    a. Step 1: Peroxidase block (EnVision® + System-HRP)
        i. Apply peroxidase block to cover the whole specimen, incubate in humidity chamber for 10 minutes, to inhibit endogenous peroxidase activity.
        ii. Wash step: Repeat step 1d.
    b. Step 2: Primary antibody.
        i. Apply diluted primary antibody to cover the whole specimen, incubate in humidity chamber for 60 minutes.
        ii. Wash step: Repeat step 1d.
    c. Step 3: Peroxidase labelled polymer (EnVision® + System-HRP).
        i. Apply labelled polymer (horseradish peroxidase labelled polymer conjugated with secondary anti-rabbit antibodies) to cover the whole specimen, incubate in humidity chamber for 45 minutes.
        ii. Wash step: Repeat step 1d.
    d. Step 4: Substrate-chromogen (EnVision® + System-HRP).

i. Apply substrate-chromogen solution to cover the whole specimen, incubate in humidity chamber for 10 minutes.
ii. Wash step: Rinse the slides gently with wash buffer using a Pasteur pipette, then place in distilled water bath for 10 minutes.

e. Step 5: Mayer`s hematoxylin counterstain.
f. Step 6: Mounting. Mount the slides in Immu-Mount (Thermo Fischer Scientific).

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