ANTAGONIST

Mycobacterium avium subspecies *paratuberculosis* is a cause of Crohn's disease

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The hypothesis that *Mycobacterium paratuberculosis* is the cause of Crohn's disease has been with us for over 80 years.[1] Yet the hypothesis remains controversial and unproved.[2–5] In spite of advances in molecular techniques such as polymerase chain reaction (PCR) which has shed light on the infectious basis of many other diseases (Whipple's disease, *Helicobacter pylori*, hepatitis C, etc.), the cause of Crohn's disease remains unknown.

The competing hypotheses are widely known:

(1) Genetic predisposition.
(2) Other infectious agents such as measles virus or an unknown agent.
(3) Abnormal autoimmune reaction to antigen(s).
(4) Environmental factors within the gut related either to dietary factors or the microbiological environment.

There is little controversy over a genetic component to the disease but this hypothesis alone cannot explain the increasing incidence. It appears most likely that the cause of Crohn's disease is a combination of a genetic predisposition to the disease and one or more of the alternative hypotheses. For the infectious disease hypothesis to be proved for any organism, Koch's postulates need to be fulfilled. It is clear that to date these have yet to be met for *M paratuberculosis*. Van Kruiningen[2] has extensively reviewed the data on culture of *M paratuberculosis* in Crohn's disease, experimental transmission of Crohn's disease, and inoculation with *M paratuberculosis*, and has found the evidence wanting. There is also no evidence of direct transmission from animal to humans, despite the frequent occurrence of *M paratuberculosis* (up to 54% in some cattle herds[3]) and Johne's disease in cattle, its detection in cows milk, and the presence of *M paratuberculosis* in a number of other animals. High risk groups susceptible to transmission of the disease would be families of cattle and sheep farmers, abattoir workers, veterinary surgeons, and possibly gastroenterologists. To date, there is no such evidence.

There are pathological similarities between Johne's disease and Crohn's disease but there are also many features that are not found. These are eloquently detailed by Van Kruiningen[2] and will not be repeated here. He suggests that the closest human disease to Johne's disease is the *Mycobacterium avium* intracellular infections of AIDS patients, not Crohn's disease.

For an agent to cause a disease such as Crohn's disease it should be reliably detectable within the tissues of diseased patients. *M paratuberculosis* has been grown from individual cases of Crohn's disease in several centres[6 7] but it has also been grown from individuals without the disease and individuals with ulcerative colitis. There are now over 24 papers addressing the detection of *M paratuberculosis* using PCR[8–31] and these are summarised in table 1. Most of these papers use the IS900 target sequence stated to be "uniquely specific" for *M paratuberculosis*.[3] Other targets claimed to be specific are GS and hspX.[3] The frequency of detection of IS900 ranges from 0% to 87.5% in non-inflammatory bowel disease tissues, 0% to 100% in ulcerative colitis, and 0% to 100% in Crohn's disease. Thus DNA from the "*M paratuberculosis* specific" IS900 sequence has been detected in patients with Crohn's disease but also frequently in patients with ulcerative colitis and normal individuals. However, other conflicting studies have shown no detectable DNA from individuals with Crohn's disease when searching for IS900. To summarise these studies, 14 of the 24 papers from table 1 found no *M paratuberculosis* in Crohn's disease against 10 which did. The negative studies contained 323 Crohn's patients and the positive studies 188. Thus the balance both in terms of the number of studies and number of patients evaluated favours the absence of *M paratuberculosis* in Crohn's disease tissues. There are a number of possible explanations for these contradictory findings.

For false positive findings

IS900 sequences are not specific for *M paratuberculosis* but can be found in other environmental or animal borne mycobacteria.[32] These results in animals were obtained using primers described by Professor Hermon-Taylor's group. This group identified the false positive bacteria as derived from mycobacteria with close sequence homology on their 16S rRNA of *M paraffinicum* (97.8%) or *M scrofulaceum* (97.1–99.45%). This increases the likelihood that the range of PCR results is due to detection of cross reacting sequences from other common mycobacterial sequences.

Cross contamination from *M paratuberculosis* cultures or previous PCR amplified sequences generated in the same laboratory can occur. In our own experience we found that our early positive results[33] could not be confirmed when we enhanced our PCR anticontamination methods.

For false negative findings

- Poor PCR design, reactions, or detection methods.
- Insensitive PCR reactions.

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Accepted for publication
5 September 2001

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Table 1 Summary of the frequency of polymerase chain reaction studies for the presence or absence of Mycobacterium paratuberculosis in Crohn's disease (CD), ulcerative colitis (UC), and non-inflammatory bowel disease (IBD) tissues. These are shown in order of date of publication

Reference	*Target*	*Non-IBD*	*UC*	*CD*
Rosenburg *et al* 1991[8]	Not stated, not IS900	0/6 (0%)	nd	0/21 (0%)
Wu *et al* 1991[9]	Pan mycobacterial sequences	nd	nd	0/20 (0%)
Sanderson *et al* 1992[10]	IS900	5/40 (12.5%)	1/23 (4.3%)	26/40 (65%)
Dell'Isola *et al* 1994[11]	IS900	7/24 (29%)	1/5 (20%)	13/18 (72%)
Lisby *et al* 1994[12]	IS900	3/28 (11%)	2/10 (20%)	11/24 (46%)
Fidler *et al* 1994[13]	IS900. Also detected non *M paratuberculosis* sequences	0/20 (0%)	0/10 (0%)	4/31 (13%)
Suenaga *et al* 1995[14]	IS900	14/16 (87.5%)	11/18 (61%)	10/10 (100%)
	GroEL	14/16 (87.5%)	11/18 (61%)	10/10 (100%)
Rowbotham *et al* 1995[15]	IS900	1/26 (4%)	0/49 (0%)	0/68 (0%)
Murray *et al* 1995[16]	IS900	0/15 (0%)	2/15 (13%)	2/9 (22%)
Kreuzpaintner *et al* 1995[17]	16S rRNA	0/1 (0%)	nd	0/4 (0%)
	Culture	0/23 (0%)	nd	0/23 (0%)
Erasmus *et al* 1995[18]	IS900	4/35 (11%)	nd	10/26 (38%)
Mishina *et al* 1996[19]	IS900 mRNA	0/2 (0%)	4/4 (100%)	8/8 (100%)
Frank and Cook 1996[20]	IS900	0/11 (0%)	nd	0/23 (0%)
Dumonceau *et al* 1996[21]	Mycobacteria	13/23 (57%)	6/13 (46%)	17/36 (47%)
	M paratuberculosis	0/23 (0%)	0/13 (0%)	0/36 (0%)
Dumonceau *et al* 1997[22]	*M paratuberculosis* 16S rRNA	0/22 (0%)	0/10 (0%)	0/31 (0%)
Al-Shamali *et al* 1997[23]	IS900	0/21 (0%)	0/6 (0%)	0/10 (0%)
	MP2	0/21 (0%)	0/6 (0%)	0/10 (0%)
Riggio *et al* 1997[24]	IS900	0/12 (0%)	nd	0/7 (0%)
Clarkston *et al* 1998[25]	IS900	0/11 (0%)	0/5 (0%)	1/21 (5%)
Cellier *et al* 1998[26]	16S rRNA any mycobacteria	2/20 (10%)	1/27 (4%)	2/47 (4%)
	IS900	0/20 (0%)	0/27 (0%)	0/47 (0%)
Chiba *et al* 1998[27]	IS900	0/3 (0%)	0/14 (0%)	0/30 (0%)
Kanazawa *et al* 1999[28]	IS900	0/13 (0%)	0/4 (0%)	0/13 (0%)
Tiveljung *et al* 1999[29]	16S rRNA	0/11 (0%)	nd	3/11 (27%)
*Ikonomopoulos *et al* 2000[30]	IS1110 element and probed 16S rRNA	nd	1/1 (100%)	7/20 (35%)
†Gibson *et al* 2000[31]	IS900	0/12 (0%)	nd	0/3 (0%)

**M paratuberculosis* sequences in sarcoidosis were also found.
†0/21 from orofacial granulomatosis.
nd, not done.

- Very low numbers of infecting organisms below the limit of detection of the assay.
- Inability to extract DNA from mycobacteria because strong walls inhibit release of DNA.
- Granulomatous versus non-granulomatous disease as *M paratuberculosis* is claimed to be more frequently found in granulomatous disease.
- Selection of wrong age groups, wrong types of material, or delay in processing material.
- Regional variations in *M paratuberculosis.*

Many of the reasons given for negative findings can be easily overcome by good study design. The use of good positive controls and demonstration of positive signals with such organisms will exclude poor PCR reaction design. Many authors have successfully amplified material from control cultured mycobacteria and strains such as LINDA isolated from a Crohn's patient so this problem does not appear to be important. Several of the assays in the negative published papers claim very high sensitivity on serial dilutions of control bacteria making it unlikely that target concentration is a problem. Other papers have reproduced the extraction methods of Professor Herman-Taylor[10] with successful detection but to no avail.[15] Others have specifically looked at granulomatous and non-granulomatous disease. Papers have appeared from all over the world with contradictory results from the same countries, making regional differences unlikely. The only way to establish the truth of the presence or absence of *M paratuberculosis* DNA is a properly funded, carefully planned, blinded, international multicentre study with good controls and free exchange of PCR methods that test multiple targets in the *M paratuberculosis* genome using agreed "optimised" methods. Cultures of "pathogenic" mycobacteria from patients with Crohn's and closely related strains of mycobacteria need to be available to evaluate the sensitivity and specificity of tests for the chosen targets. Because of the data of Cousins and colleagues[32] the positive IS900 data must be confirmed by restriction digestion or sequencing and by detection of other sequences characteristic of *M paratuberculosis*. The material evaluated must come from different hospitals and comprise fresh rapidly sampled diseased tissues from a range of granulomatous and non-granulomatous cases of Crohn's disease from the small and large intestine and from resections and biopsies. Each sample should have two parallel samples taken from well characterised cases of ulcerative colitis and age matched controls of biopsied or surgically removed non-inflammatory bowel disease material. It would be preferable for adjacent material to be available for histological assessment and for in situ hybridisation studies. Such a study needs to take place under the supervision of an independent data monitoring committee.

If DNA detection is controversial the recent claims for the detection of *M paratuberculosis* by in situ hybridisation in Johne's disease[34 35] and subsequently in human diseased tissues[36] are intriguing. If confirmed by other laboratories, this may represent very important evidence for the causality of Crohn's disease by *M paratuberculosis*. Interestingly, the results of in situ hybridisation

using an IS900 sequence found positive signals in a similar number to some of the PCR studies, namely 7/37 (18.9%) of Crohn's samples, 2/21 (9.5%) ulcerative colitis samples, and 0/22 (0%) normal samples. Signal was seen in macrophages and myofibroblasts. This study did not test the specificity of the in situ hybridisation against the cross reacting organisms described by Cousins and colleagues[32] and only derived significance by selecting idiopathic inflammatory bowel disease cases versus non-idiopathic inflammatory bowel disease, and granulomatous Crohn's disease cases versus non-granulomatous Crohn's. Further studies of this nature are urgently required to confirm or refute these results but issues of specificity must be properly addressed. Newer techniques of in situ PCR[37] and Taqman in situ PCR[38] may contribute to the debate. Sanna and colleagues[37] have developed an in situ PCR for *M paratuberculosis* using IS900 and successfully applied it to Johne's disease. Positive results in Crohn's disease would be encouraging especially if the distribution matched that of Hulten and colleagues.[36] However, multiple targets would need to be used to confirm specificity. Studies of a range of antibodies to mycobacteria and *M paratuberculosis* using immunocytochemistry have as yet failed to detect *M paratuberculosis*.[39–41]

If *M paratuberculosis* is the cause of Crohn's disease why is it that antimycobacterial agents do not eradicate the disease? To date the literature is inconclusive. A recent meta-analysis[42] found 29 papers but only eight were proper randomised placebo controlled trials and the number of patients totalled only 352 in all studies. Only one study had more than 51 patients. They stated that there was insufficient evidence to be sure of the effects of antibiotic therapy for Crohn's disease but that the combined data suggest that antibiotic therapy may help maintain remission. However, these data were based on two trials with only 89 patients in total and the hypothesis was not derived a priori. Thus we do not really know the value of antimycobacterial therapy, and the proponents of this hypothesis are open to criticism that after 80 years we have yet to see a positive, properly conducted therapeutic trial. Even if antimycobacterials improve outcome, is it due to direct action on *M paratuberculosis* or an effect on the general bacterial flora, and most importantly, do antimycobacterials cure the disease? This vitally important question is unanswered. Once the diagnostic testing trial has been completed, a major properly powered trial of effective antimycobacterial antibiotics versus broad spectrum antibiotics versus placebo in patients identified as having *M paratuberculosis* should be initiated with patients being followed by PCR to confirm the continued presence or eradication of the organism.

The beginning of large scale dissection of the pattern of RNA expression by cDNA arrays[43] of diseased Crohn's tissues, its comparison with those of other human mycobacterial infections and, when the bovine genome becomes available, of the mucosal response in Johne's disease, will provide more circumstantial evidence for or against the role of *M paratuberculosis* in Crohn's disease. Early evidence[44] is intriguing in that it points to activation of the antibacterial defensin genes 5 and 6 in Crohn's disease tissues but these are also raised in ulcerative colitis.

With a possible 90 000 people with Crohn's disease in the UK and an estimated cost of treatment of Crohn's disease to the NHS at over £200 million per year, the question of whether *M paratuberculosis* is involved in Crohn's disease needs to be resolved once and for all by large carefully designed studies of detection and treatment. More resources need to be directed at this disease to answer this important question. After 80 years the evidence for *M paratuberculosis* as the cause of Crohn's is still circumstantial and definitive studies remain to be performed.

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