Original Article

Evaluation of Immunohistochemistry Technique for Diagnosis of Extrapulmonary Tuberculosis in Biopsy Tissue Specimen as Compared to Composite Diagnostic Criteria

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Abstract

Introduction: Diagnosis of extrapulmonary tuberculosis (EPTB) has been challenging owing to its paucibacillary nature and diverse clinical manifestations. Immunohistochemistry (IHC) on biopsy specimens has presented a new perspective toward improving tuberculosis diagnosis. MPT64 is a unique antigen that has shown high sensitivity and specificity compared to other conventional techniques in its ability to diagnose tuberculosis as well as differentiate it from nontubercular mycobacteria. In this study, we aimed to analyze the utility of anti-MPT64 in the diagnosis of EPTB. **Methods:** In this cross-sectional study, conducted over a period of 1 year, 52 nonrepetitive samples from 52 participants with a presumptive diagnosis of EPTB were collected and processed. The specimens were subjected to Ziehl–Neelsen staining, GeneXpert, tissue culture by mycobacterium growth indicator tube, H and E staining, and IHC with anti-MPT64. The sensitivity and specificity of anti-MPT64 was computed against a composite diagnostic criterion. **Results:** Fifty-two consecutive participants satisfying the study criteria were recruited. The mean age of the study population was 37.35 ± 18.71 years. Lymph node specimen accounted for majority of the specimen processed (n = 20, 38.5%). The sensitivity of anti-MPT64 in the diagnosis of EPTB was 68.29%, specificity was 90.90%, positive predictive value was 96.55%, and negative predictive value was 43.47%, when composite criteria were considered standard for diagnosis. **Conclusion:** Immunohistochemical staining by anti-MPT64 is useful in establishing microbiological diagnosis of EPTB on biopsy specimens.

Keywords: Immunohistochemistry, MPT64 protein, *Mycobacterium tuberculosis*

Introduction

Extrapulmonary tuberculosis (EPTB) is a disease involving any part of the body other than lung parenchyma, including other intra-thoracic structures.[1] It constitutes about 15%–20% of all tuberculosis (TB) cases, which rises to nearly 50% when there is immunosuppression.[2,3]

A diagnosis of EPTB is usually made on clinical grounds owing to the relatively low sensitivity of existing diagnostic methods.[4-9] Being heterogeneous clinically and paucibacillary microbiologically, there are often many differential diagnoses leading to delayed/missed diagnosis.

Detection of a novel antigen called MPT64 by immunohistochemistry (IHC) is a promising new avenue to improve the accuracy of diagnosis of EPTB.[10,11]

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Methods

Study design

This was cross-sectional observational study.

Study period

The duration of the study was 1 year, 2019–2020.

Inclusion criteria

Patients of all ages with presumptive EPTB and where a

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representative sample are obtained for laboratory diagnosis (presumptive extrapulmonary tuberculosis refers to the presence of organ-specific symptoms and signs like swelling of lymph node, pain and swelling of joints, neck stiffness, disorientation, etc., and/or constitutional symptoms like significant weight loss, persistent fever for ≥2 weeks, night sweats).

Exclusion criteria

Unwilling to provide consent for the study.

Patients who received anti-tuberculosis treatment in the preceding 1 year.

Sample collection and processing

Excision biopsy/sampling of involved tissue was performed by the surgeon of the concerned specialty, 52 consecutive and nonrepetitive samples of all tissues from extrapulmonary tubercular sites from patients satisfying study criteria were collected and processed.

The tissue collected was divided into three portions. Two portions were transported to the intermediate reference laboratory (IRL) in containers of 0.9% saline for liquid culture by mycobacterium growth indicator tube (MGIT) and GeneXpert. The other sample was processed for acid-fast bacilli (AFB) smear, IHC, and histopathology examination (HPE) at our tertiary care hospital [Figure 1].

The IRL is a state-level advanced tuberculosis diagnostics laboratory established by the Government of India under the aegis of the Revised National Tuberculosis Program in India, now known as the National Tuberculosis Elimination Program (NTEP).

This study was funded by the NTEP and was approved by the Institutional Ethics Committee; the authors followed applicable EQUATOR Network guidelines during the conduct of this research project. Consent for the study was obtained from the study participants.

Procedures

a. Microscopy and staining with Ziehl–Neelsen stain (ZN) were performed on all specimens at the microbiology laboratory. These stains help to detect acid-fast bacilli in tissues. The results were reported as follows: Negative-no AFB, Scanty-1–9 AFB/100 high power field (HPF), 1+ = 10–99 AFB/100HPF, 2+ = 1–10AFB/HPF, 3+ = 10 AFB/HPF.

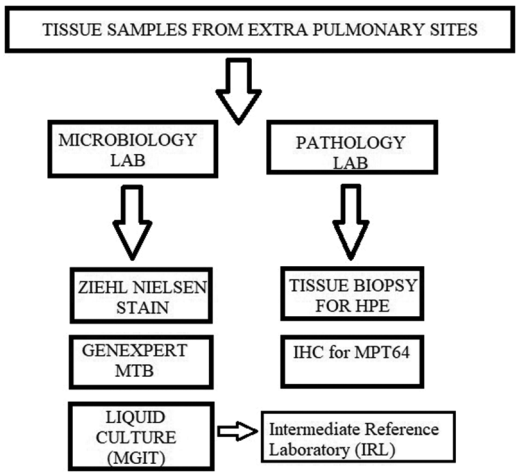

Figure 1: Schema showing specimen flow to the respective labs

b. Culture method: MGIT using Middlebrooks 7H9 broth-based culture medium with an oxygen-sensitive fluorescent sensor to indicate mycobacterial growth. Culture is the gold standard for the diagnosis of tuberculosis.

c. Molecular methods: Gene Xpert/cartridge-based nucleic acid amplification test/- This is an automated test, that uses semi-nested real-time polymerase chain reaction (PCR) to identify mycobacterial DNA. Results obtained are reported as trace, very low, low, medium, or high when mycobacterial DNA was detected. In addition, it detects rifampicin resistance mutation gene RpoB and thus gives information on Rifampicin sensitivity.

d. HPE: biopsy material obtained was prepared and fixed on phosphate buffer solution (PBS)-buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin before examination.

e. Immunohistochemical stain method: MPT64 Kit (catalog number PP140 AA).

The steps involved in the Manual IHC Staining procedure are described below:

- Cut section on charged slides/coated slides at 3–4 μ
- Fix section at 60c overnight
- Deparaffinize slides in 3 changes for xylene
- Two changes of 90% alcohol, absolute alcohol
- Wash slides in distill water
- Antigen retrieval/EDTA/CITRATE/TRIS EDTA-pH-9.0
- Cool the slides at room temperature (in retrieval solution)
- Take out the drops from the cooling wash in distilled water now add 3% hydrogen peroxide for 20 min
- Wash slides in wash buffer PBS for 5 min, 2 washes
- Now add power block for 10 min
- Wash in PBS 2 changes 5 min each
- Add primary antibody for 30 min (MTB)
- Wash 2 changes of PBS 5 min each
- Add with primary amplifier (master diagnostica) to each completely covered section and incubate at room temperature for 15 min
- Rinse Slides in 2 changes of PBS Wash Buffer 5 min in each
- Apply horse radish polymer (HRP) (master diagnostica polymer HRP) incubate at room temperature for 30 min
- Rinse in wash buffer 3 changes, 5 min in each
- Chromogen solution preparation: Add 1 drop of diaminobenzidine (DAB) Chromogen concentrates to 1 ml of DAB substrate buffer mix well. Should be prepared fresh ever time before adding incubate at room temperature for 5 min
- Abundantly wash with distilled water for 2 times for 5 min
- Counterstain with Harris hematoxylin for 1 min
- Wash in tap water
- Wash in distill water

- Dehydration in graded alcohol 70%, 80%, absolute alcohol clears in xylene
- Mount with distyrene, plasticizer, and xylene and label the slides with corresponding number.

Antigen load was evaluated, and in each section, 3 granulomas were selected. Stained epithelioid cells, giant cells, and total number of nucleated cells were accounted for. The percentage of stained cells from each granuloma was estimated. The intensity of staining was categorized as weak (1+), moderate (2+), and strong (3+). The IHC staining was seen as cytoplasmic positivity and appeared sharp and strong in a clear background.

Data collection

Sociodemographic and clinical characteristics of the patients were collected and recorded. Following specimen processing, the results obtained were collected from records of pathology and microbiology departments at a tertiary care hospital and the IRL, in a predesigned pro forma. Digitization was done in a quality-assured manner by trained data entry operators.

Sample size

Based on a previous study conducted by Mukherjee *et al*.[12] which indicated that the sensitivity of ZN stain was at least 44% as compared to IHC which was 74% and in another study by Kohli *et al*.[13] the sensitivity of IHC was 95.56% for the detection of tubercular antigen in extrapulmonary cases. The sensitivity of 44% was thus taken for sample size estimation in the present study. Considering an absolute precision of 14% and confidence level of 95%, the sample size was estimated to be a minimum of 48 subjects.

Statistical analysis

Data analysis was performed using SPSS Inc., 2009 Release, PASW, Statistics for Windows, version 18.0, Chicago, Illinois, USA: SPSS Inc. Continuous variables were described either with mean (and standard deviation) or median (and interquartile range) based on the statistical distribution of data. Categorical variables were summarized as proportion with 95% confidence interval. The key outcome variable was number diagnosed with TB on IHC MPT64 test. We thus calculated and compared positivity for each of the five tests with a composite diagnostic criterion.

Composite diagnostic criteria include: One or more positive result from either of the below-mentioned diagnostic tests is considered as composite diagnostic criteria for EPTB-Tissue smear for acid-fast bacilli, Gene Expert, Liquid culture by MGIT Method, histopathologic examination of the tissue specimen from the relevant extrapulmonary sites and or/clinically confirmed cases which are started on treatment.

Results

Fifty-two participants satisfying the study criteria were recruited. The mean age of the study population was 37.35 ± 18.71 years. The majority belonged to the age group of 18–29 years (n = 22, 42.3%) [Figure 2]. When distributed by gender, males accounted for 55.8% (n = 29), while females formed the remaining 44.2% (n = 23), the differences in their distribution by age category was not statistically significant [P = 0.908, Figure 3].

Patients were subjected to diagnostic evaluation for EPTB as per standard of practice. The distribution of samples tested by organ involved is depicted in Figure 4. The results of the evaluation are outlined in Table 1, ZN stain for AFB, was negative in all 52 specimens.

When biopsy tissues were subjected to HPE, 75% were found to have granulomatous inflammation (n = 39), 5.8% had caseating granulomatous inflammation (n = 3) and 19.2% had

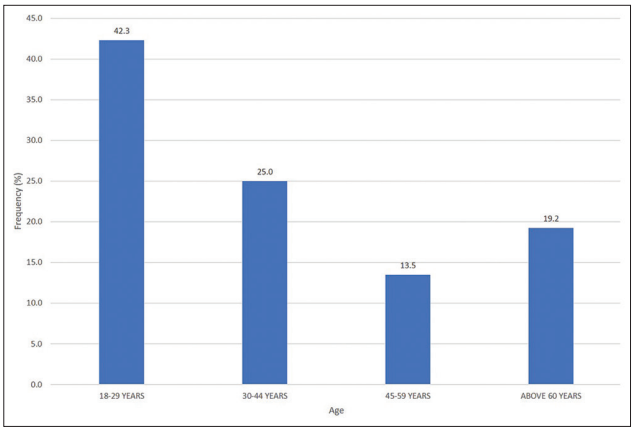

Figure 2: Distribution of subjects by age

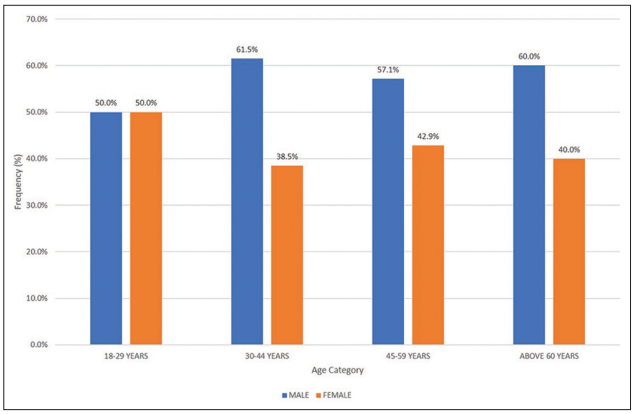

Figure 3: Distribution of gender by age category

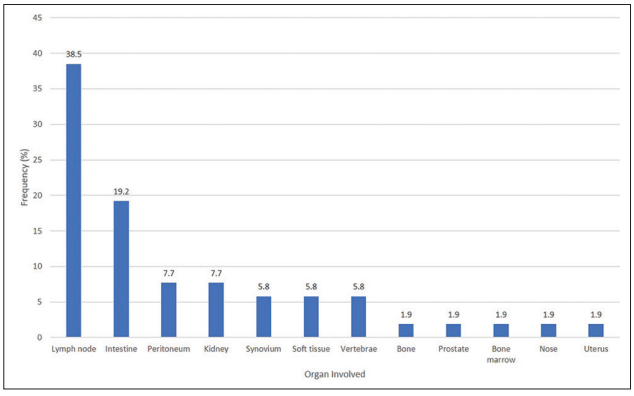

Figure 4: Distribution of samples tested by organ involved

Table 1: Depicting result of diagnostic testing by organ involved (expressed as percentage of organ involved)

Organ involved (total=52)	Histopathology finding			GeneXpert	MGIT	CC	IHC
	GI (*n*=39) (%)	CGI (*n*=3) (%)	NSI (*n*=10) (%)	Positive (*n*=5) (%)	Positive (*n*=6) (%)	Positive (*n*=41) (%)	Positive (*n*=29) (%)
Lymph node (*n*=20)	70	5	25	15	15	85	65
Intestine (*n*=10)	10	0	0	0	10	80	50
Peritoneum (*n*=4)	100	0	0	0	0	100	100
Kidney (*n*=4)	50	0	50	0	0	50	25
Synovium (*n*=3)	66.7	33.3	0	0	0	100	66.7
Soft tissue (*n*=3)	33.3	0	66.7	0	0	33.3	33.3
Vertebrae (*n*=3)	66.7	0	33.3	33.3	33.3	66.7	33.3
Bone (*n*=1)	0	100	0	0	0	100	0
Prostate (*n*=1)	100	0.0	0	0	0	100	0
Bone marrow (*n*=1)	0	100	0	0	0	100	100
Nose (*n*=1)	100	0	0	0	0	0	0
Uterus (*n*=1)	100	0	0	100	100	100	100
Overall total	75	5.8	19.2	9.6	11.5	78.8	55.8

GI: Granulomatous inflammation, CGI: Caseating GI, NSI: Nonspecific inflammation, MGIT: Mycobacterium growth indicator tube, CC: Composite criteria, IHC: Immunohistochemistry

nonspecific inflammation (n = 10), the distribution of these findings by organ involved is depicted in Table 1. All specimen was subjected to IHC using anti-MPT64, a positive result was obtained in 55.8% of the cases (n = 29/52) Figure 5.

When composite criteria were employed, 78.8% (n = 41/52) were diagnosed to have extrapulmonary tuberculosis and were initiated anti-tuberculosis treatment, the diagnosis was clinicopathological in 82.92% (n = 34/41) and microbiological in 17.07% (n = 7/41).

Among the cases that remained inconclusive despite diagnostic evaluation (n = 11/52), 27.3% were lymph node specimens intestine, renal biopsy, and soft-tissue specimen accounted for 18.2% each, nose and vertebral specimens accounted for 9.1% each [Table 1]. Three among these 11 cases had granulomatous inflammation-two intestinal mucosal and one nasal mucosal specimen, IHC for anti-MPT64 tested positive in one intestinal mucosal specimen, and the remaining 8 cases had nonspecific inflammation.

The diagnostic yield of GeneXpert, MGIT, and IHC by anti-MPT64 was calculated by comparing with composite criteria for the diagnosis of EPTB, Table 2 outlines the results for the three modalities. The diagnostic performance of IHC with anti-MPT64 was as follows, sensitivity of 68.29%, specificity of 90.90%, positive predictive value of 96.55%, and negative predictive value of 43.47%.

The diagnostic performance of IHC with anti-MPT64 in those with noncaseating granulomatous inflammation alone was as follows, sensitivity of 66.6%, specificity of 66.67%, positive predictive value of 96%, and negative predictive value of 14.28%.

Discussion

EPTB incidence has seen a rising trend in the last decade. In the European Union, there was an increase in the proportion of cases from 16.4% in 2002 to 22.4% in 2011,[8] in India,

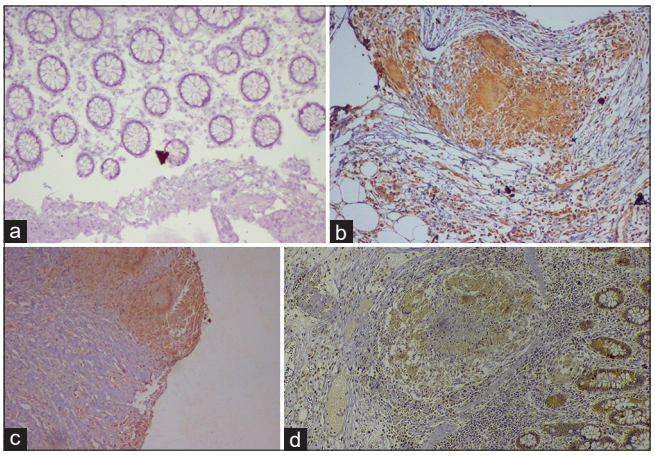

Figure 5: Immunohistochemistry images – (a) Colon biopsy - negative for MPT64 (×10)- Final diagnosis - Crohn's disease. (b) Omental biopsy, strongly positive for MPT64, seen within granulomas. (c) Synovial biopsy showing granulomas, with regions positive for MPT64. (d) Colon biopsy, revealing granulomas with MPT64 staining - 3+. MPT: Mycobacterium tuberculosis

EPTB forms 15% to 20% of all cases of diagnosed TB.[14] This trend has been attributed to HIV infection as well as improved detection rates. The incidence is higher in those carrying varying levels of immune deficiency or immune suppression.[15]

Considerable delay in diagnosis occurs in patients with EPTB, due to reasons such as its diversity in clinical presentation, inadequate resources for obtaining invasive tissue samples from inaccessible sites.[16] Thus, the need for improved modalities of diagnosis is essential to ensure an accurate diagnosis of EPTB.

Histopathological findings alone are usually inadequate to establish a diagnosis of EPTB, a potential gap thus exists, IHC with anti-MPT64 is a promising candidate to fill this void. Anti- MPT64 binds to antigen in the epitheloid cells of granulomas and thus facilitates diagnosis of TB, particularly when granulomas are non-necrotizing. The test is easily

available, cheap, robust, and rapid (results are available within 1 working day, usually). An important advantage is that diagnosis can be established even with small bits of tissue, in addition, it is less prone for contamination and does not require sophisticated equipment. This thus strengthens TB diagnosis in the absence of culture confirmation. On the other hand, the sensitivity of the test decreases with increase in the length of formalin fixation and also when the number of mycobacteria present is below that can be detected by IHC.

In the study conducted by Jørstad *et al*.[17] in a low-resource setting, the overall performance of the anti-MPT64 was better compared to other diagnostic modalities, with sensitivity and specificity of 69% and 95%, respectively, in comparison to the composite reference standard for diagnosis [Table 3]. The MPT64 test performance was best in TB lymphadenitis cases ($n = 67$, sensitivity 79%, specificity 97%) these results are similar to the findings in our study with an overall sensitivity of 68.29% and specificity of 90.90%, with the best performance being seen in peritoneal samples (100%) although only four samples were tested, lymph node specimens was second best with MPT64 testing positive in 65%**.** Hoel *et al*.,[18] in their study in a low TB prevalence setting found that overall sensitivity and specificity was 37% and 99%, respectively, the low sensitivity was attributed to low pretest possibility.

Purohit *et al*.[6] in their study, employing anti-MPT64 antibody on abdomen and cervical lymph node specimen found that its overall sensitivity was 92% and specificity was 97%. In this study, only specimens with histopathological evidence of tuberculosis were and specimens with diagnosis other than tuberculosis were used as controls. Nested PCR was used as the gold standard for diagnostic validation, this explains the higher performance compared to that in our study. In another similar study by Purohit *et al*.,[19] immunostaining with anti-MPT64 on biopsy specimens and fine needle aspirates from various organs exhibited overall sensitivity of 100% and specificity of 97% when compared to Nested PCR. Baba *et al*.,[20] in their study on 25 pleural biopsy specimens, demonstrated a sensitivity 81% and specificity of 100% compared to Nested PCR. When a clinicoradiological diagnosis and satisfactory response to the anti-tuberculous therapy was used as diagnostic comparison, the sensitivity for diagnosis with anti-MPT64 was 80% and specificity was 100%. The above studies[6,19,20] validate the potential of MPT-64 in the diagnosis of EPTB, our study further establishes its utility in a real-world setting.

Table 2: Diagnostic yield of different modalities for diagnosis of extrapulmonary tuberculosis when composite criteria were used for comparison

Diagnostic modality	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
GeneXpert	12.5	100.0	100.0	25.53
MGIT	15	100.0	100.0	26.08
IHC	67.5	91.6	96.42	45.83

MGIT: Mycobacterium growth indicator tube, IHC: Immunohistochemistry, PPV: Positive predictive value, NPV: Negative predictive value

Table 3: Comparison of the yield of anti-MPT64 in different studies

	Comparative variable	Sensitivity (%)	Specificity (%)
Jørstad *et al*.[17]	Composite reference standard	69	95
Hoel *et al*.[18]	Composite reference standard	37	99
Purohit *et al*.[6]	Nested PCR	92	97
Purohit *et al*.[19]	Nested PCR	100	97
Baba *et al*.[20]	Nested PCR	81	100
Baba *et al*.[20]	Composite reference standard	80	100

PCR: Polymerase chain reaction

All specimens tested were negative for AFB in our study, consistent with its low sensitivity of 10%–45% as indicated in other studies. The yield is variable and is largely reliant on an intact mycobacterial cell wall and bacillary load in the patient.

MGIT yield was low in our study with a sensitivity of 15% and specificity of 100% compared to composite criteria [Table 2], the low yield is due to the paucibacillary nature of EPTB and that fresh unfixed tissue with live bacilli is usually not available limiting its utility in EPTB diagnostics, in addition, long transportation time to testing laboratories potentially reduces the viability of bacilli.

GeneXpert also exhibited low sensitivity (12.5%) in our study. Variable sensitivity in extrapulmonary specimen has been recorded, it had a pooled sensitivity of 0.88 (95% confidence interval, 0.76–0.94)[9] for tissue samples of all types, which is improved on with the new GeneXpert Ultra.[3] A limitation is that formalin-fixed tissue from extrapulmonary sites cannot be processed and a concerning practice is of dropping the tissue in a container with 1% formalin with the aim of HPE which is a good tool for differential diagnosis but does not permit microbiological confirmation in a case of TB.[8]

Jørstad *et al*.[17] and Hoel *et al*.[18] in their studies have found that IHC testing for MPT64 antigen is implementable in the routine diagnostic workup of EPTB in low TB prevalence settings. Furthermore, IHC can detect fragmented bacilli as well and thus carries a higher sensitivity over direct microscopy for AFB. When compared to culture, IHC testing is quick to perform and requires less advanced laboratory equipment. Both IHC and GeneXpert have high sensitivity compared to other modalities and can give a result within a day or two. The requirement of a trained pathologist for the interpretation of IHC is its limitation.[8,21,22] Comparison of the yield of anti-MPT64 in different studies is depicted in Table 3.

The classical histopathological picture of granulomas with caseating necrosis in tuberculosis is a late phenomenon, hence early lesions showing granulomas without necrosis on HPE tend to carry ambiguity in diagnosis, making differentiation from nontuberculous etiology difficult for pathologists. Noncaseating granulomas are seen in conditions such as sarcoidosis,

nontuberculous mycobacterium, histoplasmosis, and Crohn's disease. The detection of MPT64 antigen in these situations may play a critical role in establishing or ruling out a diagnosis of EPTB. Among those with granulomatous inflammation, 64.1% (n = 25/39) tested positive for MPT64. We have appreciated the advantage of IHC in colon biopsy specimens with granulomatous inflammation, where in two cases, therapeutic trials of treatment were initiated based on the IHC result in a suspected case of Crohn's disease (MPT64-negative) and tuberculosis (MPT64-positive), the former were started on steroids and the latter on anti-tuberculosis treatment, both exhibited favorable clinical response.

The limitations of our study include a relatively low sample size as recruitment for the study was severely affected by the COVID-19 pandemic, data on the outcome of treatment were not available in all cases and a potential selection bias in samples with a higher pretest possibility being utilized for testing and analysis, this could affect accurate estimates of specificity.

Scope for future studies - the differences in yield of MPT64 across different tissue specimens draws a limitation to the widespread utility of IHC in EPTB, hence larger studies validating its utility in various specimens, incorporating control groups are necessary.

Conclusion

In the current landscape of medicine, the use of immunohistochemical markers that aid diagnosis is largely limited to that of malignancy than in granulomatous lesions. Anti-MPT64 is an effective diagnostic modality in the diagnosis of EPTB; it has improved performance compared to standard modalities used for microbiological diagnosis. In gastrointestinal disease, they can help differentiate granulomatous inflammation due to tuberculosis from others. With the burden of evidence in favor of anti-MPT64, updates in national policy and guidelines related to diagnosis and management of EPTB are the need of the hour.

Research quality and ethics statement

This study was approved by the Institutional Ethics Committee (Registration No- ECR/215/Inst/KA/2013/RR-2019). The authors followed applicable EQUATOR Network (http://www.equator-network.org/) guidelines during the conduct of this research project.

Financial support and sponsorship

This study was conducted on a fund received from the Revised National Tuberculosis Control Program, India, now known as NTEP.

Conflicts of interest

There are no conflicts of interest.

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