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MICROBIOLOGY

A *Mycobacterium* species for Crohn's disease?

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Summary

In ruminants *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the causative organism of a chronic granulomatous inflammatory bowel disease called Johne's disease (JD). Some researchers have hypothesised that MAP is also associated with Crohn's disease (CD), an inflammatory bowel disease in humans that shares some histological features of JD. Despite numerous attempts to demonstrate causality by researchers, direct microbiological evidence of MAP involvement in CD remains elusive. Importantly, it has not been possible to reliably and reproducibly demonstrate mycobacteria in the tissue of CD patients. Past attempts to visualise mycobacteria in tissue may have been hampered by the use of stains optimised for *Mycobacterium tuberculosis* complex (MTB) and the lack of reliable bacteriological culture media for both non-tuberculous mycobacteria (NTM) and cell-wall-deficient mycobacteria (CWDM).

Here we describe a Ziehl–Neelsen (ZN) staining method for the demonstration of CWDM in resected tissue from patients with Crohn's disease, revealing the association of CWDM *in situ* with host tissue reactions, and posit this as a cause of the tissue inflammation. Using the ZN stain described we demonstrated the presence of CWDM in 18 out of 18 excised tissue samples from patients diagnosed as having Crohn's disease, and in zero samples out of 15 non-inflammatory bowel disease controls.

Key words: Crohn's disease; CD; cell-wall-deficient mycobacteria; CWDM; *Mycobacterium avium* subspecies *paratuberculosis*; MAP; mycolic acid; biofilm; endospores; Koch paradox; non-replicating persistence; NRP.

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INTRODUCTION

Crohn's disease (CD) is an inflammatory bowel disease that can affect all of the digestive tract but is typified by terminal ileal involvement, transmural inflammation and non-necrotising granulomas. Despite some similarity to mycobacterial diseases, acid fast bacilli (AFB) are rarely seen in CD.[1]

There is no single reliable diagnostic test for CD, rather the diagnosis of CD rests on a combination of clinical evaluation and endoscopic, histological, radiological, and/or biochemical investigations.[2] A widely accepted view is that CD is an autoimmune disease, affecting individuals with a genetic component that predisposes to active disease. Other theories for aetiology include indirect effects of dysbiosis, smoking[3] and contributory roles of the environment.[4]

Over the last 40 years there has been a mounting body of circumstantial evidence associating *Mycobacterium avium* subspecies *paratuberculosis* (MAP) with CD. This evidence includes polymerase chain reaction (PCR) detection of insertion sequences unique to MAP,[5] raised serological responses to MAP antigens in serum,[6] and histological features that are broadly similar to those seen in Johne's disease (JD).[7]

A bacterial aetiology for Crohn's disease was first proposed by Dalziel in 1913.[8] He noted the pathological similarity of CD in humans to JD in ruminants. MAP has since been hypothesised as a causative agent for CD because of the similar clinical presentations, both in gross anatomy, and in histological features such as the presence of inflammation with associated granulomas.

A rise in the two conditions over 50 years has been documented.[9]

Some clinical trials show favourable outcomes when selected anti-mycobacterial antibiotics are evaluated as potential therapies in CD.[10,11] Response to antibiotics may suggest an association between CD and *Mycobacterium* species, but that is not conclusive proof of causality, as antibiotics may impose general changes to the gut microbiome, rather than target a specific pathogen. Thus, conclusive evidence demonstrating mycobacterial involvement in Crohn's disease remains elusive.[12]

JD is a chronic wasting disease, characterised by diarrhoea, and a progressive loss of condition. Examination of the gut tissue of affected animals often shows macrophage infiltration and granuloma formation. AFB can be demonstrated in association with granulomas. Culture of the involved areas, including lymph nodes, reliably yields MAP.

MAP, the infectious agent in JD, is a facultatively anaerobic obligate intracellular non-tuberculous *Mycobacterium* species (NTM)[13] and a member of the *Mycobacterium avium* complex (MAC), which contains other opportunistic mycobacterial human pathogens, including *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium avium* ssp *hominissuis.*

Although the other histological features make the two diseases near-identical, the primary feature differentiating JD and CD is the absence of acid-fast organisms in the tissue of CD patients.[1] However, it is important to note that examination of tissue using the Ziehl–Neelsen (ZN) stain is not particularly sensitive. There may be reasons for this, as indicated by the

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greatly reduced detection rate of *Mycobacterium tuberculosis* complex (MTB) in tissues and smears when compared with bacterial culture.[14] In the case of tissue sections, interfering substances such as formalin or xylene, present as part of the tissue processing method, may retard the ZN staining process.[15] Acid fast organisms may not always be localised in granulomatous areas. In latent MTB infections, for example, the granulomas are often paucibacillary. This may be explained by the preference shown by mycobacteria for an intracellular lifestyle. For MTB, life in granulomas is unrewarding and is associated with a reduction in numbers.[16]

Since 1883, the ZN reaction has been the primary global diagnostic stain for the diagnosis of MTB infections.[17] Following from Ehrlich's original discovery of the property of acid-alcohol fastness, the ZN stain has undergone a variety of modifications but has remained, in various formulations, a specific method for the confirmation of mycobacteria.[18] Although it has been over a century since the original discovery, an explanation of the precise mechanism of the ZN stain remains uncertain.[19] However, it is known and accepted that the ZN stain selectively stains mycolic acids, a heterogenous group of mycobacterial lipids associated with inflammation and granuloma formation.[20]

Mycolic acids have been implicated as virulence factors in some *Mycobacterium* species.[21] Free mycolic acids are found in the external biofilm of *Mycobacterium* species, demonstrating migration from the bacterial cell to the external environment. This is particularly apparent during dormancy and reverses during growth promotion.[22,23] Mycolic acids also play a role in regulating the immune response to infection by pathogenic mycobacteria, and contributing to virulence and persistence within the host.[24] Mycolic acids can directly trigger the innate immune system. In particular, mycolic acid is a pathogen-associated molecular pattern (PAMP) and may provoke synthesis and secretion of cytokines, including inflammatory cytokines such as TNF-alpha.[21,25] Mycolic acids, as well as being the specific substrates for the ZN stain, have also been shown to induce an inflammatory reaction in the host.

Cell-wall-deficient mycobacteria (CWDM), also known as L forms, are mycobacteria in which the cell wall is no longer present but the intracellular contents of the cell, which include mycolic acids, are enclosed within a semi-permeable outer membrane.[26] The outer membrane may contain mycobacterial DNA, bacterial antigens and other metabolic products, including biofilm and mycolic acids.[27] The outer membrane, unconstrained by the mycobacterial cell wall, may respond to osmotic pressure, resulting in expansion or collapse of the CWDM.[28]

CWDM have been observed in association with CD as well as other mycobacterial diseases, including tuberculosis. The CWDM form appears to be part of the life cycle of *Mycobacterium* species and is poorly understood. In CD, the CWDM form may be essential for survival of the MAP organism in adverse environmental conditions, and the absence of a cell wall may mitigate the host immune response.[29]

CWDM variants of MTB possess an outer membrane which is thicker than the parent cell wall and outer membrane combined. The loss of the cell wall may be compensated for by a thickening of the outer membranes.[30] The CWDM outer membrane is also impervious to some chemicals.[31] This may be one reason why DNA extraction from cultures of CWDM can be unrewarding.[30,32,33]

In this study, a new ZN staining method using acid-alcohol decolourisation was employed to search for CWDM in surgically resected ileocolic tissue from patients presenting at a tertiary hospital.

MATERIAL AND METHODS

Case selection

Samples were selected for use as part of a technical instruction project for a medical laboratory science degree course. A senior scientist randomly selected archived tissue samples by interrogating the laboratory information system for suitable material from 2015 through 2019.

Resected ileocolic bowel tissues were retrieved from 18 patients with a confirmed diagnosis of CD (Table 1) and 15 cases representative of non-inflammatory bowel diseases (Table 2).

In each case formalin fixed, paraffin embedded (FFPE) blocks from large resections were examined with H&E slides to confirm the presence of granulomas, prior to staining with the ZN stain. Four blocks that fulfilled these criteria were selected from the cases, which all had more than 10 FFPE blocks available. These blocks were used to cut 4 µm tissue sections. In addition, we prepared positive control tissue sections derived from JD tissues (1 paucibacillary JD and 1 multibacillary JD sheep intestine control).

Each section was stained with the conventional ZN stain used in our laboratory for detection of MTB, a new ZN stain using acid alcohol decolourisation, and a third ZN stain used for the detection of acid-fast bacilli (Table 3).

Prior to staining the tissue, sections were dewaxed to allow for aqueous solutions to penetrate, through a series of xylene and alcohol steps. After dewaxing, the section was stained by immersion in ZN stain carbol fuchsin (ZN stain; Sigma-Aldrich, USA) for 35 min at room temperature. In the new ZN staining method, decolourising was carried out using 30% concentrated hydrochloric acid (HCl) in isopropyl alcohol (IPA) for 1 min, followed by rinsing in water. The section was then counterstained with 1% methylene blue for 10 s. The section was then dehydrated, cleared, and mounted. Sections were examined with routine light microscopy at various powers and an oil immersion lens at ×1000 magnification.

For the conventional ZN stain, the same steps were followed with the exception that the decolourising step was 3% HCl in ethanol for 3–5 dips.

For the ZN method for detection of acid fastness, the same steps were followed, except that decolourisation was with 20% H_2SO_4 in distilled water for 4 min.

RESULTS

The conventional ZN stain decolouriser using 3% HCl in 97% ethanol is optimised for the detection of MTB and we observed that this decolouriser could not demonstrate the presence of CWDM. In contrast, the bacillary forms of MAP in sections from the JD sheep controls were easily visualised when either 3% HCl in 97% ethanol, or 30% HCl in IPA were used.

The use of the decolouriser consisting of a strong acid, 20% aqueous sulphuric acid, was found to adversely affect the tissue sections, making them unsuitable for microscopic examination.

The decolouriser consisting of 30% HCl in IPA performed well and we were able to demonstrate the presence of CWDM in the tissue of 18 out of 18 CD patients and in none of the 15 tissue samples from non-inflammatory bowel disease patients. JD sheep controls were positive for ZN stain in all three decolouring methods.

In the tissue sections prepared using resected tissue (mostly ileocolic, one appendix and one jejunum) from CD patients and stained using the 30% HCl in IPA decolouriser, we were able to locate CWDM using the ×60 objective lens. These CWDM were not uniformly distributed throughout the tissue sample, but scattered widely and generally associated with lymphoid clusters. They tended to be located deep in the

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Table 1 Crohn's disease tissues

Patient	Age	Gender	Sample region
1	47	F	Ileocaecal
2	21	F	Ileal
3	24	F	Ileocaecal
4	17	F	Ileal
5	13	M	Colon
6	19	M	Appendix
7	35	M	Ileal
8	51	F	Jejunum
9	25	F	Appendix
10	60	M	Ileocaecal
11	28	F	Ileocaecal
12	40	F	Ileal
13	13	F	Ileal
14	21	F	Colon plus stoma
15	82	F	Ileal
16	33	F	ileal
17	19	M	Ileal
18	26	F	Ileal

Table 2 Non-Crohn's disease controls

Diagnosis	No. cases
Colon/caecal cancer (adenocarcinoma)	11
Low grade mucinous appendiceal neoplasm (LAMN)	1
Ischaemic colitis	1
Polyps and diverticular disease	1
Burkitt lymphoma caecum	1

submucosa and muscularis propria, often amongst areas of fibrosis associated with moderate chronic inflammation and sometimes associated with inflammatory fissures. CWDM were not identified in areas of ulceration and necrosis. Macrophages were notably associated with some of the CWDM, but they were not obviously associated with granulomata. CWDM were not seen in tissue sections from the 15 control tissues. Importantly, the CWDM were absent from all 33 tissue samples when the sections were examined using the conventional ZN stain decolourised with 3% HCl in 97% ethanol.

Morphologically, the CWDM showed considerable variation in size and were occasionally in small clusters of 3–8 organisms (Fig. 1C), and rarely seen intracellularly within macrophages. Some resembled 'endospores'[34] and showed a thickened outer membrane (Fig. 1B) surrounded by a transparent margin, suggesting the presence of biofilm or an outer membrane (Fig. 1A). All CWDM seen were ZN stain positive and spherical in shape, ranging in size between a red cell and a plasma cell, and were sometimes larger than macrophages. (Fig. 1,2).

DISCUSSION

Crohn's disease is a complex disorder widely considered to have an autoimmune basis, but recent studies also point to environmental associations and a dysregulated reaction to the normal gut microbiota.[4] The CWDM form of MAP (lacking a cell wall, but enclosed in a semi-permeable membrane) is theorised by some researchers to be the infective form of MAP involved with CD;[35–37] however, CWDM have not been observed by anatomical pathologists in the course of human tissue examination.[1]

Despite the breadth of ongoing research focused on attempts to understand the relationship between Crohn's disease and mycobacteria, there is still no universally accepted explanation, either for association or causality.

This study was an attempt to use knowledge regarding the microbiology of CWDM, staining techniques and possible modes of infection to link histological appearances with microbial pathogenicity. To do this we examined archived tissue using established histological techniques, and were informed by the behaviour of CWDM, both *in vitro* and *in vivo*. In doing so, we were able to develop a variation on the ZN stain which subsequently enabled us to detect what we believe to be CWDM *in situ* in deep ileocolic, appendiceal and jejunal tissue.

CWDM are known to convert host cholesterol to mycolic acids. This metabolic pathway may represent a plausible route by which inflammation and granuloma formation could occur in the Crohn's disease patient.

Mycolic acids are widely known to be the specific target of the ZN stain. It is less well known that mycolic acids are pro-inflammatory and may be produced by mycobacteria in the dormant state.[38]

The ZN stain has been through many iterations, but the conventional ZN stain, using 3%HCl in ethanol, is regarded

Table 3 Staining method and parameters

	Conventional ZN for acid-alcohol fast bacilli	ZN for acid-fast bacilli	New ZN for acid-alcohol fast CWDM and bacilli
1	Xylene 2 min		
2	Xylene 2 min		
3	Ethanol 2 min		
4	Ethanol 2 min		
5	Water until clear		
6	Carbol fuchsin 35 min		
7	Water until clear		
8	3% HCl/ethanol (3–5 dips)	20% H_2SO_4 4 min	30% HCl/IPA 1 min
9	Water until clear		
10	1% methylene blue 10 s		
11	Ethanol 2 min		
12	Ethanol 2 min		
13	Xylene 2 min		
14	Xylene 2 min		
15	Cover slipping in DPX		

CWDM, cell-wall-deficient mycobacteria; DPX, dibutylphthalate polystyrene xylene; IPA, isopropyl alcohol; ZN, Ziehl–Neelsen.

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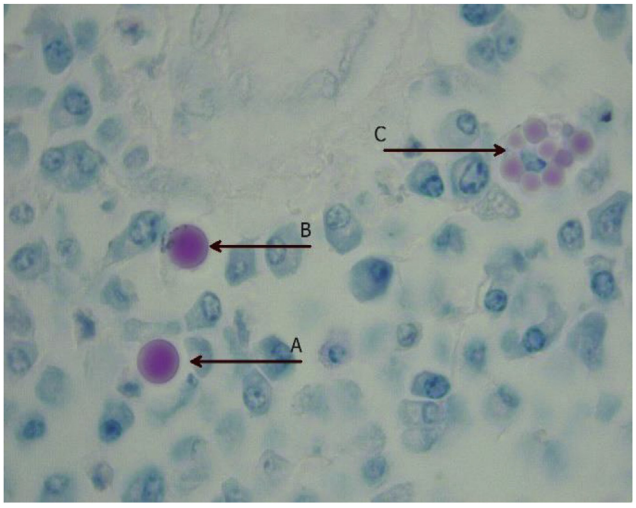

Fig. 1 Ziehl–Neelsen staining of ileal tissue from Crohn's disease patient; 1000-fold magnification showing cell-wall-deficient mycobacteria *in situ*. (A) Clear zone suggests biofilm. (B) Robust outer membrane. (C) Cluster of smaller forms.

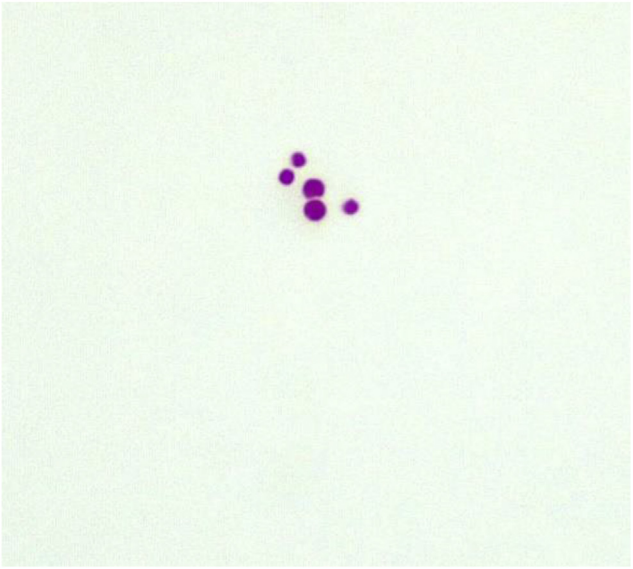

Fig. 3 Ziehl–Neelsen staining of cell-wall-deficient mycobacteria grown from the blood of a Crohn's disease patient after consecutive subcultures through supplemented 7H9 media for 7 months.

as the gold standard for rapid diagnosis of tuberculosis. This understanding is supported by widespread use of the ZN stain globally and the unquestionable reliability of the stain for the early laboratory detection of tuberculosis. The conventional method of ZN stain using 3% ethanol for decolourisation is less helpful for the detection of non-tuberculous mycobacteria, but convenience and established practice have largely ignored other methods for ZN stains.[18]

Parallel research on the growth of CWDM from the blood of Crohn's disease patients and from controls by us suggested that we revaluate the widely held perception that one ZN stain method is sufficient for all purposes.[39]

We identified that the conventional ZN stain was unsuitable for detection of CWDM in blood cultures. This insight led to the rediscovery that decolourisation with 20% H_2SO_4, after staining with carbol fuchsin containing 2.5% basic fuchsin, allowed for CWDM to be detected in supplemented 7H9 broth (Fig. 3). It was further found by us that 20% H_2SO_4 had a deleterious effect on tissue sections from Crohn's patients and also from non-inflammatory bowel disease normal controls.

This insight then led to the development of a decolourising agent consisting of 30% HCl in IPA, which provided satisfactory results when combined with carbol fuchsin containing 3% basic fuchsin. These changes allowed us to detect CWDM in the tissues in all 18 patients and none of 15 non-inflammatory bowel disease controls.

The new decolouriser also fulfilled the criteria of Barksdale and Kim[40] in confirming the acid-alcohol fast organisms detected were *Mycobacterium* species, producing mycolic acid.

Our observations of these organisms in tissue led to speculation by our group that was informed partly by the research findings of others and partly by our ongoing research into cultivation of CWDM in blood. Mycobacteria, including CWDM, will initiate inflammation *in vivo*. Direct proximity to granulomas is not mandatory, and there is ample evidence to support the contention that the downstream metabolic products of mycobacteria are toxic to the host. Mycolic acids are also associated with the production of foamy macrophages and directly initiate the primary immune response in the host.[21,41,42] Mycobacteria metabolise host cholesterol to form mainly mycolic acids and cell wall lipids, including trehalose 6, 6′-dimycolate (TDM). TDM, also known as cord factor, is found in virulent *Mycobacterium* species[21,43] where it may trigger formation of caseating granulomas.[20,44] Mycolic acids have been implicated as virulence factors in

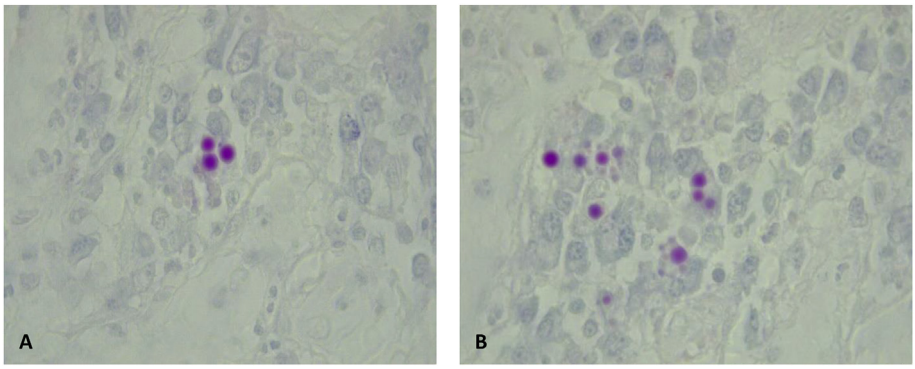

Fig. 2 (A,B) Ziehl–Neelsen staining of ileal tissue from Crohn's disease patient; 1000-fold magnification showing cell-wall-deficient mycobacteria *in situ*.

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some *Mycobacterium* species.[21] Free mycolic acids are found in the external biofilm of *Mycobacterium* species, demonstrating migration from the bacterial cell to the external environment. This is particularly apparent during dormancy and reverses during growth promotion.[21,45]

The CWDM we found in the resected tissues from CD patients may have been in the dormant or latent phase, in which mycobacteria are viable but do not replicate. In low oxygen, nutrient deficient conditions, such as occur in deep tissue or cavitary lesions, *Mycobacterium* species may assume a state of non-replicating persistence (NRP). NRP is sometimes associated with a negative ZN stain, a phenomenon referred to as the 'Koch paradox'. Thus the Koch paradox may present one possible explanation for the failure of past attempts to detect mycobacteria in tissue using the conventional ZN stain.[19]

Microbiological features observed by us included the variable sizes of the spherical CWDM, the transparent outer margin suggesting biofilm, ZN positivity and the occasional smaller clusters of CWDM. Bilateral division was also seen (not shown).

Taken together, these appearances conform to the observations of other researchers, as to CWDM morphology. In turn, this strengthened our belief that the spherical forms seen in tissue were identical to those grown in bacterial culture media from the blood of Crohn's disease patients (Fig. 3).

MAP is described as an obligate intracellular pathogen. *In vitro* blood cultures on Crohn's disease patient samples observed by us, and others, demonstrate that the CWDM will remain viable and replicate extracellularly. Our observations of tissue sections suggest that the same may apply extracellularly in the Crohn's disease patient. This observation does not diminish the possibility of involvement of a mycobacterial obligate intracellular pathogen,[46] but does raise important questions as to the life cycle of mycobacteria in the human host. If the lifestyle of *Mycobacterium* species in the host includes an extracellular phase that is additionally bereft of a cell wall, then the implications for pathogenicity are significant.

Although our preliminary observations are novel and intriguing, they also inevitably lead to questions of future directions. Over the last 30 years there have been considerable efforts to prove pathogenicity of MAP through the use of PCR on tissues and in human blood. Despite numerous papers on the subject of PCR detection of MAP in human subjects, the question of causality is still outstanding, and the PCR route has become one of confounding and diminishing returns.

Although the IS900 insertion sequence has been proposed to be unique to MAP,[5] use of PCR to detect IS900 has not been helpful in providing definitive evidence for a role for MAP in Crohn's disease. In addition, there is some evidence to suggest that IS900 may not be unique.[47,48] Numerous studies, including a recent widely ranging comparison of available analytical techniques for detection of IS900, have consistently demonstrated that IS900 is often present in both patients and controls.[49] It is of some growing concern, shared among other researchers,[50] that somewhere in the environment there may be sources of contamination with MAP, but that is far from the goal of proving causality in CD.

We are suggesting that Crohn's disease is not a consequence of a tissue invasion resulting in an infection that provokes granuloma formation, but the inevitable consequence of viable CWDM producing mycolic acids as a product of metabolism. Both the CWDM and these products of metabolism may then be taken up intracellularly by macrophages and transported to alveolar or interstitial tissue spaces where the CWDM subsequently revert to an extracellular lifestyle.[21,30]

In Crohn's disease, the genesis of inflammation may be insidious, through the presence of viable toxigenic CWDM, rather than through an orthodox infective process. Location, bacterial cell morphology and metabolism are critical to the contribution to pathology.

CONCLUSION

Our observations on tissue require validation and further research. It is not certain that the putative CWDM observed in this tissue study are derived from the bacillary form of MAP. *In situ* hybridisation of Crohn's disease tissue may provide evidence to support this contention.[36] Understanding the relevance of the observations in this paper as to the underlying factors leading to Crohn's disease will require significant involvement of biochemists, microbiologists and immunologists, with anatomical pathology support.

To clarify the relationship between CWDM and CD, efforts to culture similar CWDM forms isolated from tissues and the peripheral blood of more Crohn's disease patients should continue[32] across multiple centres.

Culture of CWDM *in vitro* is difficult but is achievable through the application of conditions that promote growth from dormancy. Culture is known to be more sensitive and specific in the laboratory diagnosis of tuberculosis,[14] and there may be a need for the further development of bacterial culture techniques to enable the demonstration of CWDM in small tissue biopsies.

Consideration should also be given to the availability of specific fluid culture media that will address the fragility of CWDM. In the laboratory, growth promotion of CWDM is stochastic and complex. The release from microbial dormancy involves the introduction of specific nutrients, oxygen and variable incubation temperatures, depending on the species of mycobacteria.[51–53]

In the era of resource utilisation controls, transfer of complex investigative research methodologies to wider users in medical laboratories is important, but will require simpler methods and commercialisation to ensure widespread adoption and utilisation.

Isolation of CWDM may lead on to accurate taxonomy, a clearer understanding of the life cycle of *Mycobacterium* species, and ultimately to improved and specifically targeted therapies. Importantly, parallel cultures of tissue biopsies and animal studies will either confirm or disprove the observations made by us in this study.

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