Mycobacterial antigen MPT64 specific polyclonal antibody production and validation for an immunohistochemistry based diagnostic test for extrapulmonary tuberculosis

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Abstract

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Background: Tuberculosis (TB) is a major health problem, especially in low- and middle-income countries. Extrapulmonary TB (EPTB) constitute 20-40% of all TB. Diagnosis of EPTB poses challenges as the routine diagnostic tests are less sensitive due to the paucibacillary nature of the disease. Therefore, there is a need to develop better diagnostic tests for EPTB. An antigen detection test based on the detection of *Mycobacterium tuberculosis* complex specific protein MPT64 by immunohistochemistry (IHC) has been developed and validated in the routine diagnostic settings (1-8). These studies show that the MPT64 antigen detection test is applicable to various forms of EPTB, including HIV positive and HIV negative cases, on biopsies, fine-needle aspirates, and cytology smears with sensitivity 70-100% and specificity 65-100% which is significantly better than the routine tests. This test is robust, feasible to implement in high TB endemic settings (2), and can help in the timely and accurate diagnosis of EPTB, preventing empirical over-treatment, morbidity, and mortality. These findings warrant the large-scale implementation of the test. However, all these studies have been carried out by the limited amount of in-house rabbit polyclonal antibody (pAb). The reproduction of an anti-MPT64 antibody with applicability on IHC is a prerequisite for large-scale use of the test. Our research group has reproduced a monoclonal anti-MPT64 antibody which gives good reactivity with enzyme-linked immunosorbent assay (ELISA) against the recombinant antigen, but it does not give good reactivity on formalin-fixed tissues. This is probably due to variable epitopes *in vivo* and changes in the antigen during the fixation process. PAbs, by virtue of their polyclonality and heterogeneity, can bind to multiple and different antigenic epitopes, and could be a suitable candidate for IHC.

Aims: The aim of this study was to reproduce the anti-MPT64 pAb, create a single batch in a large volume, validate the new pAb on formalin-fixed clinical samples, and compare the validity with Xpert MTB/RIF assay.

Material and Methods: Recombinant MPT64 protein was prepared by using a mammalian cell expression system. Rabbits were used as host animals for the generation of pAbs. An immunization strategy was designed by a pre-immunization selection of 38/180 rabbits with minimal reactivity

of their sera on the formalin-fixed tissues by IHC. The 38 selected rabbits were immunized by recombinant antigen and Titer Max Gold adjuvant by using a shorter and longer immunization protocol generating 50 ml and 90 ml of sera, respectively from each rabbit. Individual bleeds from each rabbit were tested with ELISA and IHC. Sera with good reactivity by IHC on the formalin-fixed TB positive control tissues and minimal reactivity on the non-TB tissues were further tested by making various cocktails to generate a single batch in a large volume. Various background reducing strategies were applied to achieve good specificity. These batches were tested on human clinical samples. The selected batch was validated on bacteriology confirmed (culture and/or Xpert MTB/RIF positive) EPTB (24 lymphadenitis and 21 pleuritis) and 41 non-TB biopsies by IHC. The sensitivity of the new pAb was compared with the microscopy for acid-fast bacilli and Xpert MTB/RIF assay using culture as a reference standard.

Results: All bleeds had very good reactivity with ELISA, with titer mostly around 1:200.000. With IHC, reactivity with the individual bleeds was variable, some sera gave very good sensitivity and specificity, while others were less sensitive and/or less specific due to the non-specific background staining. Among the various background reducing strategies, overnight incubation of tissue sections with 3% bovine serum albumin and 10% normal goat serum followed by further blocking with serum-free protein block gave good results with a significant reduction of non-specific staining. Among five cocktails made from sera of 25 rabbits, one cocktail consisting of sera from 10 rabbits gave the best results. The sensitivity of this cocktail was similar to the previous anti-MPT64 pAb, though the staining intensity was generally less, the signals were clearly visible. Using bacteriological confirmation as a reference standard, the sensitivity, specificity, positive and negative predictive values, and accuracy of IHC with this batch of new pAb in lymphadenitis were 88%, 80%, 72%, 92%, and 83%, respectively, and in pleuritis were 86%, 80%, 69%, 92%, and 82%, respectively. Using culture as a reference standard, the performance of the new anti-MPT64 pAb was better than AFB microscopy in both lymphadenitis and pleuritis (sensitivity 88% vs. 13%, and 89% vs. 6%), while it was better than Xpert MTB/RIF in the TB pleuritis (sensitivity 89% vs. 17%) and similar to it in lymphadenitis (sensitivity 88% vs. 88%).

Conclusion: The study shows that it is possible to reproduce pAbs that can detect the MPT64 antigen in the formalin-fixed paraffin-embedded tissue sections by IHC. The sensitivity of the MPT64 antigen detection test by using these new pAbs is better than the AFB microscopy and

Xpert MTB/RIF. This opens up the possibility of the large-scale use of this test and its inclusion in the routine diagnostics of EPTB.

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Acronyms and abbreviations

AFB	Acid-fast bacilli
AIDS	Acquired immunodeficiency syndrome
BCG	Bacillus Calmette-Guérin
cDNA	Complementary deoxyribonucleic acid
DCs	Dendritic cells
DNA	Deoxynucleic acid
ELISA	Enzyme-linked immunosorbent assay
EPTB	Extrapulmonary tuberculosis
FNAC	Fine needle aspiration cytology
HEK	Human embryo kidney
HIV	Human immunodeficiency virus
ICC	Immunocytochemistry
IFN-γ	Interferon-gamma
IHC	Immunohistochemistry
IL	Interleukin
LAM	Lipoarabinomannan
NAAT	Nucleic acid amplification techniques
N-PCR	Nested polymerase chain reaction
NSS	Non-specific staining
MTB	Mycobacterium tuberculosis
pAb	Polyclonal antibody
PCR	Polymerase chain reaction
PLcA	Phospholipase C encoding A
mAb	Monoclonal antibody
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
SS	Specific staining
TB	Tuberculosis
TBS	Tris-buffered saline

TGE	Transient gene expression
TGF-β	Transforming growth factor-beta
TGMA	Titer Max Gold adjuvant
Th	Helper Type T-cell
TNF-α	Tumor necrosis factor-alpha
Treg	Regulatory T cells
WHO	World Health Organization

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This thesis is dedicated to my parents, my brothers, my sister, and my children. I am thankful for their encouragement, love, and support, especially my oldest brother Kahtan for his faith in me and my sister Amaal for her love, support and always being there for me in difficult times.

Iman A Mohammed Ali, May 2020.

1. Background

1.1. Etiology and pathogenesis of tuberculosis

Tuberculosis (TB) is potentially a preventable and curable infectious disease, which most commonly affects the lungs and causes pulmonary TB. However, any organ in the body can be involved, and the disease is then called extrapulmonary TB (EPTB). TB is caused by an acid-fast bacillus, *Mycobacterium tuberculosis* (MTB) that is first identified by Robert Koch in 1882. From a clinical and public health perspective, TB is divided into primary and post-primary (9). Primary TB occurs when the individual is infected with MTB for the first time without prior immunity to the infection. The infection progresses to a disease within a short time, usually 2 years, of the infection (10). However, more than 90% of the immunocompetent individuals can develop a protective immune response and restrict the infection leading to the formation of granuloma (11, 12). Granuloma formation is an effective immune response mediated by adaptive immunity, which can control the infection but may not eradicate it completely (13). The mycobacteria may remain dormant as latent infection (10). Years later reactivation or reinfection in people who have developed immunity to primary TB, can result in post-primary TB disease, also known as adult-type or secondary TB (12, 14). Post-primary TB is characterized by the formation of cavities in the lungs, and contributes towards disease transmission and nearly 80% of all clinical disease (15). In high TB-endemic settings, primary TB is more common in children, while post-primary TB is more common in adults (12). Reactivation of latent infection may be triggered by factors that compromise host immunity such as, Human immunodeficiency virus (HIV) coinfection, diabetes, alcoholic liver disease, malnutrition, and use of steroids or other immunosuppressive drugs (16). In high TB endemic countries when active disease occurs in later life, it becomes difficult to ascertain whether it is due to reactivation of latent infection or a new infection with another MTB strain. Patients suffering from active pulmonary TB are the primary source of transmission of infection via minute aerosol droplets through exhalation and expectoration (16). The World Health Organization (WHO) estimated that in 2017 nearly two billion individuals were infected with MTB worldwide confirming that the way of transmission is very efficient (17).

1.2. Epidemiology of tuberculosis

The global burden of TB is high and in 2018, ten million people were estimated to have TB, and approximately 1.5 million deaths from TB were estimated (18). TB is the ninth leading cause of death worldwide and the leading cause from a single infectious agent, ranking above HIV/AIDS (18). Of all TB infections, EPTB accounts for 20% and up to 50% in HIV patients (15). The annual incidence rates of EPTB has been increasing (15).

Control of the global TB epidemic has so far failed due to the lack of effective vaccine, development of drug-resistant MTB strains, and the lack of sensitive and rapid diagnostics for all forms of TB (16). The WHO's specific targets set in the End TB Strategy include a 90% reduction in TB deaths and an 80% reduction in TB incidence by 2030, compared with 2015, but TB incidence is falling slowly at about merely 2% per year, and there are still large gaps in case detection and treatment (18). Most deaths from TB could be prevented with early diagnosis and appropriate treatment. Better diagnostic tests for all forms of TB are needed to meet the targets of the End TB Strategy.

1.3. Mycobacterium tuberculosis structure and proteins

MTB is a pathogenic rod-shaped, non-motile bacterium. It is an obligate aerobe, non-spore forming, and facultative intracellular pathogen. MTB has waxy coating on its cell surface making the cells impenetrable to Gram staining (19). Therefore, acid-fast stains are used instead of Gram staining to identify MTB under microscope. MTB has a very slow replication time, about 18-58 hours, and requires 3-4 weeks to produce visible colony on a culture medium (20). The cell wall consists mostly of lipids and carbohydrates, and it accounts for the major part of its virulence (21).

Mycobacterial proteins are either somatic proteins of cell wall and cytoplasm or secreted proteins that are produced by active secretion during the growth of mycobacterium. Among 4000 mycobacterial reported proteins, 376 are specific to MTB as they do not share homology with other proteins (22). Mycobacterial proteins filtrated from culture are mainly secreted proteins, but they also contain cell wall and cytoplasmic proteins (23, 24). Previous studies have described different methods to differentiate between antigens that are actively secreted from the mycobacterial cell from intracellular (cell wall and cytoplasmic) during culture. One method is differentiation by localization index, which is based on the measurement of secretion efficiency of

MTB proteins and groups the proteins to either secretory or cytoplasmic. Cytoplasmic proteins have by definition a localization index of zero and secretory proteins determined in MTB culture fluids with minimal lysis are given values (23). Quantitative immunologic techniques like crossed immunoelectrophoresis have been used for quantification of individual MTB proteins in sonicates of washed bacilli and culture fluids, which allows the determination of a localization index and thereby help to differentiate between cytoplasmic and actively secreted (25). Various secreted and somatic mycobacterial proteins have been characterized by these methods (23, 25). Another method is studying the release of the enzyme isocitrate dehydrogenase from MTB during its growth as a marker of autolysis (26). Five of the common mycobacterial secretory proteins 85a, 85b, 85c, MPT51, and MPT64 are containing component-specific as well as cross-reacting epitopes, but MPT64 has less homology with other secretory mycobacterial proteins (27).

1.4. Immune responses to MTB

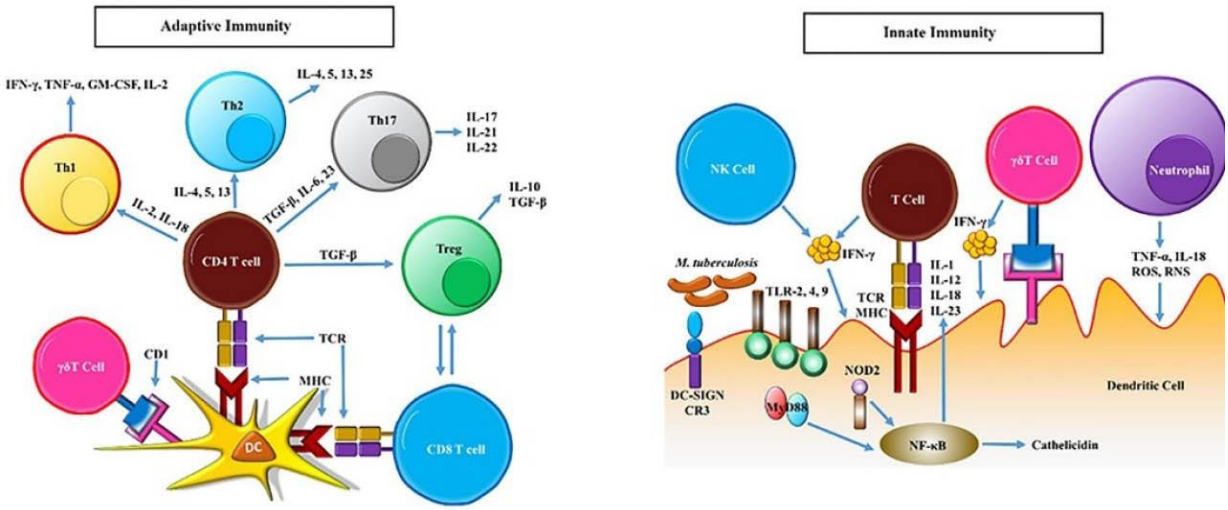

Figure 1. Cells and molecules involved in the adaptive and innate immunity against MTB.

Adapted from: Javan MR, Jalali nezhad Aa, Shahraki S, Safa A, Aali H, Kiani Z. Cross-talk between the Immune System and Tuberculosis Pathogenesis; a Review with Emphasis on the Immune Based Treatment. Int J Basic Sci Med. 2016;1(2):40-7.

1.4.1. Innate immune responses in tuberculosis

Macrophages and neutrophils are the host's first line of defense against MTB and they achieve their role of protection by phagocytosis and expression of antimicrobial peptides (28). When the MTB finds its way to a new host through inhaled airborne droplets nuclei and settles in the alveoli and finally pulmonary parenchyma, the early host response is an influx of phagocytic cells, which are the alveolar macrophages and recruited neutrophils (28). As the MTB infection is established in the lungs, the bacilli are phagocytosed by macrophages through receptor-mediated phagocytosis and also taken up by neutrophils and dendritic cells (DCs) (28). Growth of MTB can be controlled by activated macrophages but they cannot completely eradicate the pathogen neither *in vitro* nor *in vivo* (29). The main receptors on the surface of macrophage that involve in the entry of MTB or phagocytosis are complement receptors, mannose receptors, and scavenger receptors (30). Several classes of pattern-recognition receptors are expressed by macrophages and involved in the first step of recognition of MTB (30).

Neutrophils/granulocytes are permissive hosts for MTB, rapidly recruited to the site of infection that traffic MTB to draining lymph nodes (11). Lung neutrophils help to promote the adaptive immune response against MTB by delivering the pathogen to DCs in a form that makes them more efficient and faster in presenting the antigen to prime and activate naïve antigen-specific $CD4^+$ T cells (31).

MTB may modulate the immune response to its advantage by delivering signals through Toll-like receptors that can affect the innate immune susceptibility, lung pathology, immunopathogenesis, and cytokines production at the local site of infection (32).

1.4.2. Adaptive immune responses in tuberculosis

T and B cells constitute the adaptive immune response against MTB. These cells are activated by the MTB-infected macrophages and DCs of the innate immunity. DCs have a fundamental role in initiating an immune response against MTB but they are regulated at various checkpoints of the host immune response (33). They are the prime antigen-presenting cells that initiate the adaptive immune response by T cells against MTB (33). DCs start this complicated and slow process in interaction with apoptotic macrophages and neutrophils containing the MTB, which result in the

bacterial acquisition by DCs and earlier trafficking to lymph nodes and faster priming of T cells (33).

Compared with other infections, the considerable delay in the onset of adaptive T cells immune responses in TB is well recognized and partly due to pathogen inhibition of apoptosis, delayed migration of dendritic cells from the lungs to the local lymph node, and influence of regulatory T cells (Treg) and Interleukin (IL) 10 (34).

Protective immunity against MTB and delay of control of bacterial growth depends on the central mediators $CD4^+$ T-cells of helper type 1 (Th1) that activate antimycobacterial activity in mononuclear phagocytes, an activation that is mediated by cytokines including Interferon-gamma (IFN-γ) and Tumor necrosis factor-alpha (TNF-α) (35, 36). Therefore, HIV patients with low $CD4^+$ T cells are highly susceptible to TB (37). $CD4^+$ Th cells can be differentiated into Th1, Th2, Th17, and Treg cells. Th1 secrets cytokines like IFN-γ, TNF-α, IL-2, lymphotoxin, and granulocyte-macrophage colony-stimulating factor, which increase the activation of Th1cells, $CD8^+$ cytolytic T lymphocyte, and maturation and activation of macrophages/granulocytes (38). While Th2 cells produce cytokines like IL4, IL-5, IL-10, and IL-13, which stimulate B cells to produce antibodies , but at the same time, suppress the Th1 type of immune response (38). The Th17 cells produce cytokines like IL-17, IL-17F, IL-21, and IL-22, which are involved in the early phase of host defense after MTB infection through stimulation of defensin production that recruits neutrophils and monocytes to the site of inflammation (38). Differentiation of T cells is decided by different cytokines (38). Th1 cell development is promoted by IL-12, IL-18, and IFN-γ, while Th2 cell development is induced by IL-4, IL-5, and IL-13. Th17 cell differentiation is stimulated by low concentration of transforming growth factor-beta (TGF-β), IL-6, IL-22, and IL-23, while Treg cells differentiation is induced by IL-2 and high concentration of TGF-β (39, 40). TGF-β-induced Treg cells can be inhibited by IL-6, and in combination with TGF-β, promote Th17 cell differentiation (41). Regulatory mechanisms to control the inflammation, and CD4+ T cells in response to MTB infection, are necessary to inhibit host damage. Uncontrolled TNF-α and IFN-γ under conditions of infection can be unfavorable and damaging to the host (42).

Treg cells expressing FoxP3 are increased in TB infection and inhibit the production of INF-γ by memory gamma-delta T cells in response to MTB infection (43). The responsiveness of $CD4^+$ T cells can be limited through downregulation by TGF-β, which leads to suppressing the effector-

immune response (44). The balance between IL-10 and Treg cells on one side and INF-γ and TNF-α on the other side may determine whether the immune system can eradicate MTB with minimum damage to the host.

Although $CD8^+$ T lymphocytes is now generally accepted to have a role in anti-MTB immunity, it cannot compensate for absence or low $CD4^+$ T cells (15, 42). $CD8^+$ are capable of immune protection by secreting granulysin, granzymes, and perforins to kill MTB-infected cells (45).

Memory T cells are formed after that MTB find its way to human and when they encounter MTB antigen again they replicate quickly and produce many cytokines like IFN-γ, IL-2, TNF-α, lymphotoxin, and granulocyte-macrophage colony-stimulating factor (40).

The immune response to MTB results in the formation of granulomatous inflammatory lesions at the site of infection. Epithelioid cell granulomas with multinucleated giant cells and central caseation necrosis is a defining lesion of TB (13, 46). It indicates a protective immune response mounted by the host. Granuloma formation is, however, not limited to TB and is taking part in the pathogenesis of a variety of inflammatory diseases like sarcoidosis and fungal infections. Thus, histology based on the granulomatous inflammation with necrosis is not specific for TB.

1.5. Diagnostic challenges in extrapulmonary TB

1.5.1. Clinical presentation

Active EPTB has a heterogeneous range of clinical presentations and forms of disease and is regarded as a big imitator by clinicians (47). The non-specific clinical features overlap with other chronic diseases that often lead to diagnostic difficulty based on clinical features. The disease usually presents insidiously with non-specific and systemic features like fever, malaise, loss of appetite, weight loss, and night sweats (47). The localized symptoms are depending on the site of disease and the organ involved. EPTB is more common in young children and HIV coinfected individuals (48). The most common form of EPTB is tuberculous lymphadenitis followed by tuberculous pleuritis (47). Other common forms of EPTB include bone TB, peritoneal TB, urinary tract TB, and meningeal TB. The clinical presentation is usually characterized by local signs and symptoms from the involved organs with or without systemic features (47).

Tuberculous lymphadenitis is characterized by multiple and painless enlarged lymph nodes, commonly affecting the lymph nodes in the anterior cervical triangle, sometimes with fistula to the skin. Tuberculous pleuritis is characterized by chronic unilateral pleural effusion presenting with pleuritic symptoms with or without systemic symptoms.

1.5.2. Laboratory diagnosis of EPTB

The demonstration of MTB either directly or indirectly is the cornerstone of the diagnosis of EPTB. The direct diagnostic methods based on the detection of MTB or its products have low sensitivity because of the paucibacillary nature of EPTB. The indirect methods are based on the hosts immune response and measurement of this response which have low specificity.

1.5.2.1. Direct diagnostics methods

1.5.2.1.1. Acid-fast bacilli microscopy

Acid fastness is a unique feature of MTB and other mycobacterial species (49). Ziehl Neelsen staining method can demonstrate acid-fast bacilli (AFB) in both smears and tissues by direct microscopy when the bacterial load is more than 10^6 bacilli/g tissue (50). This stain makes AFB looks red/pink under microscope. Due to the paucibacillary nature of EPTB, the sensitivity of AFB microscopy is very low, between 0-40% depending on the site of EPTB (5, 6). Centrifugation of the samples and fluorochrome staining with ultraviolet microscopy can increase the sensitivity of microscopy by 10% (50, 51). The low sensitivity limits its diagnostic value even though it is cheap, easy to perform, and quick (5, 6). In addition to the low sensitivity of AFB microscopy, it cannot distinguish between MTB and atypical mycobacteria.

1.5.2.1.2. Culture methods

Culture is the gold standard method for a definite diagnosis of EPTB. It is more sensitive than AFB microscopy as fewer bacilli (10-100 bacilli/ml of sample) are needed to detect and isolate MTB for further species identification and drug-susceptibility testing (52). Solid cultures, egg-based or agar-based, are time-consuming, requiring 4-8 weeks. Liquid cultures as BACTEC system have 10 % better sensitivity than solid cultures and give rapid results within 2-4 weeks, but they are more expensive and need safe disposal of radioactive waste (53). Establishing of culture facilities need both skilled laboratory technician and advanced laboratory facilities with appropriate biosafety conditions, which limits its availability in low-resource settings. The

sensitivity of culture in EPTB is poorer as compared to pulmonary TB and vary from 20-80% (54, 55).

1.5.2.1.3. Molecular methods

Molecular methods based on nucleic acid amplification techniques (NAAT) have contributed to the development of tests like polymerase chain reaction (PCR) assays targeting different MTB genes (65 kDa, 38 kDa) or insertion sequences like IS-6110 which are in routine use. NAAT have higher sensitivity than culture methods as they can detect as few as 1-10 MTB bacilli in clinical samples and the results are available within 6-8 hours (56). NAAT can be performed on stored clinical samples either they are formalin-fixed or dried scraped material. In paucibacillary EPTB conventional PCR has low sensitivity, while nested and real-time PCR have higher sensitivity than AFB microscopy and culture (4-6). PCR assays are, however, very sensitive to contamination resulting in high false-positive results. This disadvantage has been overcome by the Xpert MTB/RIF automated molecular assay (GeneXpert). This assay is developed for the rapid detection of MTB and rifampicin resistance. The sensitivity of GeneXpert is heterogeneous and varies from 25% to 96.6% depending on the site of EPTB (57). Lower sensitivities are reported in cerebrospinal, pleural, pericardial, peritoneal, and synovial fluids (58). The main limitation of the GeneXpert, as a routine diagnostic test in EPTB, is high costs and lower sensitivity for smear-negative than smear-positive clinical samples of EPTB (59). Furthermore, this assay cannot differentiate between viable and dead bacilli and cannot provide information about drug susceptibility other than rifampicin.

1.5.2.1.4. Antigen detection tests

Antigen detection methods have been introduced and studied in recent years. Antigen assay based on detection of mycobacterial Lipoarabinomannan (LAM) in urine has shown good performance in HIV-associated pulmonary TB with a high bacterial load, but is not useful in paucibacillary EPTB (60-62). Immunohistochemistry (IHC) and immunocytochemistry (ICC) based antigen detection tests have been reported with better sensitivity and specificity than AFB microscopy in various form of EPTB as it is capable to detect MTB antigens in both intact and degraded bacilli, while AFB microscopy detects only intact bacilli (4, 5). Various mycobacterial antigens can be detected in formalin-fixed paraffin-embedded tissue, tissue aspirate, and fluids, such as mixed

mycobacterial antigens, secreted MTB specific antigen MPT64 (1-8), antigen85 (63), ESAT6 (61), Cell wall-associated antigen5 (64), LAM (65), HspX (66), TB8.4, and phospholipase C encoding A (PLcA) protein (67). The advantage of IHC and ICC over PCR is that they are robust, not sensitive to contamination, and can be performed without high-tech equipment.

1.5.2.2. Indirect diagnostic methods

1.5.2.2.1. Histopathology and Cytology

Due to the paucibacillary nature of EPTB, histological examination of specimens has been regarded as a method of choice for the diagnosis of EPTB (68). The presence of granulomatous inflammation with epithelioid cells, multinucleated giant cells, and caseous necrosis strongly suggest TB (68). However, several clinical conditions other than TB may cause the same granulomatous reaction such as fungal infections and sarcoidosis. Histological examination alone, therefore, might not lead to the correct diagnosis of TB (69). Furthermore, it is not possible to differentiate the various mycobacterial species on histological bases alone as the histological findings of tuberculous and non-tuberculous mycobacterial disease are the same. Fine needle aspiration cytology (FNAC) is recommended as first-line investigation in all accessible suspected EPTB as it is easy to perform, relatively cheap, rapid, and less invasive procedure (70). Tuberculous cytology pattern with the predominance of lymphocyte can be difficult to differentiate from other granulomatous non-tuberculous lesions and atypical TB lesions in advanced HIV disease. Therefore, explicit TB diagnosis on FNAC or body fluids is based only on bacteriological confirmation and that can only be achieved in 20-25% of EPTB cases (50).

1.5.2.2.2. Serological tests

Antibody-based serological tests like enzyme-linked immunosorbent assay (ELISA) for antibodies against MTB secretory protein superoxide dismutase are simple, inexpensive, and can be used as supplements to conventional methods to support the diagnosis of EPTB, but the negative test cannot exclude the diagnosis (71). In general, the serological tests have shown low specificity and poor reproducibility as the methods of purifying mycobacterial antigens are not reproducible (72). Therefore, they are not recommended in the diagnosis of TB in low- and middle-income countries (72).

Tuberculin skin test used in screening for TB infection is a cellular immunity mediated test that measures the delayed hypersensitivity reaction (48-72 hours) after intra-dermal injection of

tuberculin antigen (73). The reagent used in this test is the MTB purified protein derivate. However, the limitation of the tuberculin skin test in the diagnosis of active EPTB is that positive test can also be caused by Bacillus Calmette-Guérin (BCG) vaccination or previous infection with MTB or non-tuberculous mycobacteria (74) . On the other hand, patients with immunosuppressant conditions can have false-negative results (75).

An assay that measures interferon-gamma-release from the stimulated peripheral mononuclear cell *in vitro* is available as QuantiFERON TB Gold. This assay cannot differentiate between latent and active TB and thus have limited value as diagnostic tests to diagnose active EPTB, especially in high endemic areas in low- and middle-income countries (76).

There is evidence that supports the use of measurement of enzyme adenosine deaminase activity in the diagnosis of EPTB. However, this assay is not specific for TB (77).

Therefore, the lack of accurate and rapid diagnostic tests of EPTB remains one of the major challenges in the global control of TB.

1.5.3. MPT64 antigen detection test

Earlier studies in our laboratory have shown in-situ expression of nine different secreted and three somatic mycobacterial antigens, including MTB complex specific secretory antigen MPT64, by using IHC with in-house polyclonal antibodies (pAb) in various types of extrapulmonary paucibacillary TB lesions (63). These results have shown that MPT64 was consistently expressed intra-cellularly while other antigens were not detectable in the lesions where mycobacteria were below the detection limit of AFB microscopy and culture (63). This led to the hypothesis that MPT64 has a special ability of intracellular accumulation which makes it possible to detect it in lesions with only a small number of mycobacteria. An example is shown in figure 2, where MPT64 antigen is seen as reddish-brown stain in the epithelioid cells and multinucleated giant cells of a TB lesion which is negative with AFB staining and culture.

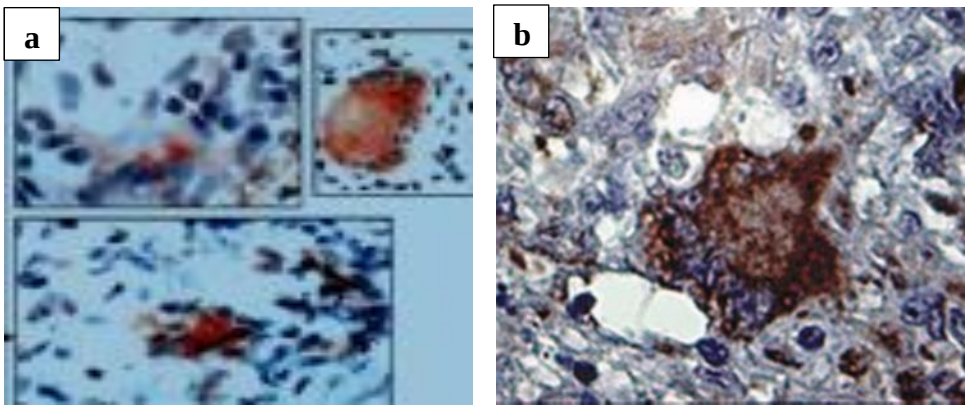

Figure 2. IHC staining pattern of MPT64 antigen in (a) pleural and (b) lymph node TB lesions. MPT64 antigen is seen as reddish-brown stain in the epithelioid cells and multinucleated giant cells of a TB lesion, which is negative with acid-fast bacilli staining and culture.

Picture (a) adapted from: 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. Applied immunohistochemistry & molecular morphology : AIMM. 2008;16(6):554-61.

Picture (b) adapted from: Mustafa T, Wiker HG, Mfinanga SG, Morkve O, Sviland L. Immunohistochemistry using a Mycobacterium tuberculosis complex specific antibody for improved diagnosis of tuberculous lymphadenitis. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2006;19(12):1606-14.

Further work confirmed the hypothesis and led to the development of a diagnostic method based on the detection of this antigen by using immunochemistry on various biological fluids, lymph node aspirates, and biopsies (2, 3, 7, 8). These studies have shown that the assay can be applied to a wide range of clinical specimens from different geographical locations including Tanzania, India, South Africa, and Norway with significantly higher sensitivity and specificity as compared to the conventional methods of AFB microscopy and solid culture. The sensitivity and specificity of the assay are shown to be similar to nested-PCR (N-PCR) (1-8). The assay performs equally well in HIV co-infected TB cases with atypical histological features (1). Table1 shows the summary of studies on the MPT64 antigen detection test.

Table 1. Summary of studies on diagnostic validity of MPT64 antigen detection test in extrapulmonary tuberculosis.

Author	Country	No. of cases	EPTB type	Reference standard	IHC/ICC with Anti-MPT64	
					Sensitivity %	Specificity %
Mustafa et al. 2006	Norway, Tanzania	55	LN	N-PCR	90	83
Purohit et al. 2007	India, Norway	153	LN, abdominal	N-PCR	92	97
Baba et al. 2008	South Africa	36	Pleural/HIV	CRS/N-PCR	80-81	100
Purohit et al. 2012	India	270	CSF, pleural, Acites, LN	N-PCR	93/96	97/96
Tadele et al. 2014	Ethiopia	118	Pleural, LN	N-PCR	88.1	89.5
Purohit et al. 2017	India, Norway	89	LN	CRS	100	97
Davidsen et al. 2018	Tanzania	152	Pleural, ascites, LN, CSF	CRS	50-100	91-97
Hoel et al. 2020	Norway	288	Extrapulmonary biopsies, aspirate, pus, fluid samples	CRS	37	99

FOOTNOTE. No, Number; EPTB, extrapulmonary tuberculosis. IHC, immunohistochemistry; ICC, immunocytochemistry; LN, lymph node; CSF, cerebrospinal fluids; CRS, composite reference standard; N-PCR, nested polymerase chain reaction; HIV, human immune deficiency virus.

MPT64 antigen is present only in MTB complex and is not detected in non-tuberculous mycobacteria (78, 79) or in BCG strains with RD2 deletions (80, 81). In other words, the MPT64 antigen detection test can differentiate between pathogenic and atypical mycobacteria. The test is

significantly faster compared to culture as the results are available within 1-4 working days depending on the sample type. These findings support the implementation of this test in a routine TB diagnostic setting. An ongoing multicenter study shows that the test is indeed implementable in the routine diagnostic setting of high TB endemic countries, and the performance of the test is better than the routine tests (3). The results from the other four sites are under analysis. These results support the view that the test should be made available for routine TB diagnostics globally. The uniqueness of the test is in the functional primary antibodies applicable on formalin-fixed material. Until now, all the studies have been carried out by the limited amount of in-house rabbit pAb. For further scale-up of the test, the reproduction of a functional antibody is the first and foremost prerequisite.

Our research group has reproduced a monoclonal antibody (mAb) anti-MPT64 which gives good reactivity with ELISA against the recombinant antigen, but it does not give good reactivity on formalin-fixed tissues. This is probably due to variable epitopes *in vivo* and changes in the antigen during the fixation process. PAbs, by virtue of their polyclonality and heterogeneity, can bind to multiple and different antigenic epitopes, and could be a suitable candidate for IHC.

2. Objectives

2.1. Main objective

The aim of this study was to reproduce an anti-MPT64 pAb for large-scale use on formalin-fixed clinical samples by using IHC.

2.2. Specific objectives

1. To design a strategy to produce a single batch in a large volume of functional anti-MPT64 pAb for application on formalin-/alcohol-fixed samples.
2. To validate the new pAb on clinical samples for diagnostic accuracy.
3. To compare the validity of new pAb with Xpert MTB/RIF assay.

3. Methods

3.1. Preparation of the recombinant protein

Recombinant MPT64 protein was prepared by the mammalian cell expression system. This system was chosen as it is able to introduce proper protein folding/posttranslational modifications (such as phosphorylation and acetylation), which are usually crucial for the activity of target protein

(82). E-coli, commonly used to produce recombinant proteins, can have posttranslational modifications of the target protein which are different from those in mammalian cells and can affect its structure and activity.

The target was MTP64 from Mycobacterium bovis (without signal sequence, with start M, linker, C-terminal His-Tag, stop codon and flanking restriction sites, 701 bp): >tr|A0A060IGQ3|24-228
MAPKTYCEELKGTDTGQACQIQMSDPAYNINISLPSYYPDQKSLENYIAQTRDKFLSAAT
SSTPREAPYELNITSATYQSAIPPRGTQAVVLKVYQNAGGTHPTTTYKAFDWDQAYRKP
ITYDTLWQADTDPLPVVFPIVQGELSKQTGQQVSIAPNAGLDPVNYQNFAVTNDGVIFFF
NPGELLPEAAGPTQVLVPRSAIDSMLAVLVPRGS AAALEHHHHHHHH *

Complementary DNA (cDNA) was synthesized and ligated into a vector suitable for transient transfection. The expression vector was added to E. coli and the correct insertion was analyzed by sequencing. The preparation of plasmid was done by the cultivation of an E. coli strain harboring the expression vector. Plasmid DNA was controlled for low endotoxin. A functional coding DNA template was introduced into the mammalian cells through non-viral methods commonly called transient gene expression (TGE). Human embryo kidney (HEK) cells were used for protein expression. The transient transfection of HEK cells was done by cultivation in serum-free suspension culture. HEK cells were transfected with INVect Transfection Reagent (Figure 3).

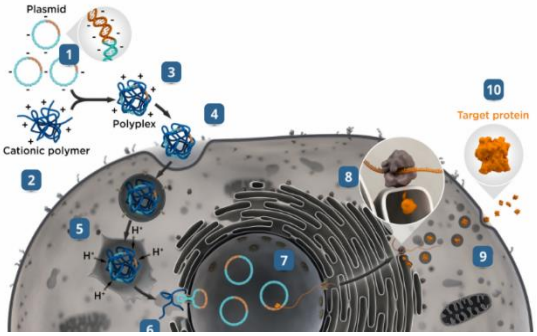

Figure 3. Summary of protocol of expression process.
Adapted from InVivo BioTech Services GmbH. https://www.transient-transfection.com/. Accessed at 11.05.2020.

The principles of the TGE method is summarized in figure 3. 1) Plasmid DNA with the genetic information of MPT64 was expressed and is negatively charged. 2) INVect transfection reagent consists of polycationic polymers. 3) Polyplexes are positively charged particles and formed as a consequence of an electrostatic reaction between plasmid DNA and INVect transfection reagent. 4) Polyplexes interact with the negatively charged cell membrane and thereby internalized through receptors on the cell membrane by endocytosis. 5) Primary and secondary amine in the polyplexes protects plasmid DNA from degradation in the

lysosome by preventing acidification of lysosome through proton adsorption leading to osmolality shift and the influx of water resulting in damage of lysosome. This is what referred to as the "proton sponge effect". 6) Plasmid DNA enters the nucleus after it has been released from polyplexes through nuclear pores or during mitosis. 7) The gene of MPT64 is transcribed into mRNA by RNA polymerase. 8) Translation of mRNA takes place in ribosomes and the MTP64 protein is formed in lumen of endoplasmic reticulum. 9) MPT64 protein is transported from endoplasmic reticulum to Golgi apparatus where MPT64 is packed into secretory vesicles that move towards the cell membrane. 10) MPT64 is synthesized.

The purification of protein was done by affinity chromatography. Dialysis was done against tris-buffered saline (TBS), pH 7.4. The expressed protein content was analyzed by SDS-PAGE which is an electrophoresis method that allows protein separation by a mass. MPT64 mas is 23 kDA. Charles River PTS Endosafe system was used to determine endotoxin. MPT64 protein was stored and delivered at 2-8 C.

3.2. Adjuvant

The commonly used adjuvant to enhance the immune response and thereby increase the production of pAb in immunization is Freund's complete adjuvant, which we could not use as it contains mycobacterial components and it was not desirable with an antibody that has specificity against other components of MTB than the target antigen MPT64. Thus, two other adjuvants were tried. The first adjuvant was lipopolysaccharides of the blue-green algae Phormium species (Biogenes, Berlin, Germany). The second adjuvant was the Titer Max Gold adjuvant (TMGA) (Sigma-Aldrich, Søborg, Denmark). The use of TGMA gave a better titer and was selected for immunization. TMGA is a type of water-in-oil emulsion. It contains three ingredients which are a block copolymer, CRL-8300, squalene (a metabolizable oil), and a unique microparticulate stabilizer. A 50:50 water-in-oil immunization emulsion with the antigens and TMGA was prepared.

3.3. Strategy for immunization and pooling of sera

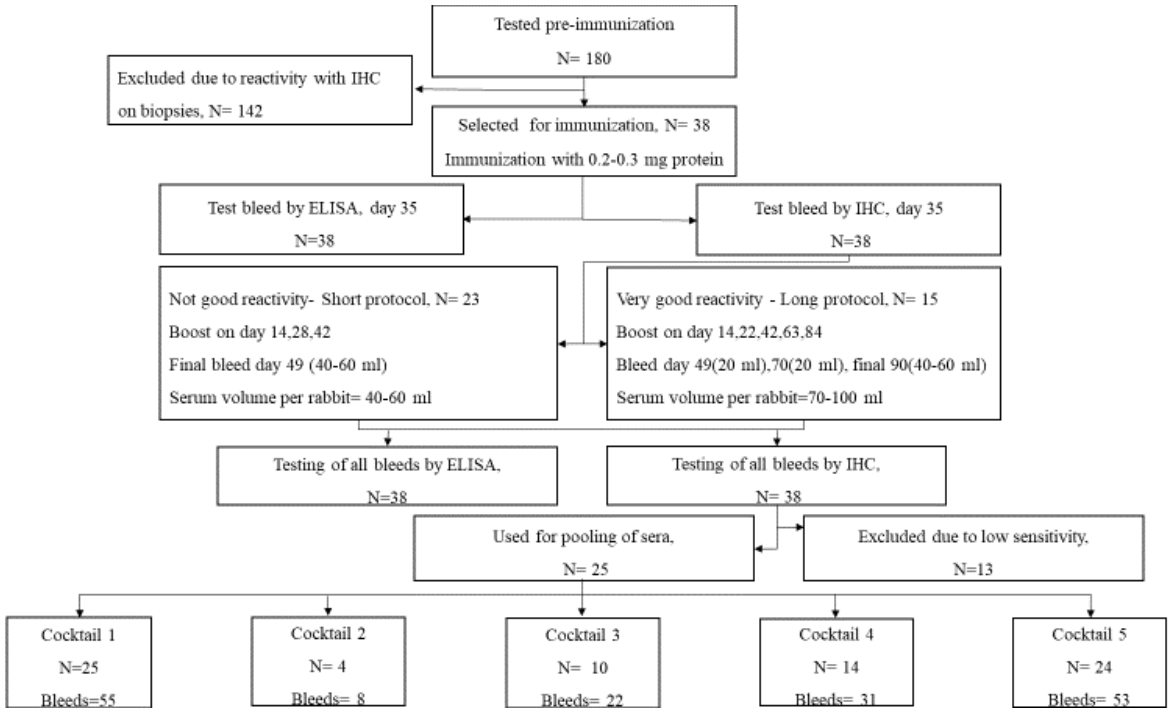

Figure 4. Immunization strategy and pooling of sera to make a functional polyclonal anti-MPT64 antibody.

FOOTNOTE. N, number; IHC, immunohistochemistry; ELISA, enzyme-linked immunosorbent assay.

Figure 4 shows the immunization plan. To minimize the non-specific reactivity, we adopted a strategy of testing the sera from several rabbits before immunization and selection of those rabbits with minimal or no reactivity by IHC. Among the 180 rabbits tested before immunization, only 38 animals were selected for immunization. Each immunization was done with about 50 μg protein per kg per animal. Injections were carried out intradermally. Boosters were given at day 14, 28, and the test bleed was done at day 35 to confirm the specific reactivity of mycobacterial antigen with ELISA and IHC. The sera from 15 rabbits gave a very good specific reactivity with IHC. These rabbits were selected for a long immunization protocol to harvest larger serum volume. In this protocol, the booster was given at day 42, 63, and 84. At day 49 and 70 bleeds were performed to harvest 20 ml serum at each time point. The final bleed was done at day 90. In the rest 23 rabbits, the specific reactivity of test bleed was not very strong, and the immunization protocol was shortened to the booster on day 42 and final bleed at day 49. All the bleeds from each animal were tested again with ELISA and IHC. All sera were preserved by 0.02% Thimerosal and 15mM

sodium azide. IHC was performed on a known TB and non-TB formalin-fixed paraffin-embedded biopsies. Sera from 13 rabbits were excluded due to unsatisfactory reactivity with IHC. Different dilutions were tried for each bleed and an optimal dilution was selected for each bleed (Table 2). The sensitivity varied among different bleeds. Further, five different cocktails were tried to make a single batch in a large volume. In cocktail 1, the sera from 55 bleeds of all 25 rabbits were mixed. Cocktail 2 was made by mixing the sera from only 8 bleeds of 4 rabbits that showed the best sensitivity and specificity on individual IHC testing. Cocktail 3 was made by combining the sera from 22 bleeds of 10 rabbits, that showed good sensitivity and specificity on individual IHC testing. In cocktail 4, the sera from 31 bleeds of 14 rabbits that gave good sensitivity, but relatively lower specificity were mixed. Cocktail 5 was made by mixing the sera from cocktail 3 and 4. All these cocktails were further tested with serial dilutions (Table 2).

3.4. Enzyme-linked immunosorbent assay

Test bleeds and final bleeds were tested with ELISA. Plates were coated with the recombinant antigen by overnight agitation of antigen in 0.05M carbonate buffer, pH 9.5 (100 µl/well) at room temperature. For blocking, wells were emptied and then incubated on a shaker with TBS containing 1% fetal calf serum (200 µl/well) for 30 minutes at room temperature. Wells were washed with TBS-TW (washing buffer tris) (300 µl/well). Different dilutions of pre-immune sera and antisera were made in TBS-TW with 1% fetal calf serum. Dilutions were added in duplicate per well (100 µl/well) and incubated in a shaker for one hour at room temperature. TBS-TW with 1% fetal calf serum was used as blank. Wells were washed four times with TBS-TW (250 µl/well). Then 100 µl/well anti-rabbit -1gG-POD (SigmaA4914), 1:20.000 diluted in TBS-TW, were incubated on a shaker for one hour at room temperature. Wells were washed again four times with TBS-TW. After that, wells were given 3,3', 5,5'- tetramethylbenzidine ready to use substrate from Kem-En-Tac Diagnostic (100 µl/well). The enzymatic reaction was stopped after 15 minutes by adding 0.5M Sulfuric acid (100 µl/well). The absorption of the yellow solution was measured using a 12-channel photometer at 450 nm (reference wavelength 630 nm)

3.5. Strategies to reduce the non-specific staining with immunohistochemistry

One major challenge we faced was the non-specific staining (NSS). Several strategies were used to reduce the NSS. A known TB biopsy and a non-TB biopsy were used to evaluate the results.

First, antigen affinity purification of pAbs was tried to improve the specificity. The recombinant MPT64 antigen was coupled with Sepharose beads and these were utilized in column chromatography to bind the anti-MPT64 pAbs. Other unwanted serum proteins and immunoglobulins were washed away. MPT64 bound pAbs were released from the column by using the acidic solution.

Secondly, sera with pAbs were absorbed overnight with the BCG Copenhagen vaccine strain which lacks the MPT64 gene to remove cross-reactive antibodies to other mycobacterial antigens than MPT64 as done previously (4). BCG sonicate was added to the undiluted sera with pAbs in 1:5 ratio, vortexed gently, and left to stand at 4 degrees Celsius for a day.

Thirdly, the sera with pAbs were absorbed with the formalin-fixed human lung and lymph nodes biopsy material. These tissues were selected because of the reactivity of the sera with pAbs with some structures in these biopsies. Non-TB lung and lymph nodes formalin-fixed, and paraffin-embedded biopsies were selected. About 10-µm thick sections were prepared from each biopsy and six sections from each biopsy were placed in a tube. These biopsies were hydrated by passing through xylene. The tissue sections were submerged in enough xylene, and vortexed to make sure that xylene was mixed with tissue sections. After ten minutes the tube was centrifuged, and the supernatant was removed. This step was repeated. Enough amount of series of declining concentration of alcohol (two times 100%, one-time 96% and 80%) was added to merge the tissue completely and then the tube was vortexed to make sure that alcohol was mixed with tissues. After three minutes, the tube was centrifuged, and the supernatant removed. The same step was repeated with distal water. The tissues were mixed with 1 ml of phosphate-buffered saline and lysed by using lysing matrix tube A (MPbio.com) which contains a garnet matrix and ¼ ceramic sphere and homogenized for 40 seconds by using Fastprep-24-5G homogenizer. This mix of beads and lysed tissue were added in the working dilution of antibodies in 1:1 ratio, vortexed, then incubated overnight in the refrigerator. The mixture was centrifuged, and the supernatant was used as the primary antibody.

Fourthly, sections on the glass slide were incubated with different protein solutions before the addition of primary antibody such that these proteins will bind to the non-specific sites in the sample. Blocking with serum-free protein block (Agilent/Dako, Glostrup, Denmark), 3% bovine serum albumin (Sigma-Aldrich, Søborg, Denmark) and 10% normal goat serum (Agilent/Dako,

Glostrup, Denmark) were tried for different times (12 minutes, 60 minutes and overnight), and in various combinations.

Fifthly, a human Fc block pure (Becton Dickinson, NJ, USA), which is a purified recombinant protein derived from immunoglobulin that reduces the binding of antibodies to Fc receptors, was tried in different dilutions. Dilutions were made by using the antibody diluent (Agilent/Dako, Glostrup, Denmark). Sections were incubated with this solution for 30 minutes after they were treated with a serum-free solution for two different durations of overnight and 15 minutes. Then the solution was taped off and the primary antibody was applied.

Finally, we also tried diluting the sera with pAbs in the sodium thiocyanate solution, and betaine (Sigma-Aldrich, Søborg, Denmark) to prevent the weaker binding of pAbs. Different dilutions of sodium thiocyanate and betaine were made by adding the defined volume of these solutions in the antibody diluent. The primary antibody was diluted in these mixtures of betaine/sodium thiocyanate and the antibody diluent.

3.6. Immunohistochemistry

Parallel, 4 µm thick sections were prepared from formalin-fixed, paraffin-embedded biopsy and cell blocks by using a sliding microtome, Leica RM 2155 microtome. The sections were placed on Superfrost Plus™ adhesion microscope slides and incubated at 58°C in hot air oven for overnight. The slides were stored at 4°C.

The detection of MPT64 antigen in the tissue sections was done by using IHC. This technique is based on the principle of antibody binding to a specific antigen. IHC can detect antigens within tissues and thus provide important information about location in addition to the identification of the Antigen. IHC was performed using the En Vision + System-HRP (Agilent/Dako, Glostrup, Denmark). The sections were deparaffinized using xylene and then treated in a series of declining concentration of alcohol to remove xylene. Then the sections were placed in distilled water for rehydration. The sections were placed in a microwave for antigen retrieval using tris EDTA buffer, pH 9 (Agilent/Dako, Glostrup, Denmark) for 20 minutes. The purpose of the process of antigen retrieval is to help to expose the epitope, which may be hidden during formalin fixation of the tissues. The sections were left to cool for 20 minutes at room temperature. Tissue sections were washed using TBS, pH 7.6 (Agilent/Dako, Glostrup, Denmark). Then pen (Agilent/Dako,

Glostrup, Denmark) was used to encircle the tissues region such that the reagent will be kept within the prescribed area. To inhibit the endogenous peroxidase activity and thereby reducing the non-specific background staining, the sections were then incubated with hydrogen peroxide (Agilent/Dako, Glostrup, Denmark) for 20 minutes. The endogenous peroxidase will otherwise react with substrate (chromogen) leading to false-positive staining. After that, the sections were washed with TBS, they were incubated overnight with a combination of 3% bovine serum albumin and 10% normal goat serum followed by serum-free protein block for 12 minutes. This was done to prevent the non-specific binding of the antibody to the tissues. Primary pAbs in 1:400 dilution (new anti-MPT64 pAb) and 1:250 dilution (previous anti-MPT64 pAb) were applied to the sections for 60 minutes. The sections were again washed with TBS and incubated with secondary antibody, labelled polymer HRP anti-rabbit (Agilent/Dako, Glostrup, Denmark) for 40 minutes. The sections were washed with TBS, and then 3-amino-9-ethylcarbazole containing hydrogen peroxide substrate (Agilent/Dako, Glostrup, Denmark) was applied for 15 minutes to visualize bound antibody. On the positive tissue sample, 3-amino-9-ethylcarbazole gave rust-red colour. In the end, the background was counter-stained with Mayer's hematoxylin for 1 minute (Agilent/Dako, Glostrup, Denmark), and the slides were mounted in Immu-Mount (Agilent/Dako, Glostrup, Denmark).

IHC staining was controlled by using two negative and one positive controls. In one negative control, the primary antibody was substituted with antibody diluent. The second negative control was non-TB tissue. The positive control was previous in-house anti-MPT64 pAb in 1:250 dilution.

3.7. Evaluation of immunostaining

Staining for mycobacterial antigen MPT64 was evaluated with light microscopy. Staining with the previous anti-MPT64 pAb was used as a comparison for intensity and pattern of staining. Criteria for selection of positive signals were based on localization of MPT64 antigen signal intracellularly in macrophages, epithelioid cells, or giant cells of granulomatous lesions or extracellularly in necrotic areas. The intensity of IHC staining of sections was evaluated based on the researcher's subjective assessment and categorized as either weak, moderate, or strong. Criteria for NSS were based on the staining of normal-looking structures in biopsies and irrelevant cells in the cell blocks.

3.8. Clinical samples for validation

Human clinical biopsies were used for the validation of the new anti-MPT64 pAb. Formalin-fixed paraffin-embedded biopsies from lymph nodes and cell blocks made from pleural effusions were used. These materials were collected as part of another project where the clinical samples were obtained from a cohort of EPTB patients. Patients of all ages with presumptive TB attending Gulab Devi Hospital outpatient clinics for enlarged lymph nodes or pleural effusion were interviewed. Patients with no history of TB treatment were enrolled (after providing informed consent) from April 2016 to August 2017. Patients were excluded if they had a history of TB treatment during the previous year. For patients with pleural effusions, fluids were aspirated using a 50 ml syringe. Excision biopsy was performed for patients with enlarged lymph nodes. Immediately, the biopsy specimen was divided into two. One half of the biopsy was placed in 10 ml of physiologic (0.9%) saline for bacteriology. The other half of the biopsy was placed in buffered formalin for histology examination. The biopsy specimen in physiologic saline was minced in a manual sterile tissue homogenizer (15 ml; 19 × 155 mm) and then transferred to a sterile 50 ml centrifuge tube. The pleural fluid specimen (35–45 ml) was transferred directly to a centrifuge tube. Then, specimens were centrifuged (3000 × g for 15 min) without decontamination and the sediment was used for smear, GeneXpert (Cepheid, Sunnyvale, CA, USA), and culture. Auramine O-stained smears were examined using a light emitting diode fluorescence microscope (83). Two slopes of Löwenstein-Jensen medium and one Mycobacteria Growth Indicator Tube (MGITe 960e; Becton Dickinson, Sparks, MD, USA) were inoculated for culture (84). GeneXpert was performed according to manufacturer protocols (85).

For patients with pleural effusions, cell blocks were prepared. The sample was centrifuged within one hour after aspiration. The supernatant was discarded, and the deposit was mixed with 10% formalin. The quantity of formalin was 10 times the quantity of deposit. The mixture was incubated for overnight fixation. After that, the sample was centrifuged. The supernatant was discarded, and the deposit was placed on filter paper. The filter paper was then placed in a cassette. Paraffin blocks were prepared in the histopathology laboratory.

3.9. Ethical clearance

The study protocol was approved by the Regional Committee for Medical and Health Research Ethics, Western-Norway (REK Vest), and the National Bioethics Committee of Pakistan (Islamabad, Pakistan).

3.10. Data management and statistical Analysis

The data was entered into Microsoft Excel and descriptive statistics were applied. To compare the differences in categorical variables, the Chi-square test was used. P-value < 0.05 was considered statistically significant. A two by two table was used to calculate Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy by using the bacteriological confirmation by culture and/or GeneXpert (Cepheid, Sunnyvale, CA, USA) as a reference method.

4. Results

4.1. Testing of individual sera with enzyme-linked immunosorbent assay

Sera from all bleeds gave very good results with ELISA. The titer was mostly around 1:200.000, which was the highest titer measured. As shown in Table 2, the titer results are homogenous, with only one exception that is not shown.

4.2. Testing of individual sera with immunohistochemistry

The sensitivity of pAbs with IHC was not as good as in ELISA. Most of the sera gave reactivity with non-specific targets in biopsies like fibrous tissue or smooth muscles, etc. This made it difficult to interpret the positive results. By dilution of the sera, this challenge was overcome to a large extent, and it was possible to differentiate specific staining (SS) from the NSS (Table 2). However, sera from 13 rabbits lost the SS as well with dilutions and were excluded from further study.

Table 2. The result of the individual sera from all bleeds of 25 rabbits tested with immunohistochemistry with different dilutions on TB biopsies and their ELISA titer.

		IHC		
Immunization protocol, bleeds per rabbit	**ELISA titer**	**Tried dilutions**	**Results**	**Working dilutions**
		500	Strong SS &weak NSS	
Short, 1	1: 200000	1000	Weak SS & no NSS	500
		500	Strong SS & weak NSS	
Short, 1	1: 200000	1000, 2000	No staining	500
		500	Strong SS & weak NSS	
		600	Strong SS & no NSS	
		1000	Weak SS & no NSS	
Short, 1	1: 200000	2000	NO staining	600
		500	Strong SS & weak NSS	
Short, 1	1: 200000	600	No staining	500
		1000	Strong SS & strong NSS	
		2000	Weak SS & no NSS	
Short,1	> 1: 200000	4000	No staining	No optimal
		1000	Strong SS & moderate NSS	
Short, 1	> 1: 200000	2000	Weak SS & weak NSS	No optimal
Short, 1	1: 200000	1000	Weak SS & strong NSS	No optimal
		1000	Strong SS &moderate NSS	
Short, 1	> 1: 200000	2000	Weak SS & no NSS	1000
		1000	Strong SS & strong NSS	
Short, 1	> 1: 200000	2000	Weak SS & moderate NSS	No optimal
Short, 1	> 1: 200000	1000	Moderate SS & Strong NSS	No optimal
Long, 3	1:200000	500	B1: Strong SS & weak NSS	B1: 500
		500 &1000	B1,2&3: Strong SS & moderate NSS	
		2000	B1,2&3: Moderate SS& no NSS	B1: 2000
			B1: No staining	
Long, 3	1: 200000	4000	B2 &B3: Strong SS & no NSS	B2&3: 4000
		500	B1,2&3: Strong SS & moderate NSS	
		600	B1,2 & 3: Moderate SS & moderate NSS	
Long, 3	1: 200000	1000 & 2000	B1,2 & 3: No staining	No optimal
		500	B1: Strong SS & strong NSS	
Long, 3	1: 200000	600	B1: Weak SS & Strong NSS	No optimal
		500	B1: Strong SS & no NSS	
Long, 3	1: 200000	600	B1: Weak SS & no NSS	B1: 500
		500 & 600	B1,2 & 3: Strong SS & moderate NSS	
Long, 3	1: 200000	1000	B1,2 & 3: Weak SS & moderate NSS	No optimal
		500	B1: Strong SS & NSS	B1: No optimal
			B1: Moderate SS & moderate NSS	
		600	B2 &3:Strong SS & weak NSS	
Long, 3	1: 200000	1000 & 2000	B1,2&3: Weak SS & weak NSS	B2 &3: 600
			B1 & 2: Weak SS & weak NSS	
Long, 3	1: 200000	1000	B3: Moderate & strong NSS	B1, 2 & 3: No optimal
		500	B1,2 & 3: Strong SS & moderate NSS	
		1000	B1,2 & 3: Strong SS &no NSS	
Long, 3	> 1: 200000	2000	B1,2 & 3: Weak SS & no NSS	B1,2 & 3: 1000
			B1: Strong SS &weak NSS	B1 &3: 200
			B2: Weak SS & weak NSS	
		200	B3: Moderate SS & weak NSS	
			B1: Moderate SS &no NSS	
			B2: Weak SS & NSS	
		500	B3: Weak SS & no NSS	
			B1: Weak SS & no NSS	
			B2: No staining	
Long, 3	> 1: 200000	1000	B3: Weak SS &no NSS	B2: No optimal
Long, 3	>1: 200 000	1000 & 2000	B1,2 &3: Strong SS & no NSS	B1,2&3: 2000
			B1: No staining	B1: No optimal
Long, 3	>1: 200 000	1000	B2 & 3: Moderate SS & no NSS	B2 & 3: 1000
		1000	B1,2 & 3: Strong SS & Strong NSS	
Long, 3	>1: 200 000	2000	B1,2 & 3: Strong SS & moderate NSS	No optimal
			B1: Strong SS & moderate NSS	B1&3: No optimal
			B2: Moderate SS & no NSS	
Long, 3	>1: 200 000	1000	B3: Strong SS & Strong NSS	B2:1000
		500 & 600	B1,2 &3: Strong SS &no NSS	
			B1 & 3: No staining	
		1000	B2: Weak SS & no NSS	
Long, 3	1:200 000	2000	B1,2 & 3: No staining	B1,2&3: 600

FOOTNOTE. ELISA, enzyme-linked immunosorbent assay; IHC, immunohistochemistry; SS, specific staining; NSS, non-specific staining; B, bleed.

4.3. Testing of cocktails with immunohistochemistry

Based on the performance of the individual sera on the biopsies, a strategy was designed to pool the sera from various rabbits and bleeds. Table 3 shows the results from various cocktails. Cocktail 3 gave the best sensitivity and specificity and was selected as the functional pAb. Figure 5 shows the staining with cocktail 3. Cocktails 1, 4, and 5 gave NSS and were not further validated on the clinical samples. Cocktail 2 gave very good results but was not validated further due to the lower amount of the sera, keeping in mind the availability of the pAbs for large-scale use.

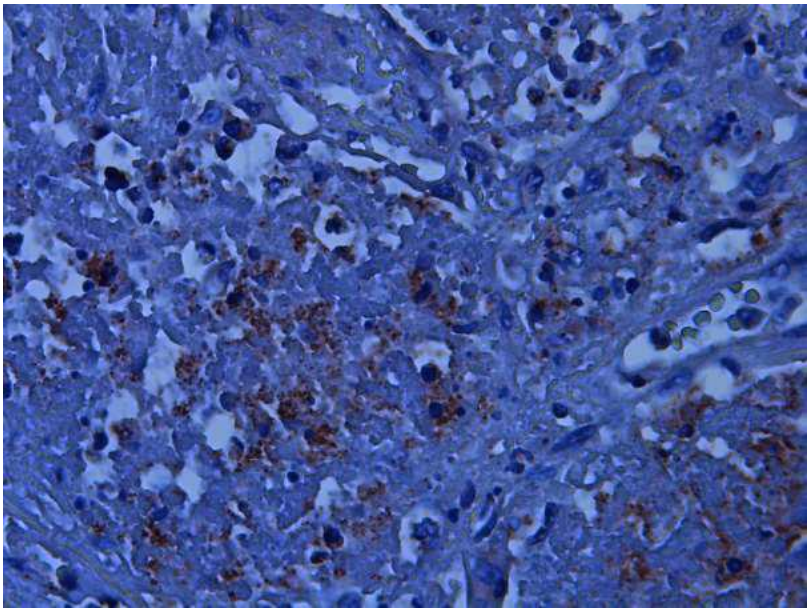

Figure 5. Immunohistochemistry staining of anti-MPT64 polyclonal antibodies (cocktail 3) in 1:400 dilution on lung biopsy with tuberculosis pneumonia. Signals are seen as reddish-brown granular staining against a blue hematoxylin background.

Table 3. Staining results with polyclonal antibody cocktails with immunohistochemistry on biopsies using different dilutions.

Dilutions	Result		Conclusion
	Positive control (lung TB biopsy)	Negative control (non-TB lymph node biopsy)	
Cocktail 1			
5, 10, 20, 40, 50,80 &100	Strong NSS	Strong staining	
200	Moderate SS & strong NSS	Strong staining	
400	Weak SS & moderate NSS	Moderate staining	
800	No SS & weak NSS	Weak staining	
1600	No staining	No staining	No optimal dilution
Cocktail 2			
50	Strong NSS	Strong staining	
100	Moderate SS & moderate NSS	Moderate staining	
200	Moderate SS & weak NSS	Strong staining	
400	Strong SS & no NSS	Weak staining	Working dilution: 400
Cocktail 3			
50 & 100	Strong NSS	Strong staining	
200	Strong SS & moderate NSS	Moderate staining	
400	Strong SS & weak NSS	Weak staining	
800	Weak SS & no NSS	No staining	
1600	No staining	No staining	Working dilution:400
Cocktail 4			
50, 100 & 200	Strong NSS	Strong staining	
400	Weak SS & Strong NSS	Strong staining	
800	Weak SS & moderate NSS	Moderate staining	
1600	No SS & weak NSS	Weak staining	No optimal dilution
Cocktail 5			
C3 400 + C4 800	Moderate SS & strong NSS	Strong staining	
C3 800 + C4 1600	Weak SS & NSS moderate	Moderate staining	No optimal dilution

FOOTNOTE. SS, specific staining; NSS, non-specific staining; C3 400, cocktail 3 in 1:400 dilution; C3 800, cocktail 3 in 1:800 dilution; C4 800, cocktail 4 in 1:800 dilution; C4 1600, cocktail 4 in 1: 1600 dilution

4.4. Strategies to reduce non-specific staining with immunohistochemistry

Table 4 shows the results of various strategies used to reduce the non-specific binding of pAbs. A positive TB biopsy and a non-TB biopsy were used for IHC. Incubation of biopsies with a combination of 3% bovine serum albumin and 10% normal goat serum by overnight incubation was the best strategy and it significantly reduced the NSS. Otherwise, all the other strategies did not work. The purification of pAbs with antigen affinity purification was not successful as it reduced the SS indicating that the epitopes in vivo in the formalin-fixed biopsies are different from the epitopes on the antigen used in the column, and the specific antibodies were lost during the process of purification. Even though the non-specific reactivity was with the various normal structures in the biopsies, the absorption of pAbs with non-TB lung and lymph node biopsies did not specifically reduce the NSS. There was an overall reduction in reactivity with both specific

and non-specific targets. Increasing the incubation time with serum-free protein block or using a pure Fc receptor blocker did not reduce the NSS either. Mixing the pAbs sera with sodium thiocyanate reduced both specific and non-specific reactivity. Mixing with betaine solution had no effect.

Table 4. Results of various strategies used to reduce the non-specific staining of polyclonal antibodies with immunohistochemistry.

Strategy	Results
PAbs + antigen affinity purification	Significantly reduced SS & NSS
PAbs + BCG Copenhagen vaccine strain	Moderate reduction of the intensity of SS, and no additional reduction of NSS
PAbs + formalin-fixed paraffin-embedded non-TB lung and lymph nodes biopsies	Moderate reduction of the intensity of SS, and no additional reduction of NSS
Sections + serum-free protein block for 12, 30 and 60 minutes, and overnight	SS unaffected, no reduction in NSS
Sections+ 60 minutes with 3% bovine serum albumin +10% normal goat serum, followed by 60 minutes serum-free protein block	SS unaffected, reduction in NSS
Sections + overnight 3% bovine serum albumin + 10% normal goat serum followed by 12 minutes serum-free protein block	SS unaffected, significant reduction in NSS
Sections + human Fc block pure	SS unaffected, no reduction in NSS
PAbs + betaine solution	No reduction in NSS
PAbs + sodium thiocyanate	Significantly reduced SS & NSS

FOOTNOTE. PAbs, polyclonal antibodies; BCG, Bacillus Calmette-Guérin; TB, tuberculosis; NSS, non-specific staining; SS, specific staining,

4.5. Validation of polyclonal antibodies on clinical samples

4.5.1. Patients characteristics

A total of 86 clinical samples were used for the diagnostic validation of pAbs. The bacteriologically confirmed TB cases with positive culture and/or Xpert were selected. Table 5 shows the characteristics of 45 patients from where the TB samples were collected. There were gender differences with a predominance of females among patients with lymphadenitis and males among pleuritis (p= 0.000). The median age was similar between the two groups. There was no statistically significant difference in systemic symptoms between the two groups. The majority (90%) of the lymph nodes biopsies had typical histological findings of necrotizing granulomatous inflammation consistent with TB. The majority (87%) of the pleuritis patients had minimal or

moderate amounts of effusion and only 13% had massive amounts. The appearance and the biochemistry of the pleural fluid were consistent with tuberculous effusion with unilateral effusion in 87%, the predominance of lymphocytes and high protein content >3g/dl.

Forty-one non-TB biopsies with various diagnoses including Reactive lymph nodes, Ovarian Papillary carcinomas, Fibroadenoma, Benign Prostatic hyperplasia, Leiomyoma, Hydrocele, Chronic tonsillitis, Corpus luteum, Myometrium-normal, Breast Carcinoma, were used as negative controls.

Table 5. Characteristics of patients with tuberculous lymphadenitis and tuberculous pleuritis.

Characteristics[1]	**TB Lymphadenitis**	**TB pleuritis**	**P-value**[5]
Age years median (range)	20 (5-44)	25 (15-70)	
Sex			0.000
Male n/N (%)	3/23 (13.0)	12/17 (70.6)	
Female n/N (%)	20/23 (87.0)	5/17 (29.4)	
Clinical symptoms n/N (%)			
Systemic[2]			0.964
Fever	13/14 (92.9)	15/15 (100)	
Weight loss	3/14 (21.4)	5/14 (35.7)	
Local[3]			
Lymph node swelling	14/14 (100)		
Pleural effusion		15/15 (100)	
Estimated amount of pleural fluid on CXR[4] **n/N (%)**			
Minimal		4/15 (26.7)	
Moderate		9/15(60.0)	
Massive		2/15 (13.3)	
Ultrasound n/N (%)			
Unilateral effusion		13/15 (86.7)	
Bilateral effusion		2/15 (13.3)	
Appearance pleural fluid n/N (%)			
Straw coloured		1/15 (6.7)	
Yellow-coloured		13/15 (86.7)	
Reddish		1/15 (6.7)	
WBC in pleural fluid			
Amount in mm^3 median (range)		310 (110-1820)	

Lymphocyte % median (range) **Protein (g/dl) median (range)**		100 (60-100) 6.8 (2.8-8.3)	
Histopathology n/N (%) Necrotizing Granulomatous Inflammation Lymphocytes & macrophages	18/20 (90,0)	NA 15/15 (100%)	

FOOTNOTE. TB, tuberculosis; n, number, N, Total number; %, percentage; NA, not available.
[1]Characteristics: Denominator is different as information was not complete for all cases.
[2]Fever was the most frequent systemic symptom.
[3]Local symptoms include symptoms from the site of disease.
[4]CXR= chest Xray.
[5]P-value= comparison between lymphadenitis and pleuritis.

4.5.2. Performance of new polyclonal antibody in comparison with the routine laboratory diagnostic tests, GeneXpert assay, and previous anti-MPT64 polyclonal antibody.

The results of all diagnostic procedures are shown in Table 6. Compared to the other tests, the new anti-MPT64 pAb test was positive in a higher proportion of specimens. The performance of the new anti-MPT64 pAb was similar to the previous anti-MPT64 pAb. In total, 86.6% of the TB cases were positive with the new anti-MPT64 pAb, compared to 6.6% with AFB microscopy, 76.2% with culture, and 60.2% with the GeneXpert. The proportion of AFB microscopy and GeneXpert positive cases were higher among lymphadenitis cases as compared to pleuritis, while the proportion of culture-positive cases was higher among pleuritis cases. Signals were detected in 8/41 (19.5%) non-TB cases tested by the new anti-MPT64 pAb.

Using culture-positive cases as the reference standard, the new anti-MPT64 pAb was compared with the GeneXpert assay. New anti-MPT64 pAb was positive in 88.2% of the cases as compared to 52.1% positivity for the GeneXpert, and 9.1% positivity for AFB microscopy.

Table 6. Results of different diagnostic tests in tuberculous lymphadenitis and pleuritis.

Samples	Number of specimens n/N (%) positive by				
	AFB Microscopy	LJ culture	GeneXpert	New anti-MPT64 pAb	Previous anti-MPT64 pAb
Bacteriologically confirmed TB cases					
Lymph node (n=24)	2/24 (8.3)	16/24 (66.7)	22/24 (91.7)	21/24 (87.5)	23/24 (95.8)
Pleural fluid (n=21)	1/21 (4.8)	18/21 (85.7)	6/21 (28.6)	18/21 (85.7)	14/21 (66.7)
Culture confirmed TB cases					
Lymph node (n=16)	2/16 (12.5)		14/16 (87.5)	14/16 (87.5)	16/16 (100.0)
Pleural fluid (n=18)	1/18 (5.6)		3/18 (16.7)	16/18 (88.9)	12/18 (66.7)
Non-TB cases* (n=41)	Not done	Not done	Not done	8/41(19.5)	Not done

FOOTNOTE. n, number; N,Total number; %, percentage; LJ, Lowenstein-Jensen; TB, tuberculosis; pAb, polyclonal antibody; AFB, acid-fast bacilli. * Non-TB cases included: Reactive lymph node, Ovarian Papillary carcinoma, Fibroadenoma, Benign Prostatic hyperplasia, Leiomyoma, Hydrocele, Chronic tonsillitis, Corpus leuteum, Myometrium-normal, Breast Carcinoma.

4.5.3. Staining pattern and comparison with previous anti-MPT64 polyclonal antibody

MPT64 antigen was stained reddish-brown within the cytoplasm of macrophages, epithelioid cells (Figure 6), and sometimes in the multinucleated giant cells or extracellularly in necrotic areas (Figure 7). Intracellular staining varied in pattern from diffuse to granular. In the cell blocks, the signals were present intracellularly in the cells morphologically consistent with macrophages (Figure 8). Compared to the previous anti-MPT64 pAb, the intensity of staining was less strong, and the NSS was more frequent (Table 7). However, it was possible to distinguish the specific signals from the NSS on the biopsies. The SS was localized in the TB lesions, and the intensity

was stronger, while the NSS was in the normal-looking structures where the TB lesions were not present.

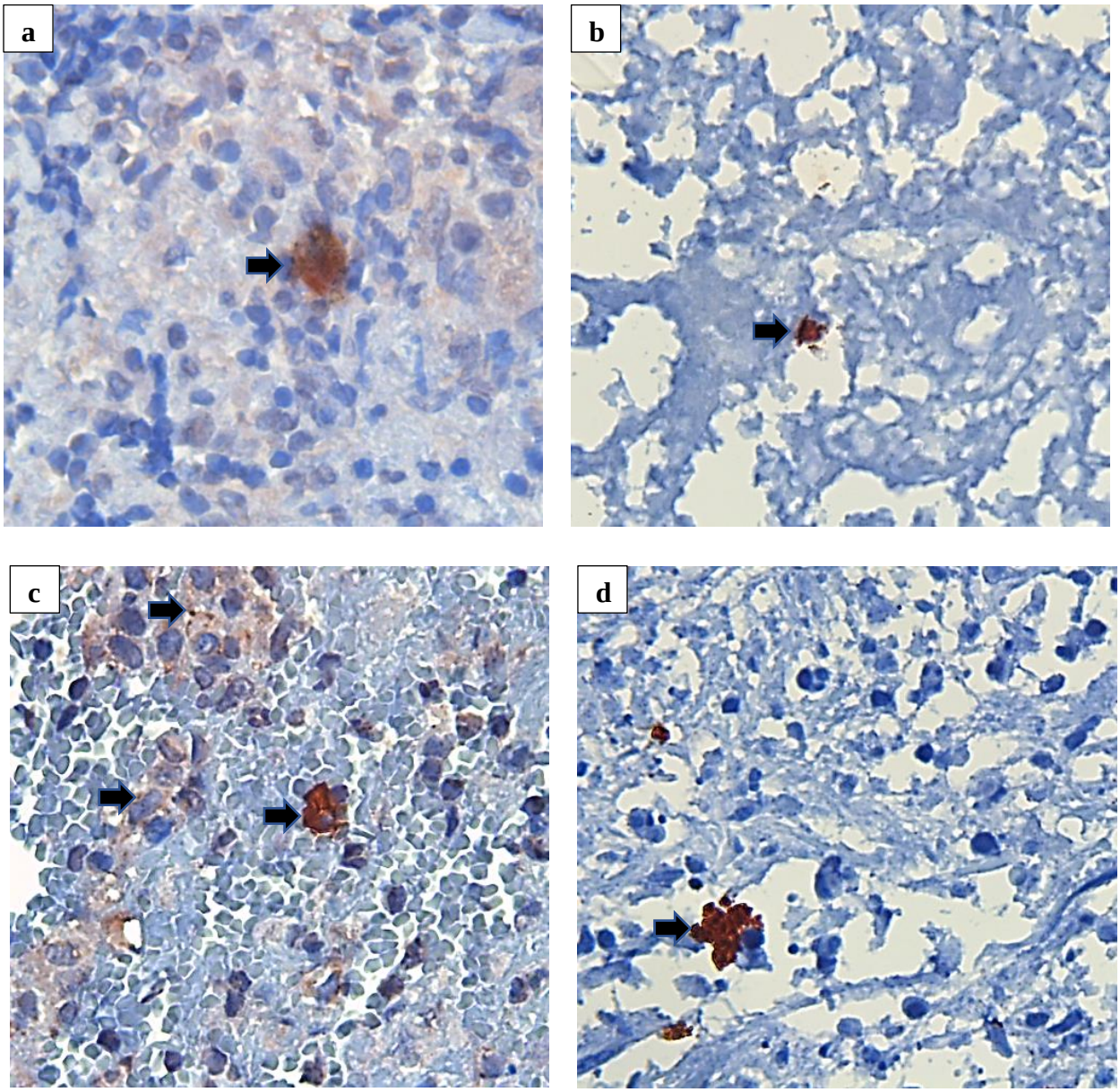

Figure 6. The staining pattern of new anti-MPT64 polyclonal antibody (a, c) and old anti-MPT64 polyclonal antibody (b, d) in tuberculous lymphadenitis from different lesions by immunohistochemistry. The positive signals are seen as reddish-brown granular staining (arrows) in the epithelioid cells of the lesions. Weak diffuse staining in a is non-specific background.

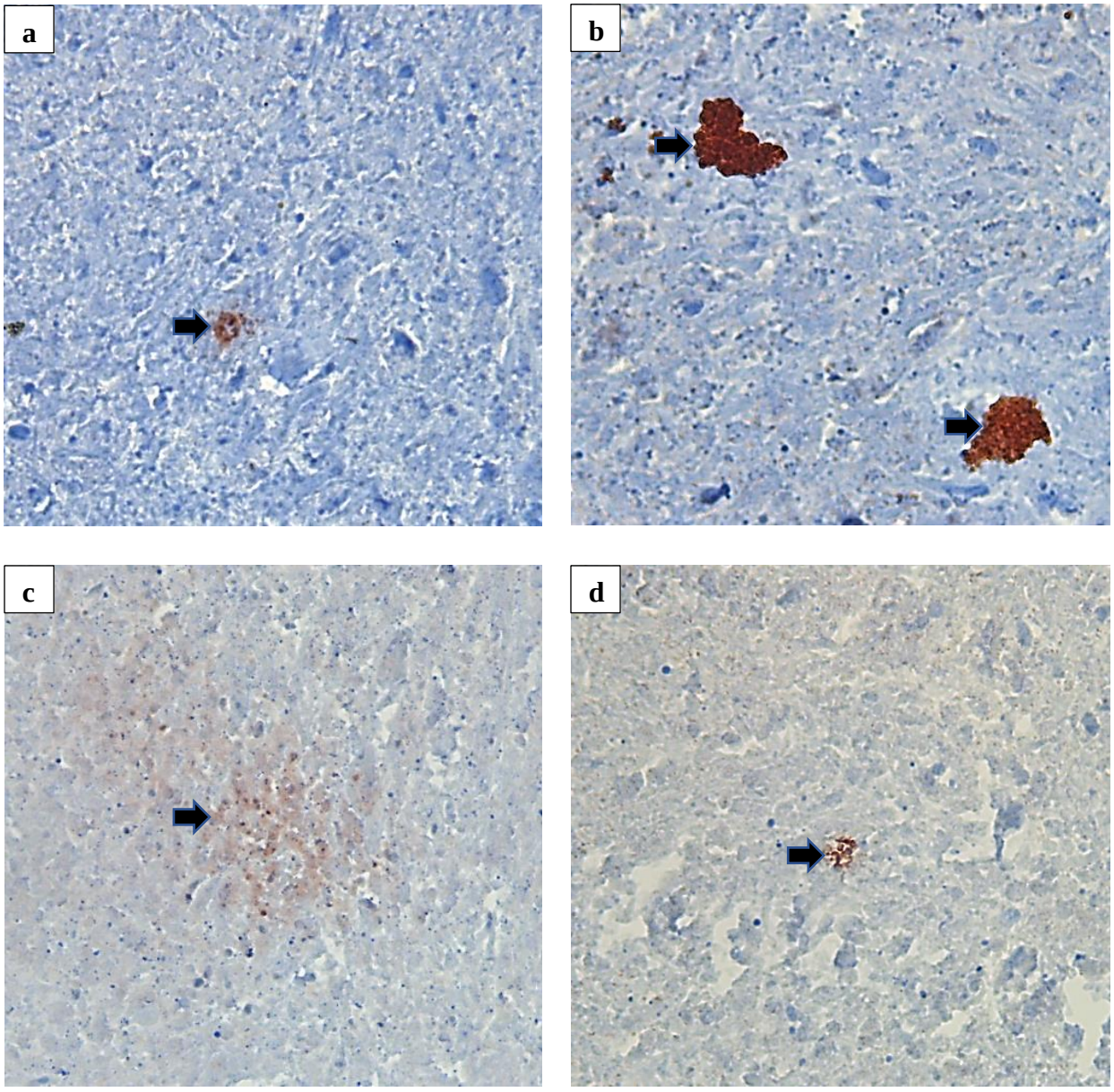

Figure 7. The staining pattern of new anti-MPT64 polyclonal antibody (a, c) and old anti-MPT64 polyclonal antibody (b, d) in tuberculous lymphadenitis from different lesions by immunohistochemistry. The positive signals are seen as reddish-brown granular (a, b, d) or diffuse (c)staining (arrows) in the necrotic area of the lesions.

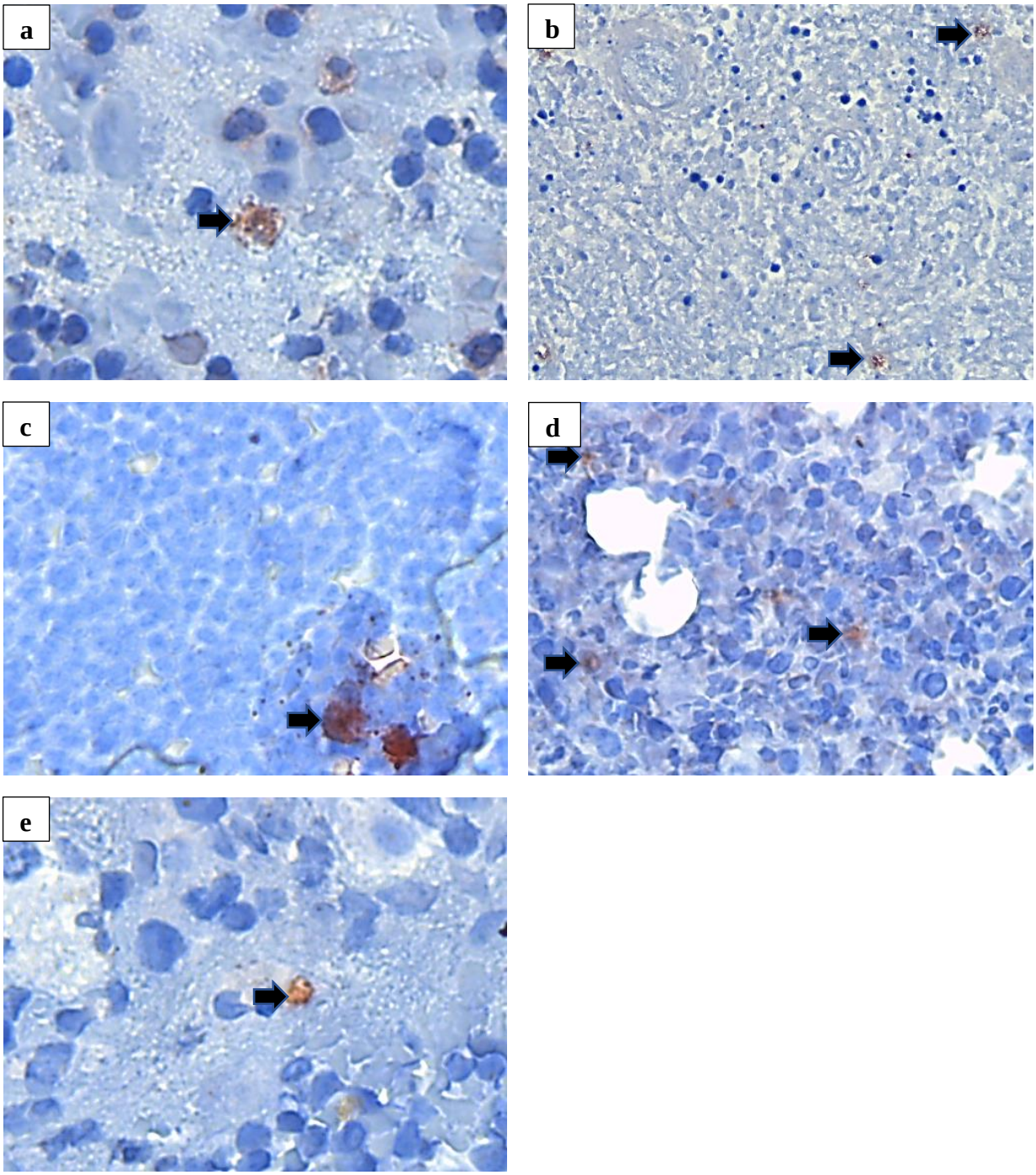

Figure 8. The staining pattern of new anti-MPT64 polyclonal antibody (a, c, e) and old anti-MPT64 polyclonal antibody (b, d) in tuberculous pleuritis from different cell blocks by immunohistochemistry. The positive signals are seen as intracellular reddish-brown granular staining (arrows).

Table 7. Comparison of intensity of staining by previous anti-MPT64 and new anti-MPT64 polyclonal antibodies by immunohistochemistry on tuberculous lymphadenitis and pleuritis specimens.

TB Cases	Number of positive cases according to intensity							
	Previous anti-MPT64 pAb				New anti-MPT64 pAb			
	None	Weak	Moderate	Strong	None	Weak	Moderate	Strong
Lymphadenitis	1	8	6	9	3	10	11	0
Pleuritis	7	3	5	6	3	15	3	0

FOOTNOTE. TB, tuberculosis; pAb, polyclonal antibody; None, no staining. Weak, moderate, and strong is based on the visual subjective criteria.

4.5.4. Diagnostic validation of the new MPT64 polyclonal antibody

The diagnostic validity of IHC with the new anti-MPT64 pAb, by using bacteriology confirmed (culture and/or GeneXpert) cases as a reference standard, showed the sensitivity of 88% and 86% for lymphadenitis and pleuritis cases, respectively with the corresponding specificity of 80% for both. Using the culture alone as the reference standard, the sensitivity of IHC with the new anti-MPT64 pAb was 88% and 89%, while for GeneXpert was 88% and 17% in lymphadenitis and pleuritis, respectively (Table 8). The performance of new anti-MPT64 pAb was significantly better than GeneXpert in the TB pleuritis patients. AFB microscopy has the lowest sensitivity of 13% and 6% in TB lymphadenitis and pleuritis, respectively.

Table 8. Diagnostic validity of immunohistochemistry with new anti-MPT64 polyclonal antibody and GeneXpert.

	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV %	NPV %	Accuracy
Bacteriological confirmation (culture and/or GeneXpert) as reference standard					
Lymphadenitis					
New anti-MPT64 pAb	88 (68-97)	80 (65-91)	72	92	83
Pleuritis					
New anti-MPT64 pAb	86 (64-97)	80 (65-91)	69	92	82
Culture as reference standard					
Lymphadenitis					
AFB microscopy	13 (2-38)				
GeneXpert	88 (62-98)				
New anti-MPT64 pAb	88 (62-98)	80 (65-91)	64	94	82
Pleuritis					
AFB microscopy	6 (0-27)				
GeneXpert	17 (4-41)				
New anti-MPT64 pAb	89 (65-99)	80(65-91)	67	94	83

FOOTNOTE. %, percentage; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; AFB, acid-fast bacilli; pAb, polyclonal antibody.

5. Discussion

In this study, we have shown a strategy to successfully reproduce a large amount of functional pAb against an MTB complex specific antigen MPT64 for application in IHC as a diagnostic test for EPTB. We have previously shown that IHC-based MPT64 antigen detection test is robust, quick, and more sensitive as compared to the routine microbiological diagnostic tests (1-8). The main aim of this study was to reproduce the primary antibody in a large amount for large-scale use of the test. The results indicate that the sensitivity of the test by using the new pAb is comparable

with the previous pAb. However, the specificity seems to be less. Due to the small sample size, the results cannot be generalized yet. These pAbs are being tested on a larger clinical sample in an ongoing project, and the results from these studies will give better evidence for the diagnostic validity of the test.

Previous studies have shown that this test is implementable in routine diagnostic settings in both high TB (3) and low TB (2) endemic settings. Furthermore, the technology of IHC is robust and widely available in most of the laboratories. AFB microscopy is quick and cheap, but it can only detect intact bacilli and it has very low sensitivity. IHC has an advantage over AFB microscopy that it can detect MTB antigen in fragmented bacilli as well, and it has thereby higher sensitivity. IHC has the potential to offer considerable improvement in the diagnosis of the most usually paucibacillary EPTB. Although culture is a gold standard for the definite diagnosis of EPTB, it is time- consuming and requires advanced laboratory facilities. IHC, on the other hand, is quick and has higher sensitivity. Both IHC and GeneXpert have high sensitivity and can give a result within a day or two. However, IHC has an advantage over GeneXpert in the sense that IHC is robust, probably cheaper, and does not require high tech equipment. One limitation of IHC is that it needs a trained pathologist for interpretation. Besides, GeneXpert sensitivity is variable in the various form of EPTB and is not recommended for all forms of EPTB (57-59).

Another antigen detection test in TB is LAM detection in urine (62). This test is only applicable for the HIV positive pulmonary TB patients with a high load of antigen that is excreted in urine (61, 62, 65). This test is not applicable for other forms of TB with relatively low antigen load, particularly EPTB which is most often a paucibacillary disease with low antigen load in the circulation. On the contrary, the MPT64 test is applicable to a variety of extrapulmonary tissues. This high sensitivity of the MPT64 antigen detection test is probably due to the intracellular accumulation of antigen in the lesions (86). The relative high load of antigen at the site of infection as compared to the bacilli is the most plausible explanation for a remarkable high sensitivity of the test despite the paucibacillary nature of the disease.

The production of mAbs would have been the ideal due to their high specificity and much less challenge with background and cross-reactivity (87, 88). Moreover, mAbs have minimal batch-to-batch variation (88, 89). However, in our laboratory, efforts to produce a functional mAb for IHC application have not been successful. Our group was able to produce a mAb which works very

well in ELISA, but it does not give good results with IHC. This could be attributed to the loss of relevant clones during the selection process, as ELISA instead of IHC was used for the selection of the relevant clones. It was not feasible to have an IHC-based system of formalin-fixed material for selection of relevant clones. Therefore, we decided to produce functional pAb. There are several advantages of pAbs over mAbs. Compared to mAbs, the main benefits of pAbs are attributed to two essential inherent features: clonal and biophysical diversity (89). The benefit of polyclonality is the possibility for pAbs to heterogeneously bind to multiple and different antigenic epitopes, resulting in higher sensitivity in certain assays. Especially in IHC, the pAbs can be a better option as the effects of tissue fixation and processing on epitopes are unknown and could be highly variable (89). The property of multiepitope binding of pAbs makes the antigen recognition possible even if some of these epitopes are affected by changes in the antigen structure (89, 90). On the other hand, mAbs may find their epitope buried, resulting in low sensitivity in detecting their antigens. It has also been reported for IHC that pAbs result in higher sensitivity when the antigens to be detected have low quantities in the sample (91). The biophysical diversity of pAbs results in greater stability when environmental challenges may lead to inactivation, lability or precipitation of other forms of antibodies (92). The storage and dilution of pAbs are in general easier than mAbs, due to the variation in their biophysical characteristics, such as charge and hydrophobicity. On the other hand, mAbs usually needs the addition of stabilizing agents to avoid aggregation, precipitation, and maintain antibody binding (92). Compared to mAbs, pAbs are relatively more resistant to changes in temperature and pH. MAbs usually need stabilizers to preserve their biological activity when they are exposed to environmental changes (93). To overcome the issue of the batch-to-batch variation from different animals, we used the strategy of pooling the sera from several animals to make a cocktail in a large volume. This strategy seems to work, and the cocktail of various antisera is stable at 4°C preserved by 0.02% Thimerosal and 15mM sodium azide.

We chose rabbits for immunization as they were most easily and widely available due to their suitable size, easy to deal with and to collect blood samples, and have long life span. Besides, they are good source of sufficient production of high-titer antiserum with high-affinity (87).

When the pAbs were tested by ELISA, they gave higher titer than when they later been tested with IHC. This can be explained by the adverse effects of formalin fixation of the tissue (89). Although

formalin fixation has many advantages, it can disrupt the 3-dimensional structures of antigen epitopes and weaken the ability of pAbs to detect the epitope. However, the polyclonality of pAbs resulting in high-avidity binding could increase the probability of antigen detection as compared to mAb (94, 95).

One of the challenges we faced with pAbs was the non-specific reactivity on the biopsies. This is inevitable due to the polyclonality and multiepitope binding of the pAbs which one hand gives higher sensitivity but oftentimes leads to high cross-reactivity with other proteins that have almost similar structures, giving non-specific background staining. In our study, we have succeeded to a large extent to overcome this negative aspect of background staining by saturating the non-specific binding sites on tissue sections with overnight incubation with bovine serum albumin and normal goat serum followed by saturation with serum-free protein block containing 0.25% casein in phosphate-buffered saline for 12 minutes. These solutions, prior to application of primary antibody, helped to reduce the non-specific binding of pAbs to the undesired sites in the tissues like connective tissue, epithelial tissue, muscles, and adipocytes by binding to them.

The negative controls in this study were selected from the archives. The criteria for selection was non-TB cases. These cases had not been tested for TB by culture or GeneXpert. We could not run these tests as material was not suitable for these tests. Therefore, we could not calculate the specificity of GeneXpert when culture was used as a reference standard. However, the probability that these negative controls would have been positive by any of these tests is very low. Another limitation of this study is that we have not tested the new anti-MPT64 pAb on non-TB cell blocks to have a direct comparison with the pleural fluid cell blocks. Furthermore, we have not tested the new anti-MPT64 pAb on the fine-needle aspirates or cell smears. The interpretation of positive signals on fine needle aspirates or cell smears can be challenging due to the non-specific background staining. In biopsies, the specific signals can be differentiated from the background staining by selecting the lesion areas where the antigen is expected to be present and ignoring the NSS in irrelevant areas of the biopsy. Further validation of these pAbs is ongoing on a larger material which will address these limitations.

In conclusion, we have shown that it is possible to reproduce pAbs which can detect the MPT64 antigen in the formalin-fixed paraffin-embedded tissue sections by IHC. It is possible to make a single batch in a large volume by making a cocktail of various sera. This opens up the possibility

of the large-scale use of this test, and its inclusion in the routine diagnostics of EPTB as it has higher sensitivity than the routine diagnostic tests.

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