Visualization of *Mycobacterium avium* in Crohn’s tissue by oil-immersion microscopy

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**Abstract**

Studies seeking *Mycobacterium avium* subsp. paratuberculosis in Crohn’s disease by PCR have generated inconsistent findings. As an alternative, microscopy offers a number of advantages, including direct visualization of organisms in tissue. Experimental infections have demonstrated that *M. avium* organisms can be seen by both acid-fast staining and species-specific *in situ* hybridization, but because they are smaller than *M. tuberculosis*, oil-immersion microscopy (∼1000 magnification) is needed. We performed a blinded search for *M. avium* in paraffin-embedded surgical resections from Crohn’s and control subjects at two centres. Specimens were coded and subjected to acid-fast staining and ribosomal RNA *in situ* hybridization for *M. avium* rRNA. Agreement between these two methods was good (42/52 patients, κ = 0.60) and similar results were observed for patients from two centers. Together, both methods provided positive results in 10 of 17 Crohn’s subjects (59%, 95% CI: 36–78), contrasting with only 5 of 35 control subjects (Odds ratio for Crohn’s vs. controls = 8.6, p = 0.002). *M. avium* organisms had an intracellular localization within inflammatory lesions, but were often observed as lone organisms outside of granulomas. Using two assays in two settings, presence of *M. avium* organisms was strongly associated with Crohn’s disease.

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**Keywords:** *Mycobacterium avium,* Crohn’s disease, Microscopy, *In situ* hybridization

1. Introduction

Crohn’s disease (CD) is a debilitating inflammatory bowel disease of unknown cause. Current etiological theories of CD focus on an interaction between host susceptibility and microbial factors, with whole genome association studies implicating genes involved in the recognition of intracellular bacteria [1–3]. Among such bacteria, *Mycobacterium avium* subsp. *paratuberculosis* (MAP) has long been investigated in CD, because of histopathological similarities between CD and veterinary MAP disease [4].

Despite decades of searching for MAP in tissue of CD patients, this hypothesis remains neither confirmed nor refuted. Much of this uncertainty stems from inconsistent results obtained from different detection methods applied [5,6]. Due to a number of challenges with isolating this fastidious organism in culture, most recent efforts have largely employed molecular methods, looking for the presence of a MAP genetic element, called insertion sequence 900 (IS900) [7,8]. However, findings from PCR-based studies have varied widely across centers [9], likely indicative of technical issues with applying non-standardized PCR and nested-PCR protocols [10]. Moreover, the finding of a positive PCR result in CD tissue poses a quantitative dilemma. A decade of experience with molecular diagnostics has shown PCR to suffer poor sensitivity in microscopy-negative mycobacterial diseases [11]. One
should not expect a high proportion of samples to be positive by PCR in the absence of microscopic evidence of infection.

An alternative possibility is that a subset of CD tissue is microscopy-positive, using a tailored microscopic technique. This possibility became evident during an evaluation of different in situ modalities for detecting mycobacteria in tissue, where we observed lone MAP organisms to be shorter and less rod-shaped than M. tuberculosis [12]. Consequently, we noted that visualization of these 1–1.5 micron coccobacilli requires oil-immersion microscopy (×1000 magnification). This observation prompted us to review the historical literature on in situ detection of mycobacteria in CD. The initial description of CD, in 1932 [13], came decades before M. avium complex (MAC) organisms were first recognized in human samples [14]. Earlier efforts to identify mycobacteria in CD employed ×400 magnification, which is sufficient to detect the long and distinctively-shaped M. tuberculosis (3–4 microns) but likely to overlook MAP. Upon further search, we could not find any published material that employed ×1000 magnification to search for short acid-fast organisms in CD, consistent with practice guidelines that recommend ×400 magnification [15].

Therefore, based on experience gained through study of natural and experimental MAP infection, we sought mycobacterial forms in CD by blinded analysis of surgically-resected samples, using oil immersion microscopy. Since established mycobacterial diseases, such as tuberculosis and leprosy, have at least a minority of cases that are microscopy-positive, we hypothesized that if MAP is present in CD, a subset of cases should be microscopy-positive.

2. Materials and methods

2.1. Clinical tissue samples and blinding

The study employed samples from two centers, the McGill University Health Centre, (Montreal, Quebec, Canada) and the McMaster University Medical Center (Hamilton, Ontario, Canada) and the study protocol was approved at each center by the hospital institutional review board. For brevity, we will refer to these throughout as Site A and Site B. At each site, paraffin-embedded blocks of surgically-resected full thickness intestinal tissue samples were collected from archives and coded prior to specimen sectioning, to perform a study that was blinded until completion of microscopy. A total of 57 samples (26 samples from 22 patients from Site A and 31 samples from 30 patients from Site B) from 52 patients were obtained. The 52 patients were categorized into three main categories; CD, ulcerative colitis (UC) and non-inflammatory bowel disease (non-IBD) according to clinical reports of the treating gastroenterologist and histopathologic review of these same samples. In total, tissue samples from 17 CD patients, 5 UC patients and 30 non-IBD patients were included in this study.

2.2. Acid-fast staining and in situ hybridization (ISH)

2.2.1. Tissue sectioning and conventional staining

For detection of acid-fast organisms within the context of histopathological changes, serial 3–4 µm sections from each paraffin embedded tissue specimen were cut, placed on silane-coated microscope slides (Sigma-Aldrich) and incubated in an oven at 50–60 °C overnight to ensure maximum tissue adhesion on the slide. The first two serial sections were subjected to haematoxylin and eosin, and acid-fast staining, respectively. Site A samples were stained using standard Ziehl-Neelsen (ZN) method for tissue by the pathology laboratory of Montreal General Hospital [16,17]. Site B samples were subjected to Fite’s stain, a laboratory variant of the ZN method for tissue samples [18].

2.2.2. In situ hybridization

A third serial section was subjected to in situ hybridization (ISH), targeting the bacterial ribosomal RNA. This method has been previously validated to identify mycobacteria in tissue, specific to the species level, based on differences between mycobacterial RNA sequences across species [12,19]. Hence, these probes are able to distinguish M. avium from M. intracellulare, M. tuberculosis, M. kansasii, and other mycobacteria, however, since MAP shares identical sequence with other M. avium organisms, they cannot be used to distinguish among sub-species of M. avium. We selected rRNA-based ISH, instead of targeting the IS900 element of MAP as has been done by others [20], because the IS900 probe has been shown by ourselves and others to be prone to the generation of non-specific signals [12,19].

Briefly, sections were de-paraffinized and then rehydrated through graded alcohol. Cells were rendered permeable by incubation in 0.02 M HCl and then in 0.01% Triton X-100. Then, proteins were depleted by incubation with proteinase K (Sigma-Aldrich) (5 µg/ml, for 30 min at 37 °C). Free nucleic acid in the cells was fixed with 4% formaldehyde to prevent washing off. Slides were washed in PBS and dehydrated through graded alcohol and air-dried, then incubated with M. avium probes (1 ng/µl), labeled at the 5′ and 3′ ends with 6-carboxyfluorescein (FAM), in hybridization buffer. Following hybridization, slides were washed and colorimetric signals were developed as described earlier [12].

2.2.3. Controls for nucleic acid staining assays

Paraffin blocks of samples of mice intravenously infected with MAP K10, which resulted in paucibacillary infection in our previous experiment, were included as a positive control in the assays. To test for non-specific binding of probes, we had two controls. First, negative control tissue samples from mice were also subjected to in situ hybridization with M. avium specific DNA probes as previously described [12]. Second, 7 CD tissues positive for both ZN and ISH were independently re-tested, probing slides processed in parallel for M. avium rRNA and M. tuberculosis rRNA [19]. These slides were then coded for reading, with the reader blinded to which molecular probe had been employed.

2.3. Reading and interpretation of microscopic results

To look for acid-fast and ISH signals, the primary reader (M.J.) first studied haematoxylin and eosin stained slides by Zeiss Axioscope microscope (Carl Zeiss), to mark areas of
inflammation (including focal and diffuse inflammation, and granulomas). Corresponding sites on ZN- and ISH-stained slides were examined by under oil immersion objective giving a final magnification of \( \times 1000 \). Before reading each test slide, a positive control slide of acid-fast stain and ISH was visualized to assure appropriate microscopic technique and remind the readers of the \textit{in situ} morphology of \textit{M. avium} organisms. Because the acid-fast stain does not provide information on the mycobacterial species, we considered a positive result to represent mycobacteria, with no speciation implied. In the case of ISH, because our probe cannot distinguish MAP from other sub-species of \textit{M. avium}, we considered a positive result to represent \textit{M. avium} complex (MAC). To be conservative in our assignment of a positive result, test sections were considered positive when mycobacteria were observed in at least 10 oil-immersion fields per sample, double the threshold typically applied in mycobacteriology laboratories. When there was no or minimal inflammation noted on haematoxylin and eosin stained slides, the entire mucosa was systematically scanned under oil immersion for at least 20 min. As a further verification of the primary reader’s interpretation, we selected 9 samples for independent assessment by a second reader (J.R.), who was blinded both to the diagnosis and the primary interpretation.

2.4. Data analysis

Prior to un-blinding study subject information, we determined the degree of agreement between ZN and ISH-based study, and between readers, using the Cohen’s kappa statistic. Having ascertained that there was moderate to strong agreement between the two methods, we chose to apply the most conservative criterion for a positive result, wherein samples positive by both staining methods (ZN and ISH) were considered positive for MAC organisms, while samples negative by one or both methods were considered negative.

Upon un-blinding patient information, we determined that we had read two different blocks for 5 patients (4 at Site A and 1 at Site B). To achieve a series based on unique patients, not samples, those controls we instead retained the most positive result for analysis. In the case of ISH, because our probe cannot distinguish MAP from other sub-species of \textit{M. avium}, we considered a positive result to represent \textit{M. avium} complex (MAC). To be conservative in our assignment of a positive result, test sections were considered positive when mycobacteria were observed in at least 10 oil-immersion fields per sample, double the threshold typically applied in mycobacteriology laboratories. When there was no or minimal inflammation noted on haematoxylin and eosin stained slides, the entire mucosa was systematically scanned under oil immersion for at least 20 min. As a further verification of the primary reader’s interpretation, we selected 9 samples for independent assessment by a second reader (J.R.), who was blinded both to the diagnosis and the primary interpretation.

3. Results

3.1. Patients

The demographic information of patients and the site of tissue samples used in this study are summarized in Table 1.

3.2. Detection of MAP in tissue samples of Crohn’s and controls

3.2.1. Acid-fast staining vs. rRNA ISH method

The two staining modalities used target different components of the bacterium, specifically the cell wall (ZN) and ribosomal RNA (ISH), and demonstrate by both methods that MAP organisms are considerably shorter than \textit{M. tuberculosis} (Fig. 1). When applying these methods to samples for the 52 analyzed patients, discordant results between acid-fast staining and ISH were obtained in 42. The 10 discordant results were ZN+/ISH− in 1 and ZN−/ISH+ in 9. Overall agreement was therefore calculated as 81% with a \( k = 0.60 \). Of the discordant samples (ZN+/ISH−), three were noted to have a few forms consistent with acid-fast organisms but had not met the minimal criterion of 10 positive oil immersion fields to be classified as ZN-positive. In contrast, in samples negative by both methods, such borderline results were not observed. To assess for inter-reader agreement, a second reader (a pathologist) read 9 samples; for which the primary reader had judged 5 to be positive. The two readers agreed in 7 of 9 instances (\( k = 0.57 \)), with the two exceptions representing samples read as positive by the primary reader but negative by the secondary reader.

3.2.2. Site A samples

Comparison of microscopy to subject classification for Site A is presented in Fig. 2a. In contrast to controls, where three-quarters of samples (9/13) were negative by one or both methods, MAC organisms were seen in about one-half (5/9) of CD surgical samples (OR for Crohn’s vs. controls = 2.8, \( p = 0.4 \)). A discordant (ZN−/ISH+) result was obtained for a further 3 CD patients, so in all, 8/9 CD patients were positive by ISH. Of the four controls with doubly positive results, two represented diseased ileum in patients clinically classified as having UC, one had colorectal cancer and one had a sigmoid volvulus.

3.2.3. Hamilton sample

Comparison of microscopy to subject classification for the Hamilton subjects is presented in Fig. 2b. In this case, over 90% (21/22) of control samples were negative by one or both methods, but MAC organisms were observed in about one-half (5/8) of CD samples (OR for Crohn’s vs. controls = 35.0, \( p < 0.001 \)). A discordant (ZN−/ISH+) result was obtained.

Table 1 Demographic information and anatomic site of tissue samples

<table>
<thead>
<tr>
<th>Site</th>
<th>Study group</th>
<th>Number of patients</th>
<th>Age (median)</th>
<th>Sex (% Female)</th>
<th>Tissue examined (Colon: Ileum: Other*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site A</td>
<td>CD</td>
<td>9</td>
<td>34</td>
<td>64.4</td>
<td>2:6:1</td>
</tr>
<tr>
<td></td>
<td>UC</td>
<td>5</td>
<td>41</td>
<td>34.4</td>
<td>2:2:1</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>8</td>
<td>70.5</td>
<td>50</td>
<td>6:2:0</td>
</tr>
<tr>
<td>Site B</td>
<td>CD</td>
<td>8</td>
<td>38.5</td>
<td>37.5</td>
<td>2:5:1</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>22</td>
<td>67</td>
<td>63.6</td>
<td>8:14:0</td>
</tr>
</tbody>
</table>

CD = Crohn’s disease, UC = Ulcerative Colitis, Other = tissue from patients with cancer, except one from Montreal with sigmoid volvulus. *Details of samples classified as other provided in results.

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for a further 2 CD patients, so in all, 7/8 CD patients were positive by ISH. Only one of the control samples was doubly positive, this consisted of the terminal ileum of a patient with colorectal cancer. In addition, one control sample harbored acid-fast forms but was negative for *M. avium* rRNA labeling; this patient had a diagnosis of colorectal cancer.

### 3.2.4. Molecular specificity of in situ hybridization results

To verify the species-specificity of our findings, seven CD samples positive by both methods (ZN and ISH) were independently re-probed, using both a *M. avium* probe and a *M. tuberculosis* probe. These samples were then coded for re-reading, blinded to previous interpretation and blinded to the identity of which probe had been used. For the slides stained with the *M. tuberculosis* probe, all 7 were called negative. In contrast, of 7 samples probed for *M. avium*, positive signals were reported in 6.

### 3.2.5. Pooled results and statistical analysis

Comparison of microscopy to subject classification for subjects combined across sites revealed that 10/17 CD patients were doubly-positive by microscopy, compared to 5/35 controls (Odds ratio for Crohn’s vs. controls = 8.6, *p* = 0.002). Comparing CD samples to non-IBD controls, the *OR* = 12.9, *p* < 0.001. Together, we visualized mycobacteria by both methods in 59% of CD samples (95% CI: 36–79).

### 3.3. Cellular localization of acid-fast organisms and ISH signals

In addition to serving as a detection method, in situ modalities permit localization of MAC organisms in the context of histopathology. Fig. 3a shows the appearance of acid-fast-stained MAP organisms in the liver of an experimentally-infected mouse, providing a control for visualization of these organisms in situ. Fig. 3b depicts tissue from a CD patient, stained and imaged in the same manner, showing the same bright pink structures in intracellular location. Of note, when comparing ZN staining to the corresponding section stained with haematoxylin and eosin, the acid-fast forms were typically not in granulomas, but rather were localized mainly in diffuse inflammatory areas in the submucosa. Fig. 3c shows the appearance of rRNA-labelled MAP organisms in the spleen of an experimentally-infected mouse, providing a control for visualization of these organisms in situ. The adjacent image demonstrates signals with similar staining in Crohn’s tissue (Fig. 3d). The same tissue, probed for *M. tuberculosis* rRNA, did not reveal these forms (Fig. 3e). *M. tuberculosis*-infected murine tissue, probed for *M. tuberculosis* rRNA, is shown as a positive control for this probe (Fig. 3f).

### 4. Discussion

Using two different staining methods, we have demonstrated that mycobacteria are strongly associated with CD in two...
Fig. 2. Comparison of the percent of microscopy results positive for patients at Site A (panel a) and Site B (panel b) as a function of assigned diagnosis. 2+: positive by both methods; 1+: positive by one method only; -/: negative for both methods.

different centres. These findings are supported by two technical considerations. First, newer methods for staining bacterial rRNA have provided an independent molecular target from cell wall based stains, permitting a study employing both techniques [19,21,22]. Second, morphometric analysis of MAP bacteria during infection has demonstrated that these organisms are smaller and less rod-shaped than the tubercle bacillus, necessitating searching at a high-power magnification for optimal visualization [12]. By applying these lessons, we were able to determine that MAC organisms are not commonly found in control tissue, in contrast to Crohn’s tissue, where these bacteria were observed in just over one-half of samples.

While these findings may have implications for the diagnosis and pathogenesis of this disease, a number of limitations of this study bear mentioning. First, subjects in our study groups were not age and tissue matched. Epidemiological observations indicate that CD affects a younger age group than colorectal cancer, in agreement with the age of the CD group in this study. Second, the site of tissue samples varied across the study groups as a consequence of the biology of these diseases; the majority of CD patients have ileal disease, unlike UC and colorectal cancer. However, when examining only colonic samples from CD patients, we still observed forms consistent with MAC by both methods in 3 of 4 samples, indicating that our findings were not ileum-specific. Third, the use of probes targeting 16S and 23S rRNA allowed us to detect MAC organisms in situ, but did not permit us to discriminate MAP (which is M. avium subsp. paratuberculosis) from other M. avium subspecies (such as M. avium subsp. avium). We accepted this limitation because our goal was to apply a probe of high diagnostic specificity (as defined by the absence of false-positive signals) rather than a probe targeting a MAP-specific element that is susceptible to non-specific binding [19,23]. Fourth, because of the small size of these organisms, it is possible that similar-staining host artifacts, such as organelles, have occasionally been interpreted as mycobacteria. We consider that the binding strategy partially addressed this concern, unless such artifacts are more common in CD tissue. We therefore re-probed samples positive for M. avium using M. tuberculosis-specific probes, and here we did not observe positive signals, arguing again that the forms we are observing are truly M. avium organisms. Finally, the detection method outlined was arduous and time-consuming, especially in the case of negative results that required examination of full tissue sections under oil-immersion for at least 20 min. Whether this method can be employed routinely in clinical laboratories remains to be determined, but we are confident that this approach is applicable to epidemiologic studies where appropriate time and effort can be devoted.

In order to look for evidence of MAC organisms in Crohn’s tissue we intentionally imposed conservative criteria, requiring signals in at least 10 oil immersion fields by both methods to assign a positive result. However, to be sure we detected all organisms present, we did not use granulomas to performed a guided search for mycobacteria [24], because organisms in paucibacillary MAP disease of ruminants and tuberculoid leprosy of humans are not confined to granulomas [25—29]. Applying the threshold of 10 positive fields, nearly one-half of CD patients were negative, indicating either the absence of mycobacteria or a bacterial burden less than 10 bacilli per milligram of tissue [12]. Consistent with this, for tuberculosis and leprosy, one-half or more of patients are microscopy-negative [30—32]. Additionally, the observation of positive results in a few controls requires further clarification. For these subjects, two possible explanations are: 1) either a small percentage of patients assigned a diagnosis other than CD harbored MAC organisms in their tissue; or 2) some of these patients had CD that was undiagnosed. Of note, 2 of the 5 control subjects with positive results had ileal disease but an assigned diagnosis of UC; if these patients instead were classified as Crohn’s disease, the Odds Ratio would increase from 8.6 to 17.1.

The data obtained in the current study substantiate that MAC organisms appear more prevalent in CD patients than controls, however these findings do not prove a causal role for MAP because of two reasons. First, the study design employed has revealed a strong association, but association is for MAP because of two reasons. First, the study design employed has revealed a strong association, but association is only one of many criteria employed in epidemiology to determine the likelihood of causality. Second, we have not formally demonstrated whether the organisms we have observed are MAP, as opposed to other sub-species of M. avium. Therefore, further study is required to determine what role, if any, these organisms might be playing in the disease process. Certain...
observations from this study do provide clues about the status of the mycobacteria we visualized. First, it can be inferred that the bacteria were alive, as rRNA is degraded upon bacterial death [33]. Second, solitary signals were frequently observed by both methods, suggesting that many of these organisms were not actively replicating. In a recent report, Woo and colleagues have described MAP persistence in macrophages in the absence of intracellular replication [34], therefore the adaptation of MAC bacteria to the host environment may involve a state of non-replicating persistence, as has been described for M. tuberculosis [35].

In conclusion, using oil-immersion microscopy, we have demonstrated MAC organisms in Crohn’s disease. Moreover, given the results of host genetic studies, the methods we have applied can be applied to look for other intracellular bacteria, using species-specific in situ hybridization directed against rRNA of candidate microbes. Further studies are indicated to determine the role of mycobacteria and other organisms in the etiopathogenesis of CD.

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