

Extrapulmonary tuberculosis - A cross-sectional study comparing the utility of Ziehl-Neelsen stain and immunohistochemistry



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ABSTRACT

Background: Tuberculosis (TB) continues to be a significant global health issue despite known causes and effective treatments. Factors such as virulent strains, drug resistance, and the human immunodeficiency virus pandemic, especially in India, contribute to its prevalence. *Mycobacterium tuberculosis*, the TB agent, can evade immune responses, persisting within host cells. A distinct feature of TB, granuloma formation, can also appear in diseases such as leprosy, histoplasmosis, and non-infectious conditions like Crohn's disease. Proper diagnosis is vital, as in resource-limited countries, TB treatment often relies on presumptive diagnoses based on imaging and symptoms, leading to high false positive rates. **Aims and Objectives:** This study aims to compare the utility of Ziehl-Neelsen (ZN) staining and immunohistochemistry (IHC) staining in suspected extrapulmonary TB lesions. **Materials and Methods:** This is a retrospective cross-sectional study conducted at the Institute of Pathology, Rajiv Gandhi Government General Hospital, Chennai, over a 2-year period from August 2015 to July 2017. Fifty cases out of the 198 suspected extrapulmonary TB lesions were selected for the study. **Results:** About 12% of cases were positive for acid-fast bacilli by ZN staining, whereas 82% of cases were positive for acid-fast bacilli by IHC staining. P value was found to be <0.001 by McNemar test which was statistically significant. **Conclusion:** Diagnosing extrapulmonary TB can be challenging because the disease presents with various clinical manifestations and the bacterial load may be low. Immunohistochemistry is superior to ZN staining for localization of the tubercle bacilli and thus can serve as an effective ancillary technique in diagnoses of extrapulmonary TB in formalin-fixed paraffin embedded tissue sections.

Key words: Tuberculosis; Extrapulmonary; Ziehl-Neelsen stain; Immunohistochemistry

INTRODUCTION

Tuberculosis (TB) remains a major global health problem, despite the identification of the causative agent 140 years ago and the advent of effective anti-TB therapy.¹ This has been attributed to many factors such as emergence of virulent strains, multidrug resistance, and rise of human immunodeficiency virus (HIV) pandemic among others.² In India, TB remains one of the leading infectious causes of mortality and morbidity.³

Mycobacterium tuberculosis, the causative agent of TB, is an intracellular microbe, capable of evading the host

immune system and persisting within the macrophages even in the face of host immune response.⁴ Granuloma formation with or without caseation necrosis, the hallmark of TB is a delayed-type hypersensitivity or Type IV hypersensitivity reaction.⁵ Granulomas, however, are not limited to TB infections, but can also occur in a variety of settings such as leprosy, atypical mycobacterial infections, schistosomiasis, histoplasmosis, cryptococcosis, and cat scratch disease, and in non-infectious conditions such as Crohn's disease, sarcoidosis, primary biliary cirrhosis, rheumatoid arthritis, foreign body granulomas, and even in neoplasms.⁶

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Thus, a definitive diagnosis needs to be made in the setting of granulomas, because in developing countries where there is a scarcity of resources, treatment of extrapulmonary TB has largely been modeled on presumptive diagnosis based on the imaging, cytological and histological findings and clinical symptoms, which carries a high false positive rate.⁷

Histopathology of formalin-fixed paraffin embedded tissue sections can provide a presumptive diagnosis of extrapulmonary TB based on the morphology of granulomas and the presence of caseation necrosis and Langhans giant cells, although non-necrotizing granulomas can also occur.^{8,9} However as mentioned earlier, it cannot reliably distinguish from other causes of granulomas which can result in under-treatment and sometimes in erroneous over-treatment.¹⁰

M. tuberculosis is acid-fast, meaning that they resist decolorization by acids, which can be utilized for their identification by special stains. Some of the stains that can be used include Ziehl-Neelsen (ZN) stain, Kinyoun stain, and fluorochrome-based stains like auramine-rhodamine stain, of which ZN stain is the most widely used.¹¹ The tubercle bacilli appear as red curved rods which can be easily identified against the blue background. ZN stain, although is rapid and inexpensive has very low and variable sensitivity which depends on the bacterial load.¹²

Immunohistochemistry (IHC) can localize the bacilli or their components in the tissue sections, for which both monoclonal and polyclonal antibodies are available. IHC offers a significant advantage over ZN in that it can detect tubercle bacilli even when there is low bacterial load and even in those who were partially treated.¹³

Aims and objectives

The aim of the study is to evaluate the diagnostic utility of IHC analysis using rabbit polyclonal *M. tuberculosis* antibody in suspected extrapulmonary TB lesions and to compare the IHC analysis with ZN staining in tissue sections.

MATERIALS AND METHODS

The approval of the institutional ethical board was obtained from the Institutional Ethical Committee, Madras Medical College under the letter number ECR/270/Inst./TN/2013. This retrospective and cross-sectional study was conducted at the Institute of Pathology, Rajiv Gandhi Government General Hospital, Chennai, for a 2-year study period from August 2015 to July 2017. Out of the 349 granulomatous lesions in extrapulmonary sites, 198 cases were of suspected TB etiology based on the clinical suspicion, presence of necrosis with or without caseation, or Langhans giant

cells which were included in the study. The remaining 151 cases were of different etiologies, namely, fungal infections, foreign body induced, autoimmune disease, inflammatory bowel disease, and vasculitis and were excluded from the study.

Data collection

Case details including age, sex, presenting complaints, details of relevant investigations, procedure done, and histopathological diagnosis of the tissues were retrieved from pathology registers. Relevant details such as imaging findings, prior history of anti-TB therapy, and immune compromised status were noted. Hematoxylin and Eosin sections of the paraffin tissue blocks were reviewed. All Out of the 198 granulomatous lesions of suspected TB etiology in extrapulmonary sites, 50 cases were selected by proportional representation of the organ systems involved, and their corresponding tissue blocks were obtained for IHC analysis of *M. tuberculosis* using polyclonal antibody and ZN staining for the visualization of the acid-fast bacilli.

Controls

Positive control

Formalin fixed, paraffin-embedded tissue section of *M. tuberculosis*-infected lung tissue which was positive for acid-fast bacilli by ZN staining was used as positive control (Tables 1 and 2).

Negative control

Formalin-fixed, paraffin-embedded tissue sections of foreign body granuloma were used as the negative control (Tables 1 and 2).

Interpretation and scoring of IHC

The IHC slides were analyzed for the presence of reaction, and cellular localization of the staining—*M. tuberculosis* shows fine or coarse granular cytoplasmic dust-like positive staining within the epithelioid histiocytes, giant cells, and in extracellular locations like areas of necrosis. The percentage of cells taking up the stain and the intensity with which they stain were also analyzed. Scores of 1+, 2+, and 3+ were assigned to mild, moderate, and strong intensity staining, respectively.

Interpretation of ZN staining

When stained with strong stains (carbol fuchsin), acid acid-fast bacilli retain their color even after treatment with strong decolorizing solutions due to the presence of the mycolic acids which takes up the stain and remains resistant to decolorization with 1% of acid-alcohol or 20% of sulfuric acid as compared to other tissues. They remain red after counterstaining with methylene blue, whereas the other tissues and microorganisms which are susceptible to acid take on the blue color. Acid-fast bacilli appear bright red curved rods and background appears blue.

Table 1: IHC

| Antigen | Vendor | Species (clone) | Positive control | Negative control |
|------------------------|-----------------|-------------------|--|------------------------|
| <i>M. tuberculosis</i> | Biocare medical | Rabbit polyclonal | <i>M. tuberculosis</i> -infected lung tissue | Foreign body granuloma |

M. tuberculosis: Mycobacterium tuberculosis, IHC: Immunohistochemistry

Table 2: ZN staining

| Vendor | Positive control | Negative control |
|-------------------|--|------------------------|
| Fisher scientific | <i>M. tuberculosis</i> -infected lung tissue | Foreign body granuloma |

M. tuberculosis: Mycobacterium tuberculosis, ZN: Ziehl-Neelsen

Statistical analysis

Statistical analysis was performed with the IBM Statistical Package for the Social Sciences. The IHC expression of MTB antigen and ZN staining were correlated and studied using McNemar test and Chi-square test.

RESULTS

The number and sites of the selected lesions are listed in Table 3.

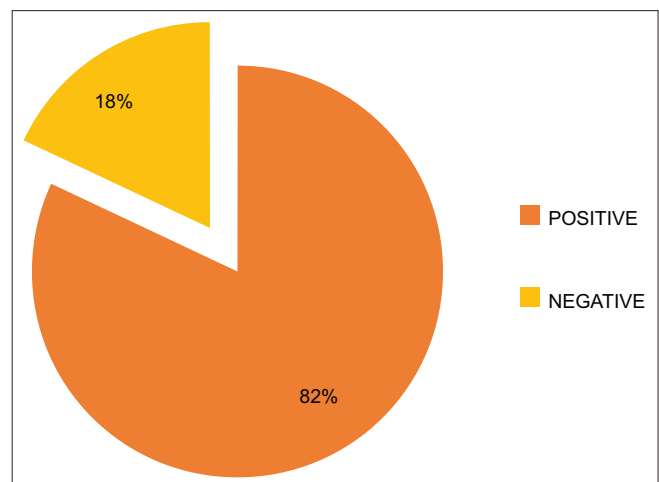
Lymph nodes accounted for the majority of extrapulmonary granulomatous lesions, constituting about 45% of all lesions, followed by intestine, soft tissues, peritoneum, and vertebra. Other rare sites of occurrence included bone marrow, breast, liver, omentum, synovium, and urinary bladder. Among the 50 cases which were selected for the study, peak incidence of granulomatous inflammation was observed in the age group of 21–40 years which constituted about 60% followed by the age group of above 40 years which formed about 32%. There was not much significant difference in the sex with males accounting for 52% and females accounting for 48% of cases. One patient was positive for HIV, who presented with cervical lymphadenopathy.

Among the 50 cases, five of the patients had previously undergone anti-TB therapy. Among the 50 granulomatous lesions, on histopathological examination, necrosis was present in 24 cases which constituted about 48% of cases. All 50 cases were subjected to IHC analysis using polyclonal anti *M. tuberculosis* antibody. Forty-one cases showed cytoplasmic positivity (Chart 1). All 50 cases were subjected to ZN staining as well. Six cases turned positive for the tubercle bacilli.

Fourteen out of 17 cases in whom there was a clinical suspicion of TB, were positive by IHC staining, whereas 27 out of the remaining 33 cases were positive by IHC staining. Two out of 17 cases with a clinical suspicion of TB were positive by ZN staining, whereas four out of the remaining 33 cases were positive by ZN staining.

Table 3: Frequency of granulomatous lesions in specific sites (numbers in brackets represent the overall figures over the 2-year period)

| Site | Frequency | Percent |
|-----------------|-----------|-----------|
| Bone marrow | 1 (4) | 2 (2) |
| Breast | 1 (5) | 2 (2.5) |
| Intestine | 8 (30) | 16 (15.2) |
| Liver | 1 (2) | 2 (1) |
| Lymph node | 22 (90) | 44 (45.5) |
| Omentum | 1 (5) | 2 (2.5) |
| Peritoneum | 4 (14) | 8 (7.1) |
| Soft tissue | 7 (26) | 14 (13.1) |
| Synovium | 1 (4) | 2 (2) |
| Urinary bladder | 1 (5) | 2 (2.5) |
| Vertebra | 3 (13) | 6 (6.6) |
| Total | 50 (198) | 100 (100) |

**Chart 1: Immunohistochemistry**

Out of the nine cases which were negative for immunohistochemistry, the tissue samples were of lymph nodes (2), intestine (2), soft tissue (1), vertebra (1), liver (1), peritoneum (1), and urinary bladder (1). The presence of necrosis had only a minimal effect on the expression of immunohistochemistry (Table 4).

Prior treatment with anti-TB drugs also had minimal effect on the IHC expression (Table 5).

Out of the 41 cases which were positive by immunohistochemistry, 19 cases had strong intensity staining (3+) and 22 cases had moderate staining intensity (2+). Three cases had mild intensity staining (1+) and were considered as negative along with the remaining six cases. About 12% of cases were positive for acid-fast

| Table 4: Necrosis – IHC cross tabulation | | | |
|--|----------|----------|-------|
| Feature | IHC | | Total |
| | Positive | Negative | |
| Necrosis | | | |
| Present | | | |
| Count | 23 | 1 | 24 |
| % within IHC | 56.1 | 11.1 | 48.0 |
| Absent | | | |
| Count | 18 | 8 | 26 |
| % within IHC | 43.9 | 88.9 | 52.0 |
| Total | | | |
| Count | 41 | 9 | 50 |
| % within IHC | 100.0 | 100.0 | 100.0 |

IHC: Immunohistochemistry

| Table 5: Anti-TB therapy * Immunohistochemistry cross tabulation | | | |
|--|----------------------|----------|-------|
| Feature | Immunohistochemistry | | Total |
| | Positive | Negative | |
| Anti-TB therapy | | | |
| Treated | | | |
| Count | 5 | 0 | 5 |
| % within IHC | 12.2 | 0.0 | 10.0 |
| Untreated | | | |
| Count | 36 | 9 | 45 |
| % within IHC | 87.8 | 100.0 | 90.0 |
| Total | | | |
| Count | 41 | 9 | 50 |
| % within IHC | 100.0 | 100.0 | 100.0 |

IHC: Immunohistochemistry

bacilli by ZN staining, whereas 82% of cases were positive by IHC staining. The $P < 0.001$ by McNemar test which is statistically significant which shows the sensitivity of immunohistochemistry is better than ZN staining (Table 6).

The images of granulomas at various sites, along with the ZN positivity and IHC staining intensity are depicted in the images (Figures 1-9).

DISCUSSION

TB is the leading cause of death among infectious diseases in India, which incidentally has the highest TB burden in the world. Early diagnosis and treatment of TB, with the disease being curable makes it all the more pertinent.¹⁴ The diagnosis of extrapulmonary TB has always been a stumbling block.

The presence of the mycobacterial antigens can be elicited by Immunohistochemistry as shown by several studies which are mentioned later and in our present study. Positive cases show coarse or fine granular cytoplasmic staining corresponding to the fragments of TB bacilli or intact bacilli can be seen within the macrophages, fibroblasts, plasma cells, lymphocytes, and even endothelial cells outside the granuloma.¹⁵

| Table 6: Comparison of IHC staining with ZN staining | | | |
|--|----------|----------|-------|
| Immunohistochemistry * ZN stain Cross tabulation | | | |
| Feature | ZN stain | | Total |
| | Positive | Negative | |
| IHC | | | |
| Positive | | | |
| Count | 6 | 35 | 41 |
| % within IHC | 14.6 | 85.4 | 100.0 |
| Negative | | | |
| Count | 0 | 9 | 9 |
| % within IHC | 0.0 | 100.0 | 100.0 |
| Total | | | |
| Count | 6 | 44 | 50 |
| % within IHC | 12.0 | 88.0 | 100.0 |

McNemar test $P < 0.001$, IHC: Immunohistochemistry

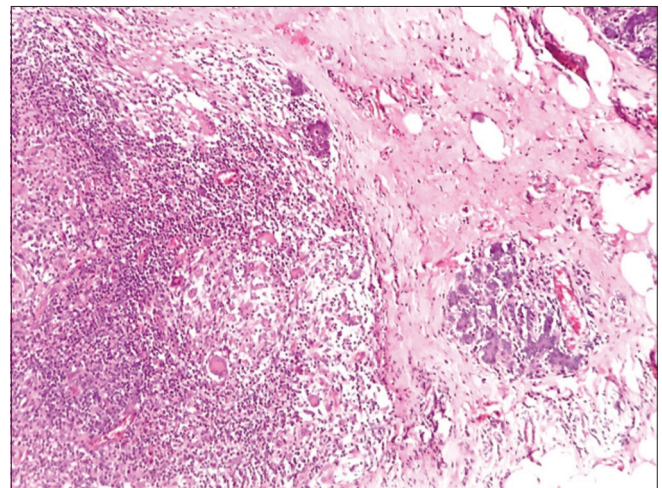


Figure 1: Breast parenchyma showing epithelioid granuloma (H&E, x100)

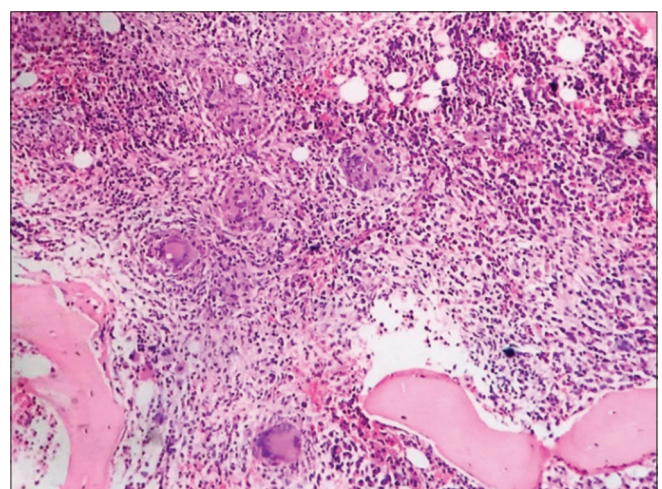


Figure 2: Bone marrow showing epithelioid granuloma (H&E, x100)

One of the major limitations of the immunohistochemistry technique is the background staining which may cause an increased false positive rate, especially if the interpreter is

Table 7: IHC positivity in extrapulmonary TB

| Study | Method and antibody used | Sample size | IHC positivity |
|------------------|--|-------------|----------------|
| Humphrey et al. | Immunoperoxidase-anti peroxidase; polyclonal anti-BCG | 59 | 77.7 |
| Barbolini et al. | Indirect avidin-biotin complex; monoclonal antibody | 23 | 100 |
| Goel and Budhwar | Streptavidin-biotin; monoclonal antibody | 36 | 100 |
| Purohit et al. | Anti-MPT antibody | 120 | 80 |
| | Anti-BCG antibody | 120 | 76.6 |
| Baba et al. | Anti-MPT antibody | 25 | 80 |
| | Anti-BCG antibody | 25 | 48 |
| Present study | Immunoperoxidase-anti peroxidase; polyclonal anti –MTB | 50 | 82 |

TB: Tuberculosis, IHC: Immunohistochemistry

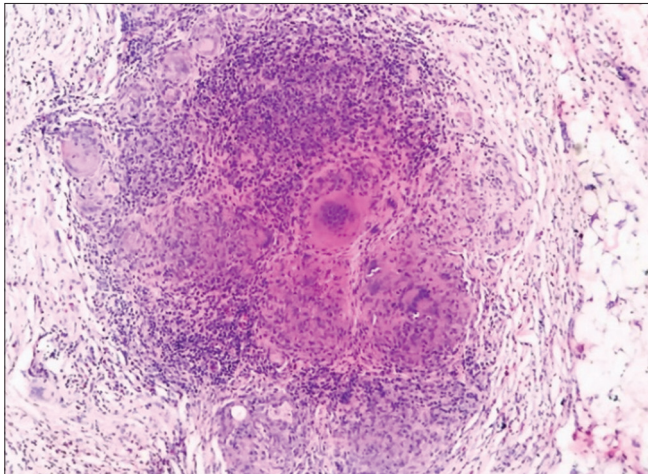


Figure 3: Peritoneum showing epithelioid granuloma with Langhans and foreign body type of giant cells (H&E, x100)

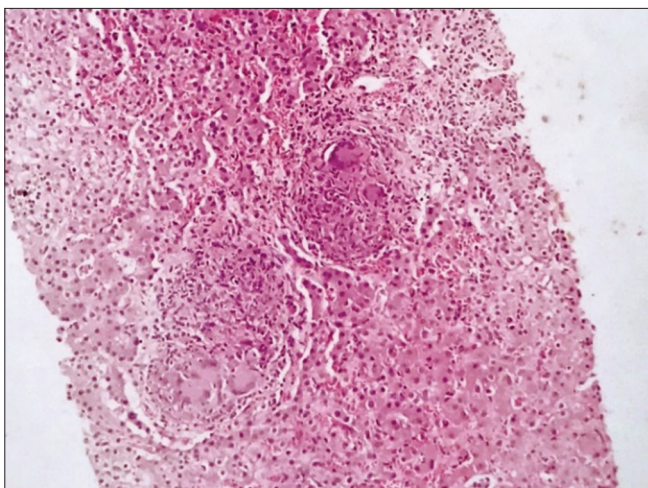


Figure 4: Liver parenchyma with epithelioid granuloma (H&E, x100)

unfamiliar with the technique. Proper antibody dilution and reducing endogenous enzyme interference with peroxide-blocking reagents are necessary to reduce the background staining.¹⁵

Another major limitation of polyclonal *M. tuberculosis* antibody is the cross-reactivity with non-TB mycobacteria like *Mycobacterium avium*, *Mycobacterium phlei*, and *Mycobacterium*

Table 8: ZN positivity in extrapulmonary TB

| Study | Sample size | Positivity rate (%) |
|--------------------|-------------|---------------------|
| Luo | 137 | 34.3 |
| Nassaji et al. | 226 | 26.1 |
| Cagatay et al. | 252 | 17.8 |
| Chakravorty et al. | 76 | 3.9 |
| Salian et al. | 60 | 25 |
| Ajantha et al. | 182 | 3.3 |
| Padmavathy et al. | 50 | 0 |
| Mukherjee et al. | 50 | 44 |
| Present study | 50 | 12 |

ZN: Ziehl-Neelsen, TB: Tuberculosis

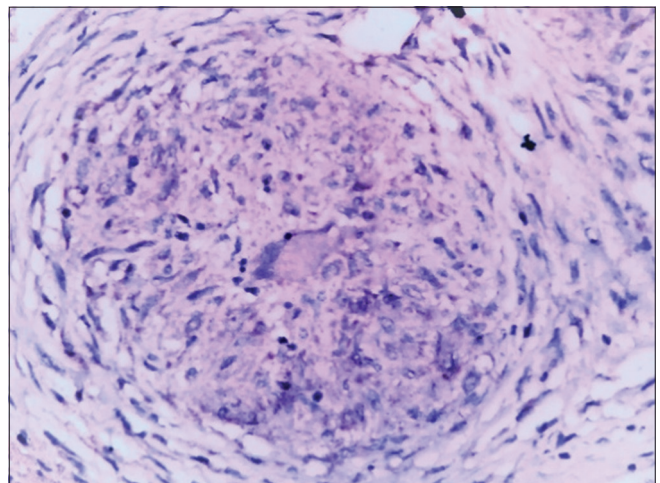


Figure 5: IHC: Mild intensity staining – Score 1 (x400)

parafortuitum, which can be overcome by using species-specific monoclonal antibody. IHC positivity in comparison with other studies is tabulated in Table 7.^{15,16} ZN staining positivity in comparison with other studies is tabulated in Table 8.¹⁷

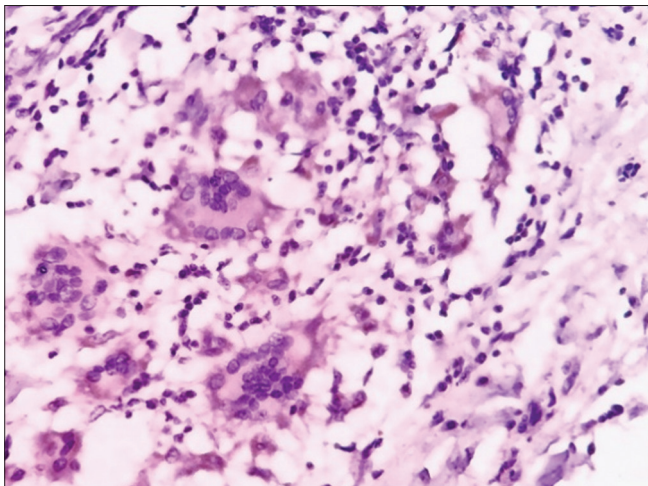
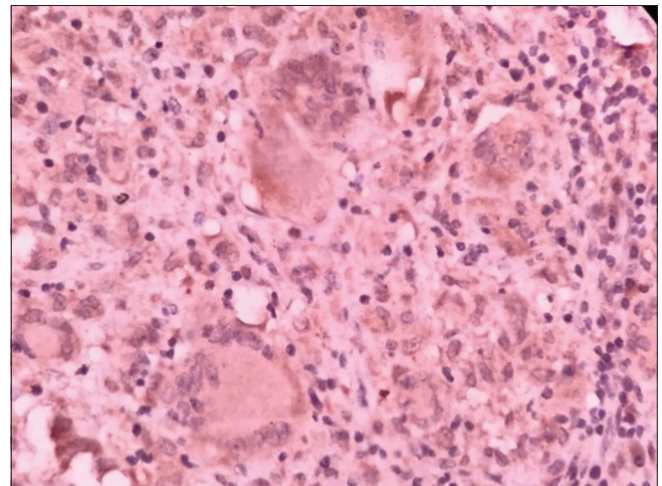
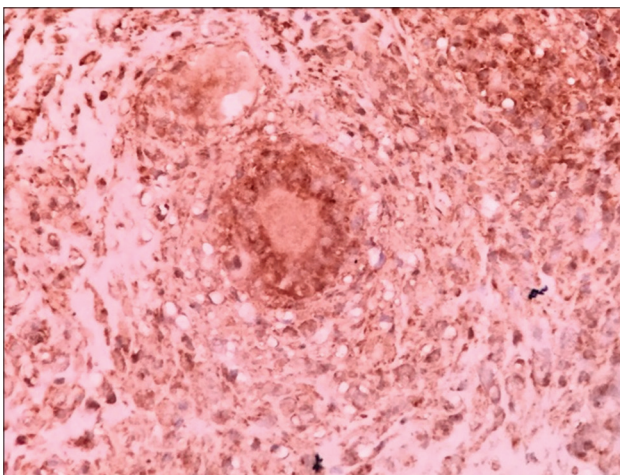
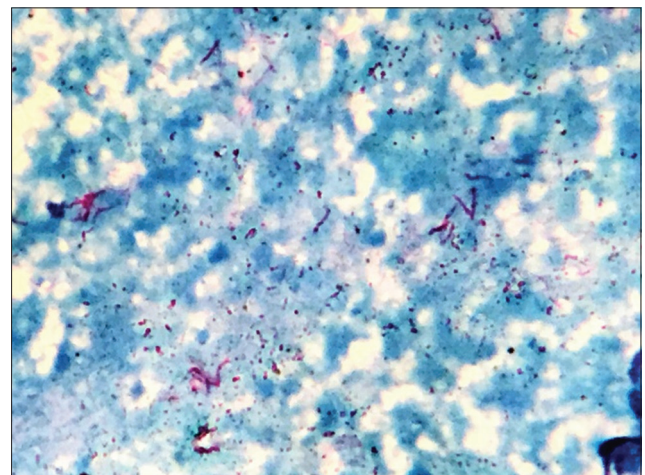
Literature has shown that studies have been done to compare the results of ZN staining and immunohistochemistry, which is shown in Table 9.¹⁸

With ZN staining, the sensitivity varies across a large spectrum from 0% to 45%, making the test less reliable. The sensitivity of the test largely depends on the load of bacilli with at least 10,000 bacilli per mL of specimen required for the stain to demonstrate the bacilli, which is

Table 9: Comparison of IHC analysis and ZN staining

| Study | Method and antibody used | Sample size | IHC positivity (%) | ZN staining positivity |
|----------------------|---|-------------|--------------------|------------------------|
| Radhakrishnan et al. | Peroxidase-anti-peroxidase; IgG anti-mycobacterial antibody | 10 | 100 | 0 |
| Mukherjee et al. | Avidin-biotin complex; polyclonal anti-BCG | 50 | 87 | 44 |
| Mustafa et al. | Polyclonal anti-BCG antibody | 55 | 64 | 0 |
| Padmavathy et al. | Indirect immunoperoxidase; polyclonal anti-BCG | 50 | 68 | 0 |
| Kohli et al. | Streptavidin-biotin; polyclonal anti-mycobacterial antibody | 100 | 72 | 23 |

ZN: Ziehl-Neelsen, IHC: Immunohistochemistry

**Figure 6:** IHC: Absent/negative staining in the granuloma (x400)**Figure 8:** Moderate intensity staining – Score 2 – Cytoplasmic granular staining within the epithelioid histiocytes and multinucleated giant cells (x400)**Figure 7:** Strong intensity staining – Score 3 – Cytoplasmic granular staining within the epithelioid histiocytes and multinucleated giant cells (x400)**Figure 9:** Ziehl-Neelsen stain showing acid-fast tubercle bacilli (bright red) in a blue background (oil immersion)

difficult in extrapulmonary TB due to the less bacillary load. Furthermore, ZN stain can only highlight the intact bacilli, not the fragmented bacilli as seen in necrosis.

With immunohistochemistry, as evidenced by studies mentioned earlier, the positivity rate has fared way better than ZN staining. In our study too, Immunohistochemistry has been shown to be a better

test than ZN staining and can highlight the intact as well as the fragmented bacilli within the necrotic areas and the IHC staining intensity remains unaffected by treatment.

Limitations of the study

The use of polyclonal Mycobacterium tuberculosis antibodies is limited by cross-reactivity with non-

tuberculous species such as *M. avium*, *M. phlei*, and *M. parafortuitum*, which can be mitigated by employing species-specific monoclonal antibodies.

CONCLUSION AND RECOMMENDATIONS

Immunohistochemistry can be a reasonable if not better alternative to ZN staining or as an adjunct in the diagnosis of extrapulmonary TB. It is a simple, robust, cheap, and sensitive test that can be employed in routine pathology laboratories where results can be available in a day, prompting early diagnosis and treatment, as opposed to the culture which takes weeks to obtain a result or polymerase chain reaction which is quite costly and not available in many of the places in developing countries. Thus in areas where Xpert MTB/RIF is not available or not affordable by the patients, IHC can be an effective alternative which can reduce the time to treatment. Besides aiding in the diagnosis, immunohistochemistry also helps in avoiding misdiagnosis, preventing the unwarranted empirical use of anti-TB drugs.

Further studies with large sample sizes employing immunohistochemistry, and comparison with polymerase chain reaction as the gold standard may be necessary to validate the sensitivity of immunohistochemistry. The use of a species-specific monoclonal antibody as opposed to the polyclonal antibody can help in distinguishing positivity due to cross-reaction with non-TB mycobacteria. Immunohistochemistry can be a useful and cheap adjunct in the rapid diagnosis of extrapulmonary TB.

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Author's Contributions:


VRG – Definition of intellectual content, Literature survey, prepared first draft of a manuscript, implementation of the study protocol, data collection, data analysis, manuscript preparation and submission of article; **KS** – Concept, design, clinical protocol, manuscript preparation, editing, and manuscript revision; **GP** – Design of study, statistical analysis and interpretation; **VTM** – Review Manuscript, review manuscript; Literature survey and preparation of Figures; and Coordination and manuscript revision.

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