Mycobacterium avium subspecies *paratuberculosis* and Crohn's disease: a systematic review and meta-analysis

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This systematic review assesses the evidence for an association between *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and Crohn's disease. We analysed 28 case-control studies comparing MAP in patients with Crohn's disease with individuals free of inflammatory bowel disease (IBD) or patients with ulcerative colitis. Compared with individuals free of IBD, the pooled odds ratio (OR) from studies using PCR in tissue samples was 7·01 (95% CI 3·95–12·4) and was 1·72 (1·02–2·90) in studies using ELISA in serum. ORs were similar for comparisons with ulcerative colitis patients (PCR, 4·13 [1·57–10·9]; ELISA, 1·88 [1·26–2·81]). The association of MAP with Crohn's disease seems to be specific, but its role in the aetiology of Crohn's disease remains to be defined.

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Introduction

Crohn's disease is a chronic inflammatory bowel disease (IBD) of unknown cause, the incidence of which is on the increase in high-income countries.[1] Since the first description of the similarities between Crohn's disease and Johne's disease in cattle in 1913,[2] it has been argued that *Mycobacterium avium* subspecies *paratuberculosis* (MAP), which causes Johne's disease, might also be a cause of Crohn's disease, and that the dysregulated immune responses are a secondary phenomenon.[3–5] Conversely, critics of the mycobacterial theory argue that MAP is a secondary invader rather than a causal factor.[6]

The association of MAP with Crohn's disease is supported by identification of MAP in patients with Crohn's disease, but not in appropriate controls. The gold standard for detection of MAP is based on isolation of the organism through culture methods.[2,7–10] However, this method is time consuming because of the organism's fastidious nature and slow growth. Molecular and serological methods are widely used alternatives, including immunocytochemistry,[11] nucleic acid hybridisation,[12] and PCR techniques.[13–15] ELISA is commonly used to investigate the immunological evidence of a MAP infection.[16–19]

A causal association of MAP with Crohn's disease would have important implications for both prevention and therapy, and is a continuing matter of concern for public-health agencies.[20–22] Since viable MAP organisms are occasionally isolated from commercial pasteurised milk,[23] the efficacy of some heat-treatment procedures of milk would have to be assessed and improved. Additionally, the search for effective treatment regimens against MAP would need to be intensified.

Our aim was to do a systematic review of case-control studies to assess the evidence that is available on MAP and its association with Crohn's disease.

Methods

Literature search

Literature searches were done in Medline (1966 to December, 2006). Keywords denoting MAP, Crohn's disease or IBD, and the study design were used: "paratuberculosis" (Medical Subject Heading, [MeSH]) or "*Mycobacterium paratuberculosis*" (MeSH) or "paratuberculosis" (free text); and "Crohn disease" (MeSH) or "inflammatory bowel disease" (MeSH) or "rectal fistula" (MeSH) or "Crohn" (free text); and "case-control studies" (MeSH) or "case-control" (free text). No language restrictions were applied. Additionally, we checked references from relevant publications and review articles.

Eligibility criteria

We included case-control studies if they compared the prevalence of MAP in patients with Crohn's disease with the prevalence in individuals free of IBD by use of PCR or ELISA. Studies comparing patients with Crohn's disease and patients with ulcerative colitis were eligible if they included another comparison group of individuals free of IBD. Studies comparing Crohn's disease patients exclusively with patients with tuberculosis or sarcoidosis were excluded. Studies were excluded if insufficient data were provided to calculate odds ratios (ORs) or if there were two zeros in the 2×2 table. Two reviewers independently assessed eligibility of publications.

Data extraction, outcomes, and definitions

We used a standardised data extraction sheet. Data extraction was done independently by two observers, and any differences were resolved by consensus. We extracted bibliographic, sociodemographic, and clinical data, aspects of study quality, and results. In studies examining several control groups, we chose controls free of IBD for the primary analysis, but, if available, also extracted data for patients with ulcerative colitis. In studies using PCR, the outcome was presence of MAP. In studies using ELISA, the outcome was presence of antibodies against MAP or the antibody titre. If several types of antibodies were assessed, we chose IgG, and if different types of antigens were assessed, we chose the antigen thought to be the most specific for MAP. The specificity of MAP detection tests used was assessed by a specialist in molecular biology (RS), who was provided with only the methods sections of the included publications.

Statistical analysis

Study results are presented as ORs with 95% CI. For studies using categorical outcome measures, calculation of an OR was straightforward. If studies measured continuous outcomes, results were converted to ORs by use of the method described by Hasselblad and Hedges.[24] This method is based on the fact that, when assuming logistic distributions and equal variances in the two treatment groups, the log OR corresponds with a constant multiplied by the standardised difference between means. An OR above 1·0 indicates a higher prevalence of MAP or higher antibody titres among patients with Crohn's disease compared with controls. The analysis was stratified by the method used (PCR *vs* ELISA). We calculated the I^2 statistic, which describes the percentage of total variation across studies that is caused by heterogeneity rather than chance.[25] Low, moderate, and high levels of heterogeneity approximately correspond to I^2 values of 25%, 50%, and 75%, respectively. There was moderate to high between-study heterogeneity and we therefore used random-effects meta-analysis to combine the results from different studies. Analyses were done in STATA (version 9.1, STATA Corporation, College Station, TX, USA).

Results

We identified 85 potentially eligible publications, and excluded 29 studies on the basis of title and abstract. 56 studies were examined in detail, of which 28 were excluded for the following reasons: 16 studies reported insufficient information to allow calculation of the OR (including small studies with no positive tests in either group),[19,26–40] two studies were excluded because the serological data from the control group were used to define the threshold for a positive antibody titre (with the consequence that the test was negative by definition in all controls),[41,42] two further studies used ineligible control groups,[43,44] and one study was a duplicate of another study.[45] In two cases, publications seemed to report on overlapping sets of patients. The report with the larger

	Year	Study size	Country	Control group	Mean* age (years)	
					Cases	Controls
Autschbach et al[54]	2005	200	Germany	Intestinal cancer, familiar adenomatous polyposis, other non-IBD-related conditions	33·9	60·8
Bernstein et al[55]	2003	43	Canada	Healthy	33·5	41·1
Bull et al[57]	2003	71	UK	Patients undergoing an ileocolonoscopy without clinicopathological diagnosis of Crohn's disease	32·6	55·5
Clarkston et al[59]	1998	32	USA	Normal colon, IBS, other functional bowel disorder	37·6	45·4
Dell'Isola et al[14]	1994	42	France	Infectious or lymphocytic colitis, polyps, malformations, angiomatosis, rheumatoid purpura, unclassified polyarthritis, autoimmune disease, genetic immunodeficiency, cystic fibrosis	5–19†	0·5–17†
Erasmus et al[62]	1995	61	South Africa	Colon cancer	..	..
Fidler et al[63]	1994	51	UK	Gut inflammation caused by ileostomies or radiation, carcinomas, vaginoplasties, Meckel's diverticulum	42	45
Hulten et al[73]	2001	58	Finland and USA	Diverticular disease, diverticulitis, ischaemic colitis, serosal abscess, cytomegalovirus colitis, acute non-specific colitis, adhesions	..	..
Lisby et al[64]	1994	52	Denmark	Colonic cancer, peridiverticulitis, ileocaecal lymphoma	34·5‡	67·0‡
Murray et al[65]	1995	24	New Zealand	Microscopic colitis, ischaemic colitis/diarrhoea, change of bowel habit, colonic polyp, anaemia, diverticular disease, rectal bleeding, IBS	35·6	58·9
Naser et al[66]	2004	43	USA	Colon cancer, diverticulitis, gastro-oesophageal reflux, healthy individuals	35·5	41·6
Romero et al[67]	2005	18	USA	Colon cancer	41·5	62·6
Rowbotham et al[15]	1995	94	UK	Rectal bleeding, diarrhoea, changed bowel habit, anaemia, IBS, colonic polyps, ascites, coeliac disease, systemic lupus erythematosus, pseudomembranous colitis	46·3	52·8
Ryan et al[68]	2002	27	Ireland	Diverticular disease, perianal sinus tissue, colon surgical scar, tubular adenoma of colon, colon carcinoma, colon tuberculosis, mediastinal node sarcoidosis, cholesterol granuloma of breast	..	..
Sanderson et al[13]	1992	80	UK	Colon cancer, gastric ulcer, sigmoid diverticulitis, small gut sarcoma	38·1	67·7
Sechi et al[46]	2005	71	Italy	IBS, gastritis, diarrhoea, adenocarcinoma, diverticulitis, screening, colitis, bleeding	..	..
Suenaga et al[69]	1995	26	Japan	IBS, colon polyps, colon cancer	39·2	54·1
Tiveljung et al[71]	1999	22	Sweden	Colon cancer, colon polyps, functional bowel disorder, ulcerative colitis	36‡	50‡

IBD=inflammatory bowel disease. IBS=irritable bowel syndrome. ..=not reported.*Unless otherwise indicated. †Range. ‡Median.

Table 1: **Characteristics of 18 case-control studies that used PCR techniques to detect *M avium* subspecies *paratuberculosis* in patients with Crohn's disease and controls**

number of patients was included in these instances.[46,47] One study with a different focus,[48] two studies that used immunoblotting,[49,50] and three studies that used culture methods,[51–53] were also excluded.

We therefore included 28 studies comprising 31 comparisons.[13–18,46,54–74] One study was done in two countries and examined different patient groups, resulting in four comparisons.[60] Reporting and methodological quality of the studies included was variable. Ten of the 28 studies did not report the sex or age distribution of patients and controls, and only 13 studies stated that laboratory staff were blinded to case-control status. 18 of the 31 comparisons used PCR techniques for the detection of MAP in tissue samples and 13 were immunological studies using ELISA.

Among the 18 studies using PCR, the median year of publication was 1999 (range 1992–2005) and the median total sample size was 47 participants (range 18–200; table 1). All studies used categorical outcomes. The median of the mean age of cases was 37·6 years (range 32·6–46·3) and of controls was 54·1 years (range 41·1–67·7). The specimens analysed were tissue samples in all but one study (serum), either obtained by ileocolonoscopy or by surgery. Studies differed regarding the site of biopsies (ileum or colon, inflamed, non-inflamed, or granulomatous tissue) and the depth of tissue extraction (full thickness samples obtained by surgery *vs* snap samples from ileocolonoscopy). The studies included the following controls: patients with gastrointestinal diseases other than Crohn's disease (predominantly colon cancer, diverticulosis, and irritable bowel syndrome) in 17 studies, and healthy individuals in one study. The prevalence of MAP DNA was higher in patients with Crohn's disease than in controls in 16 of 18 studies, resulting in a pooled OR of 7·01 (95% CI 3·95–12·4; figure 1). There was some heterogeneity in study results (I^2=44%), which was mainly attributable to the study by Bernstein and colleagues,[55] as indicated by a substantial fall in the I^2 value after exclusion of this study (I^2=10%). 12 studies compared the prevalence of MAP in Crohn's disease patients with controls free of IBD and with patients with ulcerative colitis. The ORs were similar: 6·88 (3·28–14·4) and 4·13 (1·57–10·9), respectively. We compared the results from the seven studies that used nested PCR with the other studies. Again, ORs were similar: 7·08 (2·19–22·9) and 7·02 (4·12–12·0), respectively.

The median year of publication of studies using ELISA was 1995 (range 1984–2006) and the median sample size was 93 (range 33–685; table 2). The median of the mean age was 36·6 years (range 17·9–44·8) in patients and 37·8 years (range 25·7–42·6) in controls. Outcome measures were continuous in seven studies,[16–18,61,70,72,74] and categorical in the remaining studies. All studies analysed serum samples but used different ELISA tests and MAP antigens. The protoplasmic antigen was most frequently used. One study analysed antibodies against a glutathione S-transferase fusion recombinant protein (part of IS*900*).[74] Controls were healthy individuals (seven studies or ten comparisons) or patients with other gastrointestinal conditions (three studies). The prevalence of antibodies against MAP antigens was higher in patients with Crohn's disease than in controls in ten of 13 studies (figure 2). The combined OR was 1·72 (1·02–2·90). All 13 studies also included an ulcerative colitis group; the OR for this comparison was 1·88 (1·26–2·81). Between-study heterogeneity was more pronounced in the comparison

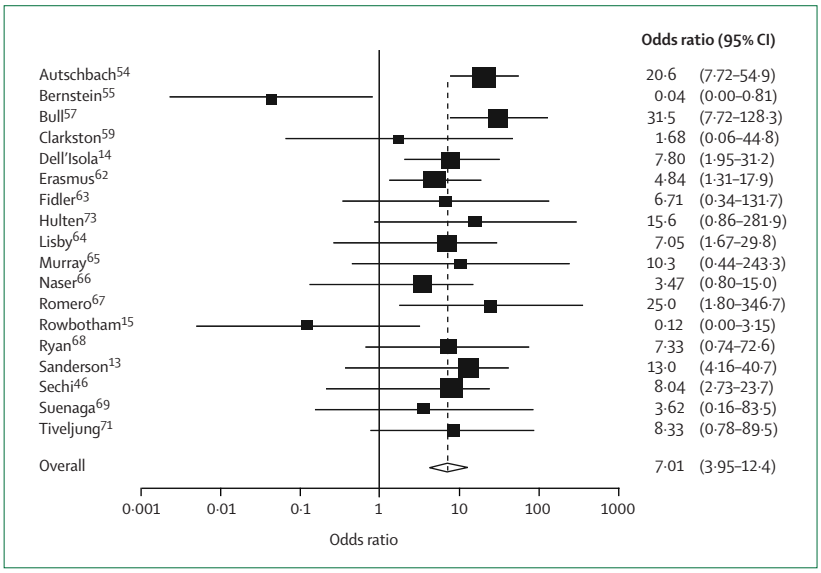

Figure 1: **Meta-analysis of 18 comparisons from case-control studies of patients with Crohn's disease versus controls, with PCR in tissue samples or blood to detect *M avium* subspecies *paratuberculosis* (MAP)**
Odds ratios (ORs) and 95% CI for each study are shown. The size of the square represents the relative weight of each study in the random-effects meta-analysis. The data are displayed on a logarithmic scale. ORs above 1·0 indicate a higher prevalence of MAP in patients with Crohn's disease compared with controls.

	Year	Study size	Country	Control group	Mean age (years)	
					Cases	Controls
Bernstein et al[56]	2004	685	Canada	Healthy	36·4	39·8
Cho et al[58]	1986	45	USA	Duodenal ulcer, diverticulitis or colon cancer	..	..
Collins et al[60]	2000	86	Denmark	Healthy	39·4	42·6
Collins et al[60]	2000	111	Denmark	Healthy	44·8	42·6
Collins et al[60]	2000	210	USA	Healthy	40·9	37·8
Collins et al[60]	2000	126	USA	Healthy	34·2	37·8
Elsaghier et al[61]	1992	47	Italy	Healthy	..	..
Nakase et al[74]	2006	94	Japan	Non-inflammatory bowel disease	35	40
Stainsby et al[16]	1993	76	UK	Healthy	..	..
Suenaga et al[70]	1999	33	Japan	Healthy	36·6	32·9
Tanaka et al[18]	1991	93	UK	Non-inflammatory bowel disease	17·9	25·7
Thayer et al[17]	1984	123	USA	Healthy	38	28
Walmsley et al[72]	1996	61	UK	Healthy	..	..

..=not reported.

Table 2: **Characteristics of ten case-control studies (13 comparisons) using ELISA tests to detect seropositivity for *M avium* subspecies *paratuberculosis* in patients with Crohn's disease and controls**

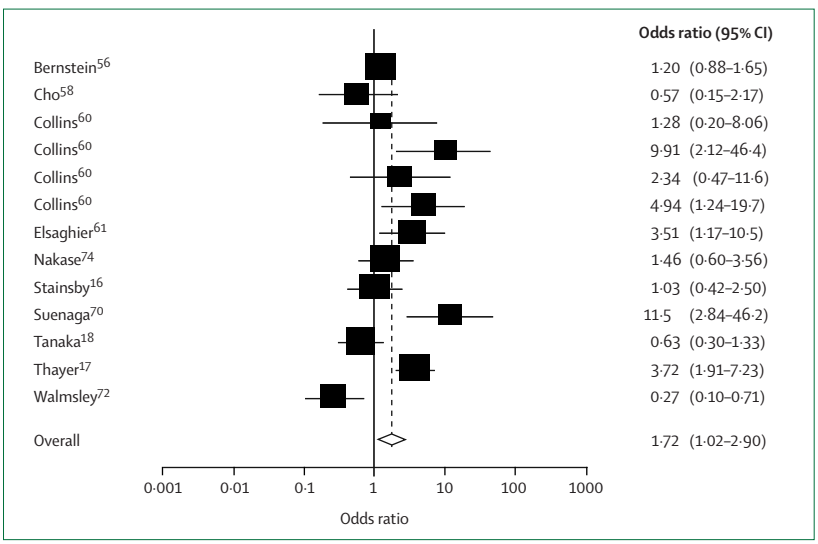

***Figure 2*: Meta-analysis of 13 comparisons from case-control studies of patients with Crohn's disease versus controls, with ELISA in serum to detect *M avium* subspecies *paratuberculosis* (MAP)**
Odds ratios (ORs) and 95% CI for each study are shown. The size of the square represents the relative weight of each study in the random-effects meta-analysis. The data are displayed on a logarithmic scale. ORs above 1·0 indicate a higher prevalence of MAP in patients with Crohn's disease compared with controls.

with controls free of IBD than the comparison with ulcerative colitis patients (I^2=75% and 44%, respectively).

Discussion

On the basis of 28 case-control studies, this systematic review and meta-analysis shows that tests positive for MAP are substantially more common in patients with Crohn's disease, independently of whether PCR in tissue samples or ELISA in serum is used, or whether patients with Crohn's disease are compared with individuals without IBD or patients with ulcerative colitis.

The results of some of the case-control studies included in our review may have been affected by confounding and bias. In well-designed case-control studies, the source population from which controls are sampled should be the same as that from which cases are also sampled.[75] This was not necessarily the case for the studies reviewed here: cases were generally recruited from specialised clinics and controls were either healthy volunteers or patients admitted with other conditions, for example bowel cancer. Furthermore, in case-control studies, it is not possible to establish whether the exposure was present before the onset of the disease or whether it represents an epiphenomenon, which may or may not influence the course of disease once Crohn's disease becomes established. The higher prevalence of positive test results in patients with Crohn's disease could be caused by a higher propensity of inflamed tissue to become infected with MAP.[3] We addressed this issue in additional meta-analyses of comparisons with patients with ulcerative colitis. If infection of inflamed mucosa is more likely, one would expect no difference in the prevalence of positive tests, or a smaller difference, when comparing patients with Crohn's disease with patients with ulcerative colitis. Interestingly, we found that tests were more often positive in patients with Crohn's disease, independently of the type of control group. This result supports a specific role of MAP or MAP-like mycobacteria in Crohn's disease, but not in ulcerative colitis. However, it is not proof of a causal role of MAP.

Most PCR studies targeted the MAP DNA insertion element IS*900*. IS*900* has long been thought to be specific for MAP, but IS*900* elements were also recently found in environmental mycobacteria. Even nested PCR systems (p90/p91; AV1/AV2) gave false-positive results.[76,77] Therefore, no entirely specific IS*900*-based MAP detection exists at present, and a positive result in the tests used in the case-control studies we analysed cannot be equated with MAP infection. The same holds true for the ELISA tests: no MAP-specific test validated for human beings is available.[50,78] Thus, although positive tests were more common among patients with Crohn's disease than among controls, this is not necessarily because of infection with MAP, but may be attributable to other, MAP-like, bacteria.

There were moderate to high levels of between-study heterogeneity. For case-control studies using PCR, heterogeneity could be attributed to a single outlying study, which contributed little weight to the overall analysis, whereas for the studies using ELISA, the sources of heterogeneity remained unclear. Confounding, bias, and differences in study populations are likely to have contributed to heterogeneity. Our focus was on the association between Crohn's disease and MAP and its specificity, rather than on exploring sources of between-study heterogeneity.

Finally, we excluded 13 studies because neither in patients with Crohn's disease nor in controls was MAP detected. One reason for this could be the method of DNA extraction: in 13 studies without detection of MAP by PCR, only three (23%) used an enzymatic step in combination with a mechanical step or sonication for DNA extraction.

In addition to the mycobacterial theory, other hypotheses exist. For example, Traunmüller[79] postulated that environmental or pathogenic mycobacteria (which are able to pass the digestive system because of their acid-fast cell wall) repeatedly stimulate the CD1 system of the intestinal epithelia and lymphoid tissues. Once the gut is pre-immunised, food additives and contaminants, with structures similar to that of mycobacterial lipid antigens, mimic these antigens and boost the immunological reaction.[79] If confirmed, this mechanism could lead to an emphasis on dietary treatment. More recently, Marks and colleagues[80] published data supporting the hypothesis that impaired innate immunity in patients with Crohn's disease predisposes to accumulation of intestinal contents, which can breach the mucosal barrier of the bowel wall. In the absence of adequate numbers of functional neutrophils for the

effective clearance of bacteria, bacteria will be taken up by macrophages to form the granulomata and chronic inflammation typical of Crohn's disease. Unfortunately, the potential role of MAP was not examined in these studies.[80]

The association of MAP and Crohn's disease, based on PCR or ELISA testing, is well established and we doubt that important further insights may be gained from additional case-control studies, such as those included in our meta-analysis. Because of the retrospective nature of case-control studies, it is not possible to ascertain whether MAP was present before the onset of the disease or whether it became established secondarily in inflamed tissues. Longitudinal studies are required to answer this question. Because of the low incidence of Crohn's disease, case-control studies nested within cohort studies are the most promising approach to the clarification of the temporal sequence of MAP and Crohn's disease, by use of ELISA in stored serum or, if available, PCR or culture in stored tissue samples. Ideally, such studies should consider genetic factors, in particular mutations of the *NOD2* gene, which have been consistently associated with Crohn's disease.[1]

Another approach to clarifying the role of MAP is the conduct of clinical trials of drug regimens efficacious against MAP. Combination regimens that include macrolide antibiotics and are given for 2 years have been proposed, based on uncontrolled studies that showed substantial benefits.[81] A randomised trial has recently been done in Australia,[3] and data presented at a conference of the Gastroenterology Society of Queensland in June, 2005, indicate that important improvements over placebo were achieved by 16 weeks.[6] Further clinical trials are needed to clarify the place of antimycobacterial combination regimens, including the importance of the duration of therapy and the role of concomitant immunosuppressive therapy.

Conclusions

A causal association of MAP would have important implications for the processing of milk and other dairy products. The occurrence of MAP in milk of productive livestock is well documented, and several studies have shown that viable MAP organisms can survive standard (high-temperature short-time) pasteurisation methods and the processes used for cheese production if high numbers of bacteria are present.[23,82–84] There are concerns that use of more thorough heat treatments, which eliminate MAP, would change the organoleptic qualities of milk and adversely affect its taste.

On the basis of this systematic review and meta-analysis, an important, causal role of MAP in the aetiology of Crohn's disease can neither be confirmed nor excluded with certainty. The organism may act as a causative agent, have a role in the context of secondary infection, which may exacerbate the disease, or represent non-pathogenic colonisation. Clearly, the accumulating evidence will need to be updated regularly to allow informed judgments on whether and when public-health action is justified.

Search strategy and selection criteria

These are described in detail in the Methods section on page 607.

Conflicts of interest

We declare that we have no conflicts of interest.

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