

Research Correspondence

Application of confocal microscopy for detecting tuberculous lymphadenitis

Tuberculous lymphadenitis, a form of extra-pulmonary tuberculosis, is diagnosed through the detection of *Mycobacterium tuberculosis* in tissue samples. Lymphadenitis may be caused by both nontuberculous mycobacteria (NTMs) and *Mycobacterium tuberculosis* complex (MTBC). Here, we highlight the advantage of using a fluorescent dye in combination with Confocal Laser Scanning Microscopy (CLSM) for identifying *Mycobacterium tuberculosis* in histological sections of lymph node tissue after correlating with Clinical, histopathological, and microbiological findings. CLSM enables the differentiation of non-specific fluorescence from the *Mycobacterium tuberculosis* fluorescence through the spectral emission method. This method proved effective even in lymph node tissue samples exhibiting weak staining using the Ziehl-Neelsen method. Overall, the integration of fluorescent dye with CLSM visualisation substantially improves bacillary visualisation, facilitates faster processing, and reduces observer fatigue due to the black background.

Keywords Auramine-rhodamine stain; Confocal microscopy; Extrapulmonary tuberculosis; Tuberculous lymphadenitis; Ziehl-Neelsen stain

Tuberculous lymphadenitis is one of the most common forms of extrapulmonary tuberculosis (EPTB) in India.¹ Accurate detection of *Mycobacterium tuberculosis* remains a significant diagnostic challenge in tuberculous lymphadenitis, as the precise aetiology of these enlarged lymph nodes is often difficult to determine based solely on clinical history, physical examination, and radiographic evaluation.^{2,3} Immunohistochemistry (IHC) using targeted antibody is among the most specific and sensitive microscopic techniques for detecting *Mycobacterium tuberculosis* in lymph node tissue and extrapulmonary tissues. Histochemical reactions using alkaline phosphatase, diaminobenzidine, and avidin-biotin-peroxidase complexes are commonly employed for visualising bacteria. A confocal laser scanning microscopy (CLSM) was used to identify *M. tuberculosis* in lymph node tissues, involving automated high-resolution scanning, image stitching, selection of necrotic and perifocal zones, and 3D reconstruction.^{1,2} Although fluorescent dyes offer superior visualisation of small structures, their use in histochemical detection of *M. tuberculosis* in patient tissues has been limited. This study aims to use the CLSM-based algorithm and fluorescent dye to visualise Mycobacteria in lymph node tissue specimens with weak Ziehl-Neelsen staining, thereby demonstrating its potential for both diagnostic and research applications.

The conventional Ziehl-Neelsen (ZN) staining method is routinely employed but often fails to detect bacilli in cases with low bacterial burdens.^{3,4}

The Auramine-Rhodamine (AR) fluorescent staining technique, when combined with confocal microscopy, provides enhanced visualisation of acid-fast bacilli (AFB) with improved sensitivity and reduced observer fatigue.^{1,3} The current study evaluated the significance of confocal fluorescence microscopy using AR staining in diagnosing tuberculous lymphadenitis and compared its performance with the conventional ZN method.

This study was undertaken at the department of Central Research Facility and Pathology, Dr. D. Y. Patil Medical College, Hospital and Research Centre, Pune, Maharashtra, India, a tertiary care hospital. A total of 18 patients with a clinical suspicion of lymph node tuberculosis were included in the study from December 2024 to July 2025. Informed consent was obtained from TB patients prior to surgery.

The study was approved by the Institutional Ethics Committee, Dr D. Y. Patil Vidyapeeth, Pune, Maharashtra, India. The patient's age ranged from 13 to 66 yr. The study consisted of 6 males and 12 females. The lymph node tissue removed after surgery was used for further histological investigation, following informed consent, and the patients were assured of confidentiality regarding their identity.

A total of 18 patients (8 males and 10 females) aged 13–66 yr presenting with lymphadenopathy were enrolled. Clinical features included an evening rise in temperature, lymphadenopathy, and weight loss. Lymph node biopsies were obtained and processed for both ZN and AR staining. For ZN staining,

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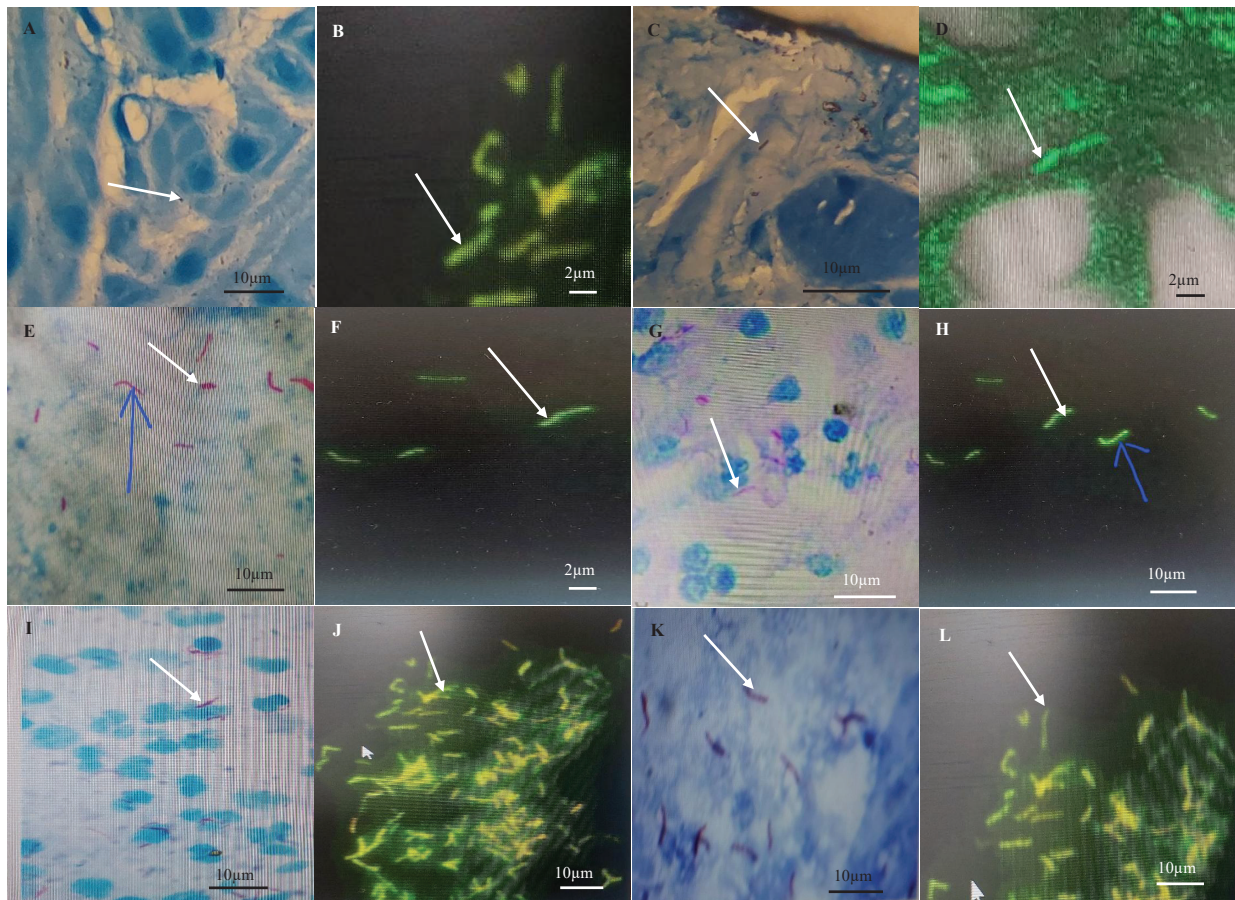


Figure. Tubercle bacilli, (A, C, E, G, I, K) pink indicated by arrow, seen in the first image with ZN stain (100X), using light microscopy shown with white background, and green bacilli (B, D, F, H, J, L) seen in Auramine Rhodamine stain (40X) using Confocal laser scanning microscopy with black background (the portions of the images were zoomed in, and the ImageJ software was used for processing of confocal images).

slides were screened under a light microscope using an oil immersion objective (100 \times). For Auramine–Rhodamine staining, slides were observed using a Carl Zeiss LSM 710 (Carl Zeiss, Germany) confocal laser scanning microscope at 40 \times magnification. Auramine excitation was done using a 405 nm diode laser, and rhodamine was excited using a 561 nm diode laser. The presence of bright green or yellow-orange, fluorescent bacilli were considered positive for *M. tuberculosis*, as correlated with clinical, histopathological, and microbiological findings. Of the included 18 cases, 13 were positive for *M. tuberculosis* by both ZN and AR staining, while five were negative by both methods.

The study included patients aged 13 to 66 yr, comprising eight males and ten females. The most common clinical manifestations were lymphadenopathy (61.7%), weight loss (51.7%), and fever with evening rise (46.7%). Cervical lymph nodes were observed as the most frequently involved group (62.5%). Cytomorphological evaluation most often revealed

caseous necrosis accompanied by lymphocytes and histiocytes, a pattern consistent with tuberculous pathology. Five cases were negative on both ZN and AR staining; these included patients later diagnosed with squamous cell carcinoma, ovarian carcinoma, and autoimmune disorders such as Sjögren’s syndrome.

ZN-stained slides revealed pink acid-fast bacilli at 100X magnification, labelled as 1a-6a, whereas AR-stained slides showed bright green bacilli at 40X, which were zoomed in for the interested regions against a dark background, labelled as 1b-6b, as seen in **Figure**. The Image J software was used for image processing. Confocal imaging enabled zooming into regions of interest (ROI) for confirmation, facilitating easy detection.

Confocal laser scanning microscopy, when used in conjunction with Auramine-Rhodamine staining, enhances diagnostic accuracy in tuberculous lymphadenitis. The bright fluorescence of bacilli

शोध-संदेश

ट्यूबरकुलस लिम्फैडेनाइटिस, जो कि फेफड़ों के बाहर होने वाली टीबी का एक रूप है, की पहचान ऊतक नमूनों में माइक्रोबैक्टीरियम ट्यूबरकुलोसिस (टीबी का जीवाणु) की उपस्थिति से की जाती है। यह संक्रमण नॉन-ट्यूबरकुलस माइक्रोबैक्टीरिया तथा माइक्रोबैक्टीरियम ट्यूबरकुलोसिस कॉम्प्लेक्स के कारण हो सकता है। इस अध्ययन में फ्लोरोसेंट डाई के साथ कॉन्फोकल लेज़र स्कैनिंग माइक्रोस्कोपी के उपयोग को प्रभावी पाया गया, जिससे चिकित्सीय, ऊतक-पैथोलॉजी तथा सूक्ष्मजीव संबंधी निष्कर्षों के साथ मिलान कर जीवाणु की सटीक पहचान संभव हुई। यह तकनीक सामान्य प्रकाश-दीप्ति और जीवाणु की विशिष्ट दीप्ति में अंतर करने में सक्षम है। यह विधि उन नमूनों में भी कारगर सिद्ध हुई, जहाँ पारंपरिक रंगाई विधि (ज़ील-नील्सन) से रंगाई कमजोर थी। अतः, फ्लोरोसेंट डाई एवं कॉन्फोकल माइक्रोस्कोपी का संयुक्त उपयोग जीवाणु की बेहतर पहचान, तेज़ प्रक्रिया तथा पर्यवेक्षक की थकान में कमी लाने में सहायक है।

against a dark background reduces observer fatigue and enables examination at lower magnifications, unlike the Ziehl–Neelsen (ZN) technique, which requires an oil-immersion objective.^{1,4}

In the present study, the Confocal microscopy using the Auramine-Rhodamine technique demonstrated 100% concordance with ZN staining, identifying all 13 ZN-positive cases and correctly excluding all five ZN-negative cases. Notably, bacillary visualisation was markedly improved with confocal fluorescence microscopy using Auramine-Rhodamine staining. The procedure also demonstrated greater time efficiency, as it omits the heating step required for ZN staining.^{5,6,7,8}

These findings are consistent with previous studies, which report a 10-20% increase in detection rates with fluorescent staining compared to conventional light microscopy.^{1,5,6} Additionally, the ability of confocal laser scanning microscopy to provide optical sectioning and three-dimensional imaging facilitates more accurate identification of true bacilli and reduces the likelihood of false-positive interpretations caused by artefacts or debris.⁹ Future investigations involving larger sample sizes are warranted to further validate the sensitivity and diagnostic value of Auramine-Rhodamine staining combined with confocal laser scanning microscopy.

The present study's findings indicate that Auramine-Rhodamine fluorescent staining of lymph node biopsy tissue, examined by confocal laser scanning microscopy, is a reliable adjunct to conventional Ziehl–Neelsen staining for the diagnosis of tuberculous lymphadenitis. This technique offers distinct advantages, including enhanced bacillary visualisation, faster processing, and reduced observer fatigue due to the black background. Although the sensitivity of AR staining is comparable to that of ZN staining, its superior visualisation capacity is valuable in cytology-based samples, such as fine-needle aspiration (FNAC).^{1,10} Further large-scale studies in Indian laboratory settings are recommended to assess the diagnostic utility and cost-effectiveness of incorporating these methods into routine practice.

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Bhagyashri Patil-Takbhate,¹ Charusheela Gore² and Srikanth Tripathy^{1*}

¹Central Research Facility and ²Department of Pathology, Dr. D. Y. Patil Medical College, Hospital and Research Centre, Dr. D. Y. Patil Vidyapeeth, Pune, Maharashtra, India

*For correspondence:
director.medicalresearch@dpu.edu.in

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