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Rapid and Specific Diagnosis of Extrapulmonary Tuberculosis by Immunostaining of Tissues and Aspirates With Anti-MPT64

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Background: Extrapulmonary tuberculosis (EPTB) constitutes about 15% to 20% of all cases of tuberculosis (TB). The confirmation of EPTB has always been a challenge to laboratory personnel. We aim to evaluate the diagnostic potential of immunostaining with anti-MPT64 in various EPTB specimens.

Materials and Methods: We studied a total of 51 TB cases and 38 non-TB control specimens comprising of fine-needle aspirates and formalin-fixed biopsies. These were investigated using a combination of the Ziehl-Neelsen method, the Lowenstein-Jensen culture, immunostaining with anti-MPT64 and anti-BCG, and nested-polymerase chain reaction (PCR) for IS6110. Results of all the tests were compared using nested-PCR as the gold standard.

Results: Diagnostic validation of immunostaining for anti-MPT64 was performed using nested-PCR as the gold standard. The overall sensitivity, specificity, and positive and negative predictive values for immunostaining with anti-MPT64 were 100%, 97%, 97%, and 100%, respectively.

Conclusions: Immunostaining using anti-MPT64 is a rapid and sensitive method for establishing an early and specific diagnosis of *Mycobacterium tuberculosis* infection. The technique is simple to be incorporated into routine pathology laboratories.

Key Words: anti-MPT64, immunostaining, tuberculosis, diagnosis

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BACKGROUND

Extrapulmonary tuberculosis (EPTB) has been a major cause of suffering from time immemorial. Any organ system in the body can be affected by tuberculosis (TB). Molecular genetic evidence of infection with the *Mycobacterium tuberculosis* complex is available in Egyptians mummies in the form of Pott's spine.[1] EPTB constitutes about 15% to 20% of all cases of TB with a wide variability range (4% to 48%) among the reporting countries of notified TB cases.[2–4] Its annual global incidence has been increasing in the last decade due to the changing TB control practices, the population growth, and the spread of human immunodeficiency virus (HIV). The rate of EPTB is reported to be > 50% in patients with HIV coinfection.[5]

The diagnosis of EPTB has always been a problem for clinicians. As per WHO, the diagnosis of EPTB should be made on the basis of a culture-positive specimen or caseating granuloma on biopsy or strong clinical evidence consistent with active EPTB.[6] However, it is difficult to demonstrate *M. tuberculosis* with routine techniques such as culture and staining for acid-fast bacilli (AFB) due to the pauci-bacillary nature of EPTB. In 35% to 65% of the patients with EPTB, Ziehl-Neelsen (ZN) staining[7] or Lowenstein-Jensen (LJ) culture are negative. Also, the results of the tests assessing TB infection such as tuberculin skin test or interferon-gamma release assays may be misleading.[8] Secondly, routine cytology and histology cannot distinguish between the diseases caused by TB or non-TB mycobacterium, or chronic inflammatory conditions. TB, thus, remains undiagnosed in 20% cases even after multiple biopsies or aspirations.[9–11] Furthermore, EPTB is difficult to diagnose due to the extremely wide clinical presentation depending on the organ affected, the disease stage, and the immune response of the host. Most often, the possibility of EPTB is lately put into the differential diagnosis during the

evaluation of a patient. The EPTB diagnosis is thus most often made by the integration of several nonspecific results from various investigations. The need to perform invasive techniques for most of the EPTB cases to obtain specimens further complicates the diagnostic protocol of EPTB. In the past decade, newer techniques such as radiometric and nonradiometric assays together with improved molecular methods for identifying *M. tuberculosis* have an improved diagnostic capacity, but the use of these techniques in resource-constrained settings where TB remains an important public health problem is limited. The decision for an empirical course of antibiotic treatment in this situation, thus, often precedes evidence-based diagnosis.These problems highlight the need for an improvement in the diagnostic methods of EPTB.

We previously described the use of immunostaining to detect *M. tuberculosis* complex-specific mycobacterium antigen MPT64 from lymph node fine-needle aspirates and biopsies from tuberculous lymphadenitis, abdominal TB, and tuberculous pleural, ascitic, and cerebrospinal fluids.[12–14] Immunostaining using anti-MPT64 showed a high specificity (95%) and sensitivity (85%).[12,13] The technique is robust and yields consistent results in different settings and in HIV patients.[14,15] The aim of this study was to extend the previously published research by testing the diagnostic potential of immunostaining using anti-MPT64 on additional sites of EPTB, and extension of the method to the lesser invasive sampling procedure of fine-needle aspirates from different sites.

MATERIALS AND METHODS

Patients and Specimens

The study was performed at Ujjain Charitable Hospital, Ujjain, India, from July 2007 to March 2010. Fine-needle aspirate smears and surgical biopsies were collected from the Department of Pathology. Detailed clinical results were obtained from the patients or from the records. Surgical biopsies from non-TB (control) lesions were also obtained from the Department of Pathology, Haukeland University Hospital, Bergen, Norway. Two known pulmonary TB biopsy specimens from the archive with a high bacterial load were used as known positive controls. The diagnostic categories included are shown in Table 1. The collected specimens were processed for ZN stain, LJ culture (not for all biopsies), cytology or histology examination, immunostaining with anti-BCG and anti-MPT64, and nested-polymerase chain reaction (PCR) for IS6110. The final diagnosis of TB was established on the fulfillment of one of the following criteria: the presence of AFB on ZN stain and/or *M. tuberculosis* on culture and/or positive IS6110-based nested-PCR. Specimens were classified as non-TB when all the above-mentioned criteria were negative. Biopsies or aspirates from patients with pulmonary TB, patients below 14 years of age, and those on corticosteroid therapy or on immunosuppressive therapy were excluded from the study.

The decision for aspiration or biopsy was based on the clinical necessity and not for the sake of participation in the study. Informed written consent was obtained from the patients, and the patients were ensured of confidentiality. Ethical approval was obtained from the Institutional ethical committee at Ujjain Hospital and the regional ethical committees in both Norway and India. The performance indices (the sensitivity, the specificity, and the positive and negative predictive values) were calculated and compared on an accuracy matrix.

Fine-Needle Aspirate and Biopsy

Fine-needle aspiration from mass was performed under sterile condition using a 22-G needle. One part of the aspirate was sent for culture and the other part was used for preparing multiple smears. The slides were air dried, and stained with May-Grunwald-Giemsa stain for cytological examination, ZN staining, and 3 to 5 smears were ethanol fixed and used for PCR and immunostaining. On microscopic examination, the cytological findings were classified according to the amount of necrosis, the type of cells and their arrangement into necrotic granulomas, necrotic material showing mainly degenerated neutrophils, lymphocytes, and epithelioid cells, and nonnecrotic granulomas.[13]

The biopsies were fixed in 4% phosphate-buffered formaldehyde for conventional paraffin embedding, followed by hematoxylin-eosin and ZN staining by the heat carbol fuschin method. Subsequent sections were collected on 3 special capillary gap slides (Dako A/S, Denmark) for immunostaining and 5 to 6 thick sections in Eppendorf tubes for PCR. Contamination was prevented by cleaning the blade with 96% ethanol after sectioning each sample; negative controls were sectioned first, followed by test blocks and positive control blocks. The biopsy sections were examined for the presence and the type of epitheloid granulomas (well-organized and nonorganized) and the presence or the absence of necrosis.[12] A part of the specimen was sent for mycobacterial culture on LJ media before formalin fixation. Some biopsies were, however, not sent for culture.

Immunostaining

Immunostaining was performed using the Dako kit (EnVision + System-HRP; Dako, Glostrup, Denmark) on all the smears from the aspirates and the tissue sections to demonstrate the presence of mycobacterial antigens MPT64 and BCG as described in detail previously.[12,13] In brief, the alcohol-fixed aspirate smears were hydrated through decreasing grades of alcohol, and washed in phosphate-buffered saline.[13] For biopsy, tissue sections were first deparaffinized and hydrated; then, microwave antigen retrieval using citrate buffer, pH 6.2, at 750 W for 10 minutes, and at 350 W for 15 minutes was performed. The sections were cooled at room temperature for 20 minutes. All the slides (aspirate and biopsy) were then incubated with hydrogen peroxide for 5 minutes. to inhibit the endogenous peroxidase activity, followed by incubation with primary antibodies: (i) rabbit polyclonal anti-BCG (Dako, Hamburg, Germany) at 1/5000 dilution for 1 hour after treating the sections with 3% bovine serum albumin for 3 minutes; (ii) in-house rabbit polyclonal

TABLE 1. The Performance of Diagnostic Tests on Various Tuberculosis Specimens (N = 51)

Tuberculosis Specimens	No. Specimens Positive by			
	Lowenstein-Jensen Culture	Ziehl-Neelsen Stain	Staining With Anti-BCG	Staining With Anti-MPT64
Aspirate				
Pleural mass (n = 4)	2	0	4	4
Lung mass (n = 3)	1	0	3	3
Renal mass (n = 2)	NA*	0	2	2
Abdominal mass (n = 4)	0	0	4	4
Breast lump (n = 2)	1	0	2	2
Inguinal lymph node (n = 5)	2	0	5	5
Axillary lymph node (n = 5)	2	1	5	5
Ileocecal mass (n = 5)	0	0	5	5
Soft tissue mass (n = 3)	2	2	3	3
Chest wall abscess (n = 3)	1	1	3	3
Biopsy				
Epididymal mass (n = 2)	0	0	2	2
Renal (n = 2)	NA	0	2	2
Bone (n = 3)	NA	0	3	3
Breast (n = 2)	NA	0	2	2
Intestinal (n = 4)	NA	0	4	4
Axillary lymph node (n = 2)	1	1	2	2

*Results not available.
NA indicates not available.

absorbed anti-MPT64 antibody at 1/250 dilution for 1 hour. Anti-MPT64 was absorbed with an MPT64-non-producing BCG strain as detailed previously to remove cross-reactive antibodies.[15] The bound antibodies were recognized with a secondary antibody anti-rabbit dextran polymer conjugated to horseradish peroxidase for 45 minutes. Positive signals were visualized as reddish brown with a 3-amino-9-ethylcarbazol and hydrogen peroxide-containing substrate and counterstained with Harris hematoxylin for 1 minute; slides were then mounted in an aqueous immunomount (Sigma Chemical) for microscopy. All incubations were carried out at room temperature, and the sections were washed thoroughly with 0.05 M trisbuffered saline-tween 20 between incubations. In every experiment, 1 positive and 2 negative controls [in one the primary antibody was substituted with antibody diluents and in the other an irrelevant rabbit polyclonal antibody (anti-HCG) was used to evaluate the specificity of the reaction] were included. The intensity of staining was evaluated and categorized as weak, moderate, and strong staining on the basis of subjective assessment.

Nested-PCR Assay

The DNA extraction procedure was followed as described in detail previously for biopsy and aspirates.[16] For aspirates, in brief, with "wet-scratching," the smears were treated with 100 µL of proteinase K in tris-EDTA digestion buffer and the washing was collected in an eppendorf tube, which was treated with the slurry of acid-washed RNase-free zirconium-silica ceramic beads of 0.5 g of 0.1 mm diameter and then processed in the Hybaid Ribolyser shaker. The sample was incubated at 80°C for 20 minutes and centrifuged at 5000g for 2 minutes. The supernatant was used for PCR amplification or it was stored at −20°C until use. For biopsy, the deparaffinization, the digestion, and the extraction of DNA was performed as per the procedure described previously.[12,16]

From each specimen, the extracted DNA was amplified in triplicate by nested-PCR for a 123-base pair fragment from IS6110 in the first round of the reaction with the primer of coding sequence 5′CCTGCGAGCGTAGGCGTCGG3′ and 5′CTCGTCCAGCGCCGCTTCGG3′. The amplified product served as a template for a second round of PCR to amplify a 92-base pair fragment with the primers of the coding sequence 5′TTCGGACCACCAGCACCTAA3′ and 5′TCGGTGACAAAGGCCACGTA3′ as mentioned previously in detail.[12] The PCR mix consisted of 5 µL eluted DNA + 25 µL of HotStarTaq master mix (Qiagen, West Sussex, UK) + 0.25 µL of each 100 µM primer stock solution + distilled water to make a final volume of 50 µL. For nested-PCR, 1 µL of first PCR product was used as the template. Positive and negative controls and random sample retest were processed in all the PCR assays.

RESULTS

A total of 89 specimens (52 aspirates and 37 biopsies) with a confirmed diagnosis of either TB (n = 51) or non-TB (n = 38) were analyzed in the study (Table 1). Non-TB specimens were chronic nonspecific lymphadenitis (n = 29) biopsies showing fungal (n = 2) and parasitic granuloma (n = 2) and malignancy (n = 5).

AFB Microscopy, Culture, and PCR

AFB were detected by ZN staining in 05/51 (10%) specimens and *M. tuberculosis* was cultured in 12/40 (30%) TB specimens. PCR, ZN staining, and culture were negative in all non-TB specimens. Nested-PCR was performed for comparison and to calculate the diagnostic indices of immunostaining.

Microscopy

Biopsies revealed either well-organized and/or poorly organized granulomas, whereas the smears showed

various cytomorphologic changes such as granulomas without caseation necrosis, necrotizing granulomas having variable degrees of necrosis with a few well-organized epitheloid cells or giant cells, or tuberculous abscess such as a large amount of necrotic material with neutrophils, lymphocytes, and a few scattered epithelioid cells. Control specimens showed respective changes as described in Table 1.

## Immunostaining and Validation of the Test

Immunostaining with anti-MPT-64 was seen as reddish brown granular staining in the cytoplasm of mononuclear cells and multinucleated giant cells (Figs. 1, 2). In caseous necrotic areas of TB lesions, occasional but strong signals were detected. The staining pattern was predominantly granular with anti-MPT64, whereas it was predominantly diffuse with anti-BCG antibodies with a similar localization.

Results of various tests were compared between the TB and the non-TB specimens. Positive immunostaining with anti-MPT64 was seen in all TB specimens and in 1 control specimen, thus yielding a sensitivity of 100% and a specificity of 97%, whereas anti-BCG was positive in all TB specimens and in 6/38 (15·7%) control specimens with a sensitivity of 100% and a specificity of 84%. When a diagnostic validation of immunostaining was performed using PCR as the gold standard, the overall sensitivity, specificity, and positive and negative predictive values for immunostaining with anti-MPT64 were 100%, 97%, 98%, and 100%, respectively, and the corresponding values for anti-BCG were 100%, 84%, 88%, and 100%, respectively.
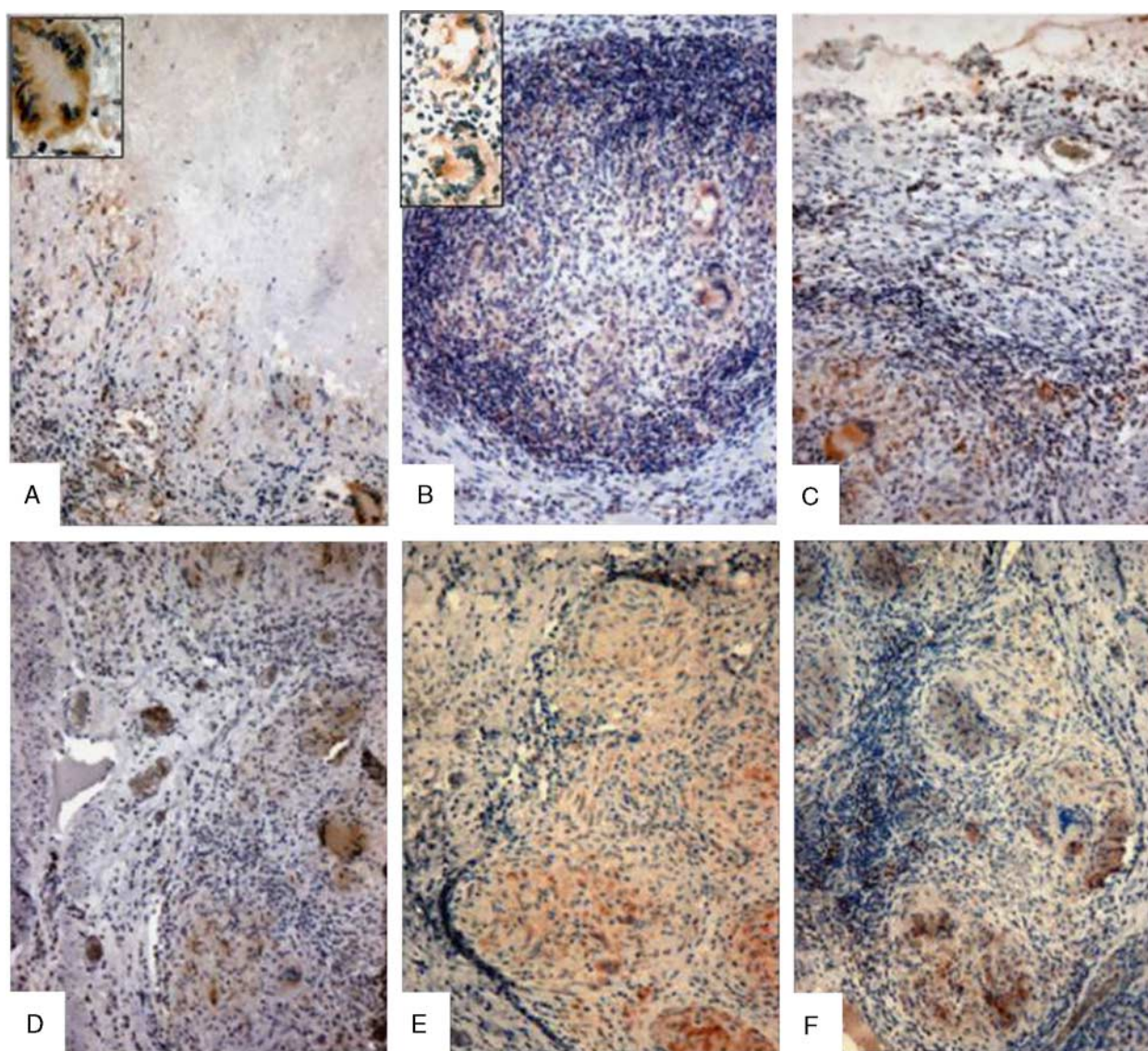
FIGURE 1. Immunohistochemical staining with anti-MPT64 in tuberculosis lesions from caseating granuloma in the axillary lymph node (A), noncaseating granuloma from the intestinal wall (B), tuberculous mastitis (C), renal tuberculosis (D), tuberculousepididymitis (E), and tuberculous osteomyelitis (F). Insets show staining in giant cells at a higher magnification.
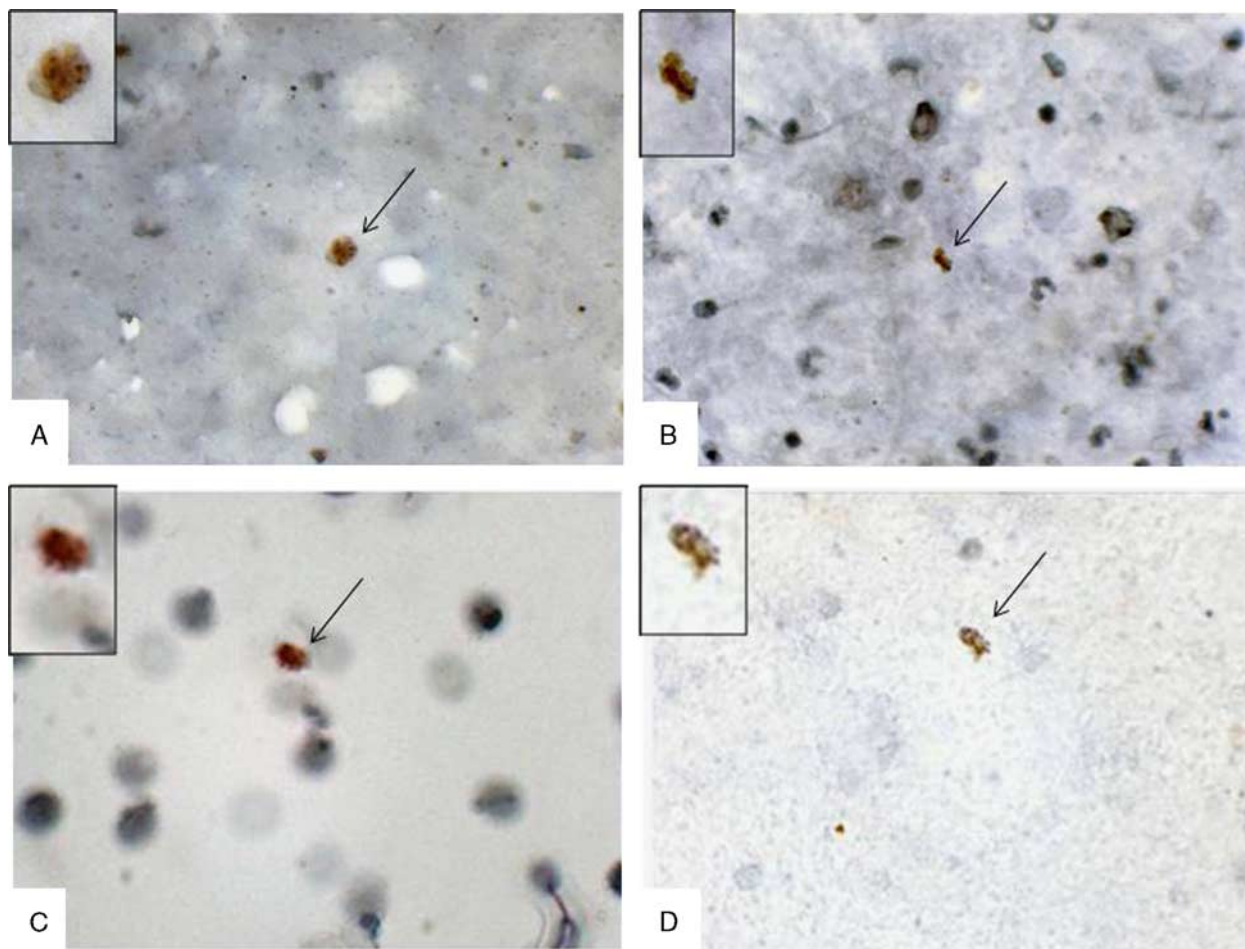
FIGURE 2. Immunocytochemical staining with anti-MPT64 on cell smears (as shown by arrows) from fine-needle aspirates of tuberculosis lesions from a breast lump (A), a chest wall abscess (B), a pleural mass (C), and an Abdominal mass (D). Inset shows strong and granular intracellular staining with anti-MPT64.

An overall comparison showed a significantly ($P < 0.001$) higher sensitivity of immunostaining with anti-MPT64 as compared with ZN stain and LJ culture for the diagnosis of TB without compromising the specificity of the test. The immunostaining was positive in all granulomas without necrosis and necrotic material, whereas the ZN staining was negative in all granulomas and positive in only 01/12 necrotic material (Table 2).

DISCUSSION

We have previously shown the importance of immunostaining using anti-MPT64 for the diagnosis of EPTB.[12–15] In the present study, however, we have shown that using anti-MPT64 immunostaining, it is possible to confirm TB with a consistently high sensitivity and specificity in different suspected TB lesions having equivocal histology or cytology findings in resource-limited settings where newer molecular diagnostic or conventional culture facilities are not available.

The strength of immunostaining using anti-MPT64 is that it is simple, rapid, robust, sensitive, provides clear

and intense signals, makes it possible to differentiate *M. tuberculosis* complex infection from atypical mycobacteria at early stages of disease, is equally effective for aspirates and biopsies, and needs minimum equipment and technical skill personnel. It can easily be availed in any clinical pathology laboratory. One of the most important advantages is that even very tiny specimens are suitable

TABLE 2. Positive Results of Staining Methods in Various Cytological Patterns Among Tuberculosis Specimens (N = 51)

Microscopy Pattern	Anti-MPT64 Staining	ZN Staining
Aspirate (N = 36)		
Necrotic material (n = 12)	12	1
Necrosis with granuloma (n = 5)	5	3
Granuloma (n = 18)	18	0
Biopsy (N = 15)		
Granuloma without caseation (n = 10)	10	0
Granuloma with caseation (n = 2)	2	1
No well-formed granuloma (n = 3)	3	0

for processing, which is extremely important as it is very difficult to get sufficient specimens from inaccessible EPTB lesions. Thus, immunostaining with anti-MPT64 should be a diagnostic procedure in the routine evaluation protocol of EPTB cases in the surgical pathology laboratory.

Our results show that immunostaining with anti-MPT64 is much more sensitive than ZN microscopy and culture.[12,13] Although immunostaining is a bit more technically demanding with extra cost as compared with ZN staining, the disadvantages are balanced by the sensitivity, the speed, and the ease with which specimens can be evaluated at a lower magnification ($\times 10$ or $\times 40$) rather than using oil immersion during microscopy. Also, immunostaining is significantly faster compared with culture as the results are available within 1 working day. Also, this assay has a sensitivity and specificity comparable to that of nested-PCR, with the advantage of being simple, robust, and not sensitive to contamination and does not require sophisticated equipments, which make it suitable for implementation in low-income and middle-income countries with relatively modest laboratory facilities. These findings warrant the implementation of this assay in routine TB diagnostic settings in low-income and lower-middle income countries where an estimated third of new EPTB cases are missed even with sophisticated technologies such as Xpert MTB/RIF automated molecular assay for accurate diagnostic assays.[17–20] As the new WHO guidelines on research priorities aim to identify factors that can enhance the delivery and the implementation of new diagnostics, this assay may be feasible and therefore should be validated at various centers.

The most important advantage of the assay is that we could confirm granulomatous and necrotic lesions confidently not only on biopsy, but also with cytology material, which is very important in EPTB cases (Table 2). Our findings once again proved that the high negative prediction of anti-MPT64 immunostaining (100%) would help to exclude mycobacterium infection in equivocal lesions (granulomatous lesions without necrosis), which prevent empiric anti-TB treatment. The high positive predictive value of anti-MPT64 (97%) helped us reach an accurate diagnosis, which is essential in low-endemic countries and in AIDS-prevalent areas. We previously proved consistently higher performance indices of the anti-MPT64 antibody on various EPTB clinical specimens from different geographical locations including low-TB and high-TB endemic settings and HIV samples (Tanzania, India, South Africa, and Norway).[12–15] The assay performed equally well in HIV-coinfected TB cases with atypical histologic features.[14]

We overdiagnose 1 control specimen as tuberculous lesion with anti-MPT64 immunostaining. However, misdiagnosis and overdiagnosis were more often observed with anti-BCG immunostaining. Probably, the lower specificity with anti-BCG, the only commercially available antibody for TB, due to cross-reactivity with other infectious organisms[21–23] could be the reason for the restraint of immunostaining as the routine assay for the evaluation of granulomatous lesions in the surgical pathology laboratory. However, immunostaining with anti-MPT64 antibodies provides sharp and strong signals with a clear background compared with anti-BCG antibodies, making interpretation easier and permitting a more confident diagnosis of *M. tuberculosis* complex organism. Because of the low sensitivity of the culture in pauci-bacillary EPTB, we used positive nested-PCR to define TB cases and for the validation of the immunostaining assay. All culture-positive cases were positive with nested-PCR and immunostaining. As the sensitivity and the specificity of the anti-MPT64 immunostaining assay was very similar to that of nested-PCR, we once again prove its reliability for the confirmation of TB in resource-constrained settings. A further multicenter study with a larger number of specimens may help decide its use in clinical practice as a standalone and replacement of the ZN stain test for EPTB cases.

In conclusion, our study provides the insight for the feasibility of using the technique as a standalone diagnostic technique for better management of EPTB patients in settings where culture or molecular facilities are not available. We believe that immunostaining using anti-MPT64 for the identification of *M. tuberculosis* complex infection combined with routine cytology/histology and clinical examination is a sensitive, relatively simple, robust, and specific technique that can easily be incorporated into routine pathology laboratories to diagnose EPTB.

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