

Advances in Methods for Diagnosis of Chronic Mycobacterial Infections of Gastrointestinal Tract

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ABSTRACT

Abdominal tuberculosis (TB) is an important public health problem in developing countries. Because of overlap in the signs and symptoms of the chronic mycobacterial diseases like intestinal tuberculosis (ITB), Crohn's disease (CD), ulcerative colitis, and other inflammatory diseases, there is a need to arrive at a specific diagnosis. Several investigations like computed tomography scan, different endoscopy procedures, ascitic fluid adenosine deaminase (ADA), tuberculosis polymerase chain reaction (TB-PCR), GeneXpert, laparoscopy, etc., are being increasingly used to diagnose TB. Advances in imaging methods and direct access to affected sites by endoscopy have made significant contribution in improving the diagnosis. A combined evaluation of clinical features, endoscopy, histology, and response to treatment has been recommended to differentiate between CD and ITB. Various studies show that clinical features and histopathology, especially granuloma characteristics, have a major role in moving toward specific diagnosis of these conditions. Development of a large number of probes and gene amplification (different variants of PCR and isothermal methods) for TB and other mycobacteria has provided very powerful tools. If used properly they can significantly help in arriving at specific diagnosis of chronic mycobacterial diseases of intestinal tract. Detection of mycobacterial genetic/antigenic components in biopsies by *in situ* hybridization (ISH), *in situ* PCR, and immunohistochemistry (IHC) has been observed to be quite useful in differentiating ITB from CD. A number of newer methods based on expression of angiotensin converting enzyme (ACE), aptamers and biosensors have already appeared on the horizon and have potential diagnostic as well as therapeutic value for various forms of TB including abdominal TB. While many of these approaches/techniques have shown promise, they have not been adequately studied to become part of diagnostic strategy for clinical settings in countries like India.

Keywords: Crohn's disease, Diagnostic methods, Immunohistochemistry, *In situ* hybridization, *In situ* polymerase chain reaction, Intestinal tuberculosis, Probes.

How to cite this article: Katoch VM. Advances in Methods for Diagnosis of Chronic Mycobacterial Infections of Gastrointestinal Tract. J Gastrointest Infect 2017;7(1):26-31.

Source of support: Nil

Conflict of interest: None

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INTRODUCTION

Members of genus *Mycobacterium* cause a variety of afflictions in human beings as well as other animals. While leprosy has characteristic features, there can be a lot of overlap among pathology and clinical symptomatology of diseases caused by *Mycobacterium tuberculosis* as well as many other mycobacteria. Several mycobacterial species have been known to be associated with acute and chronic infections of abdomen and gastrointestinal (GI) tract.^{1,2} Due to therapeutic relevance, the chronic mycobacterial intestinal diseases, TB, and CD deserve special attention. Besides the disease caused by *M. tuberculosis*, there has been tremendous interest about the role of *Mycobacterium paratuberculosis* in the etiology of CD.

Abdominal TB is considered as an important public health problem in developing countries and has been listed as the sixth most frequent site for the extrapulmonary involvement. It can involve any part of the GI tract, including appendix, peritoneum, and hepatobiliary system.³⁻⁵ It has been mentioned that abdominal TB comprises approximately 1 to 3% of all cases of TB and approximately 12% of extrapulmonary tuberculosis (EPTB).⁶

It is believed that *M. tuberculosis* reaches the GI tract via hematogenous spread, ingestion of infected sputum, or direct spread from infected contiguous lymph nodes and fallopian tubes. The clinical manifestations of abdominal TB are varied and can mimic many other disease processes including malignancy,⁷ often causing delay in diagnosis. Countries like Korea and India have guidelines/criteria to diagnose ITB and EPTB including abdominal TB.^{8,9} For timely and proper management, we need to differentiate TB from CD, ulcerative colitis, various phenotypes of inflammatory bowel disease (IBD), malignancy, etc. While ulcerative colitis has chronic diffuse and continuous mucosal inflammation of the colon, CD is a heterogeneous entity comprising several different phenotypes and may involve the entire GI tract. It has been mentioned that most of the clinicians from China, Japan, and Korea use their own national guidelines for IBD diagnosis and those from other Asian countries follow the European Crohn's Colitis Organization's guidelines.¹⁰ Whichever guidelines one may follow, major emphasis has been on resolving the overlap in the presentation of CD and ulcerative colitis. A

change in diagnosis from CD to ulcerative colitis during the first year of illness occurs in about 10 to 15% of cases, which shows the importance of timely and specific diagnosis of these conditions.¹¹ In case of ITB, the most common site of involvement is the ileocecal region, which has been attributed to the low levels of digestive activity, relatively increased physiological stasis, higher rate of fluid and electrolyte absorption, and more lymphoid tissue at this site.⁵ Primary gastric TB also poses a diagnostic challenge; it may mimic peptic ulcer disease, IBD, neoplasia (including malignancy), and other infectious diseases.^{7,12} Abdominal TB has been described to be presenting broadly in three morphological forms: ulcerative, hypertrophic, and combination of both ulcero-hypertrophic form.⁵ Besides common signs and symptoms like fever, weight loss, and abdominal pain, clinical presentation is influenced by these morphological varieties. In HIV patients as well as in some other patients, GI bleeding may be an important manifestation.¹³

There has been extensive clinical experience about diagnosis as well as medical and surgical management of abdominal TB. A lot of information is available from India also,^{14,15} and this work done in the past should serve as a foundation for the future. If diagnosed early, response to standard medical therapy has been described to be excellent in abdominal TB.¹⁶ Like any other disease, clinical suspicion will be the key to success in case of GI tuberculosis (GITB). Mishra et al¹⁷ have stressed on combinatorial diagnostic approach for rapid detection and characterization of GITB. Despite the use of combination of several methods, diagnosis can be confirmed in a maximum of two-thirds of GITB cases.¹⁸ This shows a big gap and scope for innovation and translation on this aspect. The search of various databases shows that recent published data from India is very limited for abdominal TB and it will be appropriate to develop guidelines based on real experience from different parts of the country.

Like other forms of TB, serology cannot be used with confidence to support the diagnosis of TB. The situation is relatively better for tests used to measure cell-mediated immunity/delayed type of hypersensitivity, such as tuberculin and gamma interferon.^{19,20} It has been observed that the T-SPOT.TB assay can be a useful tool for diagnosing EPTB in both the immunocompetent and the immunocompromised patients, including those suffering from tuberculous meningitis, pericarditis, lymphadenitis, and ITB.²¹

Times have been changing for the better. Advances in optics—endoscopy and imaging methods—have contributed significantly to improvements in the diagnosis of GI TB. Several investigations like CT scan, capsule endoscopy, balloon enteroscopy, laparoscopy, ADA,

TB-PCR, GeneXpert, etc., are being increasingly used to diagnose TB.¹⁶

Endoscopy is now considered as an investigation of choice for GITB as it allows for visualization and appropriate sampling of tissue for histology and culture. Such investigations are well complemented by radiological imaging. Findings from endoscopy and radiological imaging are being used by many to diagnose ITB; it is implicit that success will depend on the stage of the disease and the time at which investigations are carried out.²² When colonoscopy is done, it is important to be aware of subtle endoscopic findings that are characteristic for ITB. It has been reported that recognition of such findings may lead to a correct diagnosis of ITB at an early stage.²³

Demonstration of Mycobacterial Components in Tissues

Though the access to affected tissue is still not optimum in case of GITB, it is becoming better and better with the passage of time. Techniques for immunological and molecular detection of *M. tuberculosis* components have become very sensitive and specific. It has been observed that IHC staining with monoclonal antibodies specific to *M. tuberculosis* may be an efficient and simple diagnostic tool in addition to classic examination methods for the diagnosis of ITB.²⁴

During the last three decades, a large number of molecular probes and gene amplification techniques (PCR as well as isothermal) have been developed for the detection of *M. tuberculosis*. *In situ* hybridization and *in situ* PCR methods have also been described for several infectious diseases including TB. While gene amplification assays have been extensively used for pulmonary and certain extrapulmonary forms, experience is limited in case of GITB. The real-time PCR assay has been reported to be highly sensitive and specific for the detection of *M. tuberculosis* complex deoxyribonucleic acid (DNA) in histopathological specimens of GITB.²⁵ Multiplex PCR has also been reported to be highly sensitive and specific for both the ITB and peritoneal TB groups. When combined with histopathology, multiplex PCR could detect 97.5% of all the cases in the ITB group.²⁶ It has been reported that gene amplification methods can vastly improve the detection of *M. tuberculosis* in specimens from abdominal tuberculosis.²⁷ Even then sensitivity of detection remains very low and suggests the need of improving these assays as well. As drug resistance in EPTB has been recently identified as a major problem in India,²⁸ application of molecular methods for detection of drug resistance in such specimens assumes great importance.²⁹ There is, however, very little information in the public domain

about drug resistance in GITB, and the need to generate data from different settings in India is highly desirable.

GeneXpert in ITB: This gene amplification technique has been found to be quite useful for the diagnosis of TB as well as detection of rifampicin resistance. This has been adopted by several national governments, including India, for their national programs. Most of the published experience on GeneXpert is on pulmonary TB. A limited published literature shows that it can also be used for specimens from GITB.³⁰ In a recent report, GeneXpert was found to be useful in confirming the diagnosis of TB of intestine, which was clinically mimicking malignancy.⁷ It is expected that in future more data will emerge on the actual potential of this assay for diagnosis as well as for detection of drug resistance in GITB.

Crohn's Disease vs Intestinal Tuberculosis

Distinguishing CD from ITB has been recognized to be clinically challenging, but is important for patient management. Clinicopathological similarities of ITB with CD have been highlighted as problems in differential diagnosis.^{18,31} A combined evaluation of clinical features, endoscopy, histology, and response to treatment has been recommended to differentiate between CD and ITB.³²

There are different approaches/steps for differentiation of CD from ITB, such as follows:

- *Clinical profile:* It has been observed that weight loss and mucosal nodularity are associated with ITB, whereas abdominal pain and excessive intestinal involvement are more commonly associated with CD.³³ Night sweats, longitudinal ulcers, and granulomas are considered important features to differentiate CD from ITB. Clinical parameters like fever, bleeding per rectum, diarrhea, and duration of symptoms have been reported to have highest accuracy in differentiating CD from GITB.³⁴
- *Granuloma characteristics:* Granuloma is an important feature of chronic mycobacterial diseases including TB.³⁵ Analysis of certain features of granulomas has been observed to be important in differentiating GI tuberculosis from CD. Granulomas exceeding 300 µm in maximal diameter, >5 granulomas/section, and confluent granulomas were observed to be more frequently present in ITB than in CD ($p < 0.05$).³⁶ In another study, granulomas in ITB cases were also observed to be larger (mean widest diameter 508 ± 314 µm; range 100–1100 µm) than those in CD cases.³⁷ While such granuloma characteristics appear to be promising markers to distinguish CD from ITB, clearly there is a need to have multicentric studies to decide on cutoffs for correlating with the level of evidence.

- *Anti-Saccharomyces cerevisiae antibodies (ASCA):* In some studies, ASCA were reported as good biomarker for the diagnosis of CD.³⁸ On the contrary, ASCA immunoglobulin (Ig)G and ASCA IgA were not observed to be of help to differentiate between ITB and CD in the study reported by Makharia et al.³⁹
- *Tissue ACE:* Assessment of tissue ACE expression has been reported to be helpful for the differential diagnosis of CD and ITB.⁴⁰ More data need to be generated before recommending this for clinical application.
- *T-SPOT.TB:* This enzyme-linked ImmunoSpot assay has also been a valuable assay in differentiating ITB from CD, particularly in the diagnostic exclusion of ITB based on its high specificity and negative predictive value.⁴¹

Mycobacterium paratuberculosis as causative agent of CD: For quite some time, CD was considered as a nonspecific chronic transmural inflammatory disease associated with a frameshift mutation in the *NOD2* gene. However, several researchers have observed the presence of *M. paratuberculosis* within the intestinal tissues of CD patients and linked it with the etiology of the disease. The rate of detection of *M. avium* subsp. *paratuberculosis* in individuals with CD has been reported to be highly significant, thereby implicating this mycobacterial pathogen in disease causation.⁴²

Morphological characteristics of M. tuberculosis vs. M. paratuberculosis: As *M. paratuberculosis* is much smaller than *M. tuberculosis*, this differentiation has been suggested as an important characteristic relevant for presumptive identification of these pathogens in the tissues, using oil-immersion microscopy ($\times 1000$ magnification).⁴³

Immunohistochemical staining: IHC staining of biopsy specimens with anti-VP-M660 antibody has been reported to be a simple and fast technique with 73% sensitivity and 93% specificity for establishing an early differentiation of TB from CD.⁴⁴

Multiple antigenic peptide (MAP)-specific DNA: The frequency of MAP-specific DNA in biopsies by quantitative PCR has been found to be significantly higher in CD patients (23.2%, $p = 0.03$) as compared with controls (7.3%). However, no significant difference in intestinal MAP presence was observed between ITB patients (12.5%, $p = 0.6$) and controls (7.3%). Using IHC for detection of MAP antigen, the prevalence of MAP in CD was 2.9%, 12.5% in ITB patients, and 2.4% in controls. Thus, there is an apparent problem with the detection of *M. paratuberculosis* directly or through IHC.⁴⁵

Fecal TB-PCR: Fecal TB-PCR has been suggested as a good screening test to distinguish ITB from CD.⁴⁶ The TB-PCR test combined with characteristic histopathologic features have been reported to be useful in the differential

diagnosis of ITB and CD in endoscopic biopsy samples.⁴⁷ A recent meta-analysis concluded that PCR for *M. tuberculosis* is a promising and highly specific diagnostic method to distinguish ITB from CD.⁴⁸

In situ hybridization: ISH is a sensitive and specific method to demonstrate the presence of DNA/RNA of any pathogen directly in diseased tissue. Sechi et al⁴⁹ used an adapted ISH technique to detect IS900 *M. paratuberculosis* DNA in paraffin-embedded tissue from CD tissue samples. Demonstration of the presence of cell wall-deficient *M. avium* subsp. *paratuberculosis* in 35 of 48 paraffin-embedded tissue specimens from 33 patients with CD by ISH with IS900 as a probe⁴⁹ should be considered as a landmark in progressing toward establishing the etiology of CD. In another study, Sechi et al⁵⁰ were also able to identify *M. paratuberculosis* DNA in around 69% of the paraffin-embedded intestinal samples of CD patients analyzed.

Subsequently *M. avium* subsp. *paratuberculosis* was detected in a majority of Sardinian CD patients.⁵¹ Pulimood et al⁵² have also opined that *in situ* PCR can be potentially useful to differentiate ITB from CD, if sensitivity is improved.

Aptamers in Diagnosis and Therapeutics

Aptamers, the small biomolecules (oligonucleotides or peptides) that bind to specific targets, have been considered as highly promising tools for diagnosis and therapy as well. They can be selected from a large pool of randomly chosen biomolecules or can be selectively designed. Aptamers can have very high affinity and also have capacity to penetrate deep into the tissues.

Aptamers for diagnosis of tuberculosis: During recent years, aptamers have been considered promising tools for developing point-of-care diagnostic assays for TB. Though no specific use of aptamers in improving the diagnosis of GITB has been investigated, the potential is immense. Species-specific aptamers to effectively capture or discriminate *M. tuberculosis* strains have been developed.⁵³ Mozioglu et al⁵⁴ selected single-stranded DNA (ssDNA) aptamers that recognize *M. tuberculosis* H37Ra through systematic evolution of ligands by exponential enrichment (SELEX). Tang et al⁵⁵ also generated ssDNA aptamers "antibodies" against mannose-capped lipoarabinomannan of the predominant clinical epidemic *M. tuberculosis* Beijing genotype strains by SELEX technique. Anti-*M. tuberculosis* aptamers have been found to be successful in differentiating *M. tuberculosis* from closely related species.⁵⁶

Aptamers in imaging: For almost one decade, aptamers have been explored for the imaging of tumors.^{57,58} Lele⁵⁹ and his colleagues at Haffkine Institute have started studies on the use of labeled aptamers in TB imaging and

highlight the importance of this approach to monitor the disease activity in the tissues.

Biosensors: While promising leads are already there, this knowledge needs to be available in the field. The development and testing of biorecognition agents (antibodies and aptamers) and design of electrocatalysts, researchers in biosensor design may need to evolve focused research efforts toward development and deployment of low-cost biosensors.⁶⁰ Published data show that Mtb36 aptamers are highly selective for *M. tuberculosis*, and can be used in an aptamer-based biosensor approach for the detection of *M. tuberculosis*.^{61,62} Such low-cost biosensors will have immense potential for TB. It is hoped that biosensors for field application will be available in future for EPTB, including abdominal TB.

Aptamers in therapy of tuberculosis: Strong basis for development of aptamers as novel and strong anti-TB agents has been recently described.⁶² Aptamers have also been experimentally found to be useful in animals (mice and Rhesus monkeys) in reducing progression of TB infection.^{56,61} One of the mechanism of action could be better presentation of *M. tuberculosis* antigens through dendritic cells.⁵⁶

Looking at the Future

This mini review shows that the potential of several techniques in improving the diagnosis of GI mycobacterial infections has been demonstrated. This presentation may or may not be comprehensive, but it is not difficult to conclude that potential of newer approaches, including molecular methods, has not been adequately explored for GITB and CD especially in our Indian settings. There is a great need to have India-specific guidelines by pooling of recent experiences involving clinical, histopathological, imaging, molecular, and immunological techniques targeting demonstration of mycobacterial components in the tissues by using molecular and immunological reagents. Use of aptamers with appropriate biosensors and other imaging techniques can become a game changer for all forms of TB, especially EPTB. Of course, GITB needs to be specifically addressed. During recent years, the Indian Council of Medical Research (ICMR), New Delhi, has initiated multicentric studies on these and related aspects. In addition, in-depth studies will be required using these molecular methods on epidemiology of diseases like CD in humans and Johne's disease in animals to prove or rule out the zoonotic link.⁶³ There are joint ICMR and Indian Council of Agricultural Research (New Delhi) mechanisms, as part of intersectoral coordination through Government of India, Department of Health Research, to undertake such studies. The future is, thus, full of opportunities to make the diagnosis of GITB and CD much more

robust than it is today and develop good evidence-based guidelines for their effective management.

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