Detection of *Mycobacterium avium* Subspecies *paratuberculosis* from Patients with Crohn's Disease Using Nucleic Acid-Based Techniques: A Systematic Review and Meta-analysis

I. Abubakar, MD, PhD, D. Myhill, PhD,* S.H. Aliyu, MD,† and P.R. Hunter, MD**

Abstract: This study is a systematic review and meta-analysis of studies using nucleic acid-based techniques to detect *Mycobacterium avium paratuberculosis* (MAP) in patients with Crohn's disease (CD) compared with controls. Database searches were conducted and risk difference estimates were calculated using meta-analysis. Fifty-eight studies were reviewed, 47 of which were included in the analysis. The pooled estimate of risk difference from all studies was 0.23 (95% confidence interval [CI], 0.14–0.32) using a random effects model. Similarly, MAP was detected more frequently from patients with CD compared with those with ulcerative colitis (risk difference 0.19, 95% CI, 0.10–0.28). Year of study, assay type, and inclusion of children explained some but not all of the observed heterogeneity. The data confirms the observation that MAP is detected more frequently among CD patients compared with controls. However, the pathogenic role of this bacterium in the gut remains uncertain. Our analysis demonstrates that there is an association between MAP and CD, across many sites, by many investigators, and controlling for a number of factors; however, this association remains controversial and inconclusive. Future studies should determine whether there is a pathogenic role.

(Inflamm Bowel Dis 2007;00:000–000)

Key Words: *Mycobacterium avium* subspecies *paratuberculosis*, etiology, Crohn's disease

The histopathological and clinical similarities between human chronic granulomatous enteritis,[1,2] intestinal tuberculosis, and animal paratuberculosis[3] has led to the suspicion that *Mycobacterium avium* subspecies *paratuberculosis* (MAP) has a role in the etiology of Crohn's disease (CD). The literature on the pathogenesis, pathophysiology, and clinical management of CD has evolved considerably over the last decade due to advances in molecular biology and genetics. Despite considerable medical debate, the etiology of CD remains an enigma. However, there is a consensus that CD results from a sustained immune response arising from an environmental stimulus in a susceptible host.[4] MAP has been postulated to be a potential stimulus at least in a subset of patients.[5]

Unlike *Mycobacterium tuberculosis*, MAP is fastidious and slow-growing, with nutritional dependence for the iron chelating compound mycobactin.[5] In vitro culture techniques have improved significantly in the last decade. Grant et al[6] compared Mycobacterium Growth Indicator Tube (MGIT) and BACTEC culture media for the recovery of MAP from milk and found both media were able to detect 10 organisms per mL of milk. However, despite these improvements in culture media it remains a difficult organism to isolate from humans, for unclear reasons that may include mycobacterial dormancy or low bacillary burden. Similarly, serological assays are unlikely to provide the evidence required, as they are not reliable for the diagnosis of Johne's disease, a disease of animals caused by MAP.[7]

A number of studies have reported the more frequent detection of the insertion sequence IS900 in CD patients compared with controls using polymerase chain reaction (PCR) assays.[5,8] In contrast, other authors found no association between CD and the detection of IS900.[9,10] The 2 main methods that have been used include PCR and in situ hybridization (ISH) assays. Although other IS900-like elements have been described in environmental mycobacteria,[11,12] the entire IS900 gene is unique to MAP.[13] The short fragments of the IS900 sequence targeted in various studies may not be unique, as evidence of partial cross-hybridization between *M. avium* strains has been reported in AIDS patients.[14] Multiplex PCR simultaneously targeting several fragments of the IS900 gene has been developed to overcome this difficulty.[15] In addition, crosshybridizing elements can be distinguished

Received for publication May 1, 2007; Accepted August 6, 2007.

From the *School of Medicine, Health Policy and Practice, University of East Anglia, Norwich, UK, †Department of Infectious Disease Medicine and HPA Microbiology Laboratory, Addenbrooke's Hospital, Cambridge, UK.

Reprints: Dr. Ibrahim Abubakar, School of Medicine, Health Policy and Practice, University of East Anglia, Norwich NR4 7TJ, UK (e-mail: i.abubakar@uea.ac.uk).

DOI 10.1002/ibd.20276

Published online in Wiley InterScience (www.interscience.wiley.com).

from IS900 by optimized sample processing and through the design of primer pairs.[16]

Various reviews have been conducted examining the MAP hypothesis that suggest that although there is insufficient evidence, a causal link in a subset of CD patients cannot be excluded.[17] There has, however, been no formal systematic review and meta-analysis assessing all the studies using PCR and ISH assays. We present a systematic review of all published studies and, where appropriate, meta-analysis of studies using PCR and ISH to detect MAP in patients with CD and controls.

MATERIALS AND METHODS

Search Strategy

Database searches of MEDLINE (1966 to January 2006), Embase (1980 to January 2006), Biosis (1993 to January 2006), Cochrane Library (2006 Issue 1), and Web of Science were conducted using keywords for the organism (*Mycobacterium avium paratuberculosis,* paratuberculosis, *Mycobacterium paratuberculosis*) and "Crohn's disease." Other databases searched include Database of Abstracts and Reviews and Conference proceedings (World Congress of Gastroenterology, British Gastroenterology Society, United European Gastroenterology Week and American College of Gastroenterology). Bibliographies of published articles including previous reviews were checked for additional studies. The search was limited to studies conducted on human beings and no language restrictions were used. We contacted authors who have published studies relevant to PCR and ISH assays for the detection of MAP in CD patients and relevant material requested.

Inclusion Criteria and Data Extraction

All titles and abstracts from each of the searches were examined. The full text of each article was obtained and reviewed if the study compared the frequency of detection of MAP among CD patients with controls using PCR or ISH assays. A reviewer (I.A.) extracted data from each study. Any study without a control group was excluded.

Quality Assessment

The quality of each study was appraised using a form adapted from http://www.medepi.net/meta/. No studies were excluded on the basis of quality, but rather the criteria were used to check for evidence of heterogeneity in the analysis.

Statistical Analysis

Risk difference was estimated from the selected studies and pooled using meta-analysis. If significant heterogeneity ($P < 0.20$) between study results was evident using a chi-square test, a random effects model was used to calculate the summary risk difference. Meta-regression analysis was used to investigate the effect of heterogeneity due to study characteristics including measures of quality. This allowed us to assess the extent to which covariates affect the random effects summary measure estimate. Factors likely to result in differences in the ability of assays to detect MAP were agreed on a priori such as the inclusion of children, study year, reported inclusion of patients on immunosuppressive therapy, use of nested PCR, mechanical disruption and laser dissection, blinding, use of positive and negative controls, and the presence of granulomas in tissue samples. Publication bias was assessed based on funnel plots and a P-value of less than 0.20 using Begg's test.[18] Statistical terms used in the article are explained in the box. All analyses were conducted using the Stata software (v. 9; StatA Corp., Texas).

Risk difference: the main outcome measure used in this study, is defined here as the difference between the risk in the Crohn's disease group (proportion of positive results) and the risk in the control group (proportion of positive results). The risk difference describes the actual difference in the probability of a positive result observed among Crohn's disease patients and controls.

Funnel plot: a method used to assess publication bias. It is a simple scatterplot of the study effect, risk difference in this case, estimated from individual studies against some measure of each study's sample size (standard error of the risk difference in this meta-analysis). In the absence of bias the plot should resemble a symmetrical funnel.

Statistical heterogeneity: excessive variation between studies beyond what would be expected by chance alone. The difference is usually due to clinical and/or methodological diversity among the studies.

Random effects model: assumes that the effects being estimated in the different studies are not identical, but follow some distribution. The center of this symmetric distribution describes the average of the effects, while its width describes the degree of heterogeneity. This type of model is usually used in the presence of heterogeneity.

Ethical Considerations

This study is a systematic review of the literature with no patient contact and does not raise any ethical issue of note.

RESULTS

The study selection procedure is outlined in Figure 1. Fifty-eight full text studies were reviewed, 47 of which were included in the analysis. Two studies reported the assessment of both a PCR and an ISH assay.[19,20] Therefore, a total of 49 studies were analyzed. The median study population size was 51 individuals, with a range of 5 to 200.

Study Characteristics

Table 1 summarizes the characteristics of the included studies. The assessment of study quality was limited by the

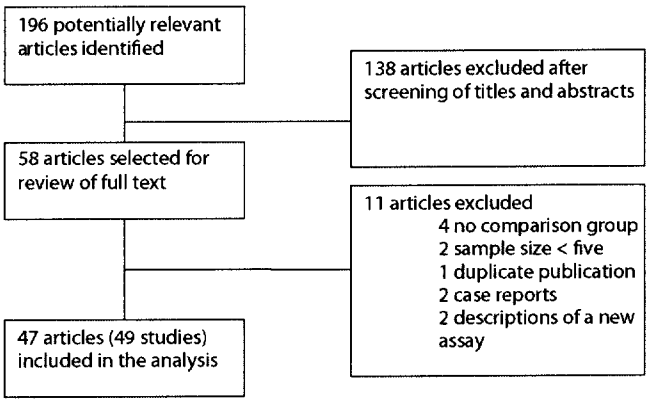

FIGURE 1. Flow chart outlining the study selection process.

amount of information reported in published articles; however, additional data were obtained from authors (10/47) where possible. The studies are reported from laboratories in 15 countries. Forty-three used PCR, while 6 studies reported analysis of in situ or DNA hybridization. In 2 studies the only available comparison group were patients with ulcerative colitis (UC).[21,22]

Forty-two PCR studies used the IS900 target sequence, while 1 used 16s rRNA.[23] Four hybridization assays used the insertion sequence IS900 as the target. The methods used for DNA extraction also differed. Table 1 outlines the variation in quality between studies. Several measures that may enhance detection of MAP in humans, such as mechanical disruption of tissue and the use of nested PCR, were not used in many studies. Six studies had an additional step of mechanical disruption.[16,24–27] Eleven (25.6%) PCR studies and 1 (16.7%) hybridization study reported blinding of the investigator as to the source of tissues or samples. All studies describe measures to reduce the probability of cross-contamination; hence, we could not investigate this as a source of heterogeneity.

All Studies

A meta-analysis of all 49 studies (including 2 studies with both PCR and hybridization tests) revealed evidence of significant heterogeneity. The pooled estimate of risk difference was 0.23 (95% confidence interval [CI], 0.14–0.32) using a random effects model. The studies were divided into the 2 main test groups (PCR and hybridization assays).

Sensitivity analysis was carried out by excluding studies with no evidence of MAP from any CD patient or control. Thirty-two studies detected the organism in at least 1 person. Among these studies a risk difference of 0.33 (95% CI, 0.22–0.45) was estimated. Figure 2 shows the forest plot of the risk difference from the reviewed studies.

PCR Studies

Thirteen studies showed a risk difference of zero. Three studies showed a negative risk difference suggesting greater detection of MAP in control specimens, although only 1 was statistically significant. The full range of risk difference estimates varied from 0.21 to 0.73.

There was statistically significant evidence of heterogeneity (chi-square = 468.99, $P < 0.001$). The summary measure of risk difference from a random effects model was 0.20 (95% CI, 0.12–0.28) and remained almost identical after excluding the study by Kreuzpaintner et al,[23] which did not use IS900 (0.20; 95% CI, 0.12–0.28). Similarly, exclusion of any single study did not significantly alter the summary measure. When the analysis was limited to studies investigating tissue samples only (i.e., excluding blood), a risk difference of 0.23 (95% CI, 0.10–0.29) was observed.

Hybridization Studies

There was significant evidence of heterogeneity (chi-square = 301.29, $P < 0.001$); therefore, a random effects model was used. The summary measure of risk difference was 0.43 (95% CI, 0.01–0.84).

Detection of MAP in CD Compared with UC

Thirty-eight studies had data comparing samples from CD and UC patients. The pooled risk difference was 0.19 (95% CI, 0.10–0.28).

Investigating Heterogeneity and Publication Bias

We used a regression analysis model to investigate potential sources of heterogeneity. Factors considered a priori to be potential sources of heterogeneity were chosen either because they relate to the ability to detect MAP or can lead to a bias in estimating the measure of association. Table 2 shows the results of the meta-regression model for all studies, PCR assays and hybridization tests. The year of study, type of assay, and inclusion of children in the study were associated with the outcome measure. The positive association suggests that more recent studies, those that include children and PCR studies, were more likely to report a positive result.

The funnel plots for publication bias showed evidence of asymmetry (Fig. 3) (Begg's test; $z = 3.08$, $P = 0.002$).

DISCUSSION

The results suggest that there is sufficient evidence for the presence of MAP in the gut of patients with CD. A summary risk difference of 0.23 (95% CI, 0.14–0.32) was calculated, which remained consistent after sensitivity analysis excluding various sets of studies. Earlier studies have suggested that MAP may also be found among controls but recent investigations using appropriate extraction techniques and more sensitive assays have revealed a significantly higher

TABLE 1. Characteristics of Studies and Risk Difference for the Detection of *Mycobacterium paratuberculosis* in Crohn's Disease Patients Compared to Non-Inflammatory Bowel Disease Controls.

Study, Year (References)	Assay Type (Target Sequence)	Inclusion of Children	Mechanical Disruption	Granuloma	Controls	Immunosuppression	Blinding	Cases (Positives/ Total)	Controls (Positives/ Total)	Risk Difference (95% Confidence Interval)	Location
PCR assays											
Rosenberg 1991[40]	IS900	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	(0/21)	(0/6)	0.00 (–0.20–0.20)	Oxford, UK
Sanderson 1992[41]	IS900	Yes	No	Yes	Yes	Not stated	No	(26/40)	(5/40)	0.53 (0.35–0.70)	London, UK
Moss 1992[42]	IS900	Not stated	No	Yes	Yes	Not stated	Not stated	(6/18)	(1/6)	0.17 (–0.20–0.54)	London, UK
Wall 1993[43]	IS900	Not stated	No	Not stated.	Yes	Not stated	No	(6/28)	(0/13)	0.21 (0.03–0.39)	Surrey, UK
Cellier 1993[44]	IS900	No	Yes	Not stated	Not stated	Yes	No	(0/45)	(0/18)	0.00 (–0.08–0.08)	Paris, France
Dell Isola 1994[25]	IS900	No	Yes	Not stated	Yes	Yes	Yes	(13/18)	(9/30)	0.42 (0.16–0.69)	Paris, France
Lisby 1994[45]	Nested	Yes	No	Yes	Yes	Yes	No	(11/24)	(3/28)	0.35 (0.12–0.58)	Copenhagen, Denmark
Fidler 1994[46]	IS900	No	No	Yes	Yes	Not stated	Yes	(4/31)	(0/20)	0.13 (–0.01–0.27)	Surrey, UK
Rowbotham 1995[36]	IS900	Yes	No	Not stated	Yes	Yes	Not stated	(0/68)	(1/26)	–0.04 (–0.13–0.05)	Leeds, UK
Murray 1995[37]	IS900	No	No	Not stated	Yes	Not stated	Yes	(2/9)	(0/15)	0.22 (–0.06–0.50)	Palmerston N, NZ
Suenaga 1995[47]	IS900	No	No	Yes	Not stated	Not stated	Not stated	(10/10)	(14/16)	0.13 (–0.08–0.33)	Kochi, Japan
Erasmus 1995[48]	IS900	Not stated	Not stated	Yes	Yes	Not stated	Not stated	(10/26)	(4/34)	0.27 (0.06–0.48)	Tygerberg, SA
Kreuzpainter 1995[23]	16s rDNA	Yes	No	Not stated	Not stated	Yes	No	(0/4)	(0/1)	0.00 (–0.66–0.66)	Hamburg, Germany
Mishina 1996[49]	RT IS900	No	No	Not stated	Yes	Yes	Not stated	(8/8)	(0/2)	1.00 (0.55–1.45)	New York, US
Dumonceau 1996[50]	Nested IS900	No	No	Not stated	Yes	Not stated	Not stated	(0/36)	(0/23)	0.00 (–0.07–0.07)	Brussels, Belgium
Frank 1996[9]	Nested IS900	No	No	Yes	Yes	Not stated	No	(0/23)	(0/11)	0.00 (–0.13–0.13)	Michigan, US
Gan 1997[51]	IS900	Not stated	No	Yes	Yes	Not stated	Not stated	(17/36)	(3/20)	0.32 (0.10–0.55)	Chengdu, China
Al Shamali 1997[52]	IS900	No	No	Various types	Yes	Not stated	Not stated	(0/10)	(0/21)	0.00 (–0.14–0.14)	Kuwait, Kuwait
Huatian 1997[53]	IS900	Not stated	No	Yes	Yes	Not stated	No	(17/36)	(3/20)	0.32 (0.10–0.55)	Chengdu, China
Riggio 1997[54]	Nested IS900	Not stated	No	Oral tissue–yes	Yes	Not stated	Not stated	(0/7)	(0/12)	0.00 (–0.20–0.20)	Glasgow, UK

TABLE 1. *(Continued)*

Study, Year (References)	Assay Type (Target Sequence)	Inclusion of Children	Mechanical Disruption	Granuloma	Controls	Immunosuppression	Blinding	Cases (Positives/ Total)	Controls (Positives/ Total)	Risk Difference (95% Confidence Interval)	Location
Clarkston 1998[55]	IS900	No	No	Yes	Yes	Not stated	Yes	(1/21)	(0/11)	0.05 (–0.11–0.20)	Kansas City, US
Kallinowski 1998[56]	IS900	No	No	Not stated	Yes	Not stated	Not stated	(0/21)	(0/24)	0.00 (–0.08–0.08)	Heidelberg, Germany
Del Prete 1998[57]	IS900	Yes	Not stated	Stool samples	Yes	Not stated	Yes	(15/32)	(1/41)	0.44 (0.27–0.62)	Bari, Italy
Chiba 1998[58]	Nested IS900	Yes	Not stated	Yes	Yes	Not stated	Not stated	(0/30)	(0/3)	0.00 (–0.33–0.33)	Akita, Japan
Cellier 1998[24]	Nested IS900	No	Yes	Yes	Yes	Yes	No	(2/47)	(2/20)	–0.06 (–0.20–0.09)	Paris, France
Kanazawa 1999[10]	IS900	No	No	Not stated	Yes	Not stated	Not stated	(0/13)	(0/13)	0.00 (–0.14–0.14)	Hirosaki, Japan
Collins 2000[59]	Nested IS900	No	No	Not stated	Yes	Yes	Yes	(15/79)	(3/48)	0.13 (0.02–0.24)	Wisconsin, US
Schwartz 2000	IS900	Not stated	No	Not stated	Yes	Not stated	Not stated	(10/27)	(1/15)	0.30 (0.08–0.53)	US
Gibson 2000[60]	IS900	Not stated	No	OFG	Yes	Not stated	Not stated	(0/24)	(0/12)	0.00 (–0.12–0.12)	Glasgow, UK
Hulten 2001b[19]	IS900	No	No	Yes	Yes	Not stated	Yes	(9/36)	(0/22)	0.25 (0.10–0.40)	Houston, US
Ryan 2002[38]	Nested, CSLM IS900	Not stated	No	Yes	Yes	Not stated	Not stated	(6/15)	(0/12)	0.40 (0.14–0.66)	Cork, Ireland
Fujita 2002[31]	IS900	No	Not stated	Yes	Not stated	Not stated	Not stated	(0/16)	(0/18)	0.00 (–0.11–0.11)	Tokyo, Japan
Ellingson 2003[61]	IS900	Not stated	Not stated	Yes	Yes	Not stated	Not stated	(0/35)	(0/21)	0.00 (–0.07–0.07)	Iowa, US
Bernstein 2003[62]	Nested IS900	No	No	Yes	Yes	Not stated	Yes	(0/24)	(6/28)	–0.21 (–0.38—0.05)	Manitoba, Canada
Bull 2003[16]	Nested IS900	Yes	Yes	Yes	Yes	Yes	Yes	(34/37)	(9/34)	0.65 (0.48–0.83)	London, UK
Naser 2004[8]	Nested, IS900	No	Not stated	Blood samples	Yes	Yes	Yes	(13/28)	(3/15)	0.26 (–0.01–0.54)	Orlando, US
Sechi 2004b[20]	IS900	Not stated	Not stated	Not stated	Yes	Not stated	Not stated	(8/82)	(0/20)	0.10 (0.01–0.19)	Sassari, Italy
Sechi 2005[27]	IS900	Not stated	Not stated	Not stated	Yes	Not stated	Not stated	(25/37)	(7/34)	0.47 (0.27–0.67)	Sassari, Italy
Sechi 2005[28]	IS900	Yes	Yes	Not stated	Yes	Yes	Not stated	(25/30)	(3/29)	0.73 (0.56–0.90)	Sassari, Italy
Romero 2005[63]	Nested CSLM IS900	No	No	Not stated	Yes	Yes	No	(10/12)	(1/6)	0.67 (0.30–1.03)	Orlando, US

TABLE 1. *(Continued)*

Study, Year (References)	Assay Type (Target Sequence)	Inclusion of Children	Mechanical Disruption	Granuloma	Controls	Immunosuppression	Blinding	Cases (Positives/ Total)	Controls (Positives/ Total)	Risk Difference (95% Confidence Interval)	Location
Autschbach 2005[29]	Nested IS900	No	Yes	Yes	Yes	Yes	Yes	52/100)	(5/100)	0.47 (0.36–0.58)	Heidelberg, Germany
Pooled risk difference										0.20 (0.12–0.28)	
Hybridization											
Butcher 1988[64]	pMB21 & pMB22	Not stated	No	Not stated	Yes	Not stated	No	(0/17)	(0/4)	0.00 (–0.27–0.27)	London, UK
Yoshimura 1988[65]	DNA DNA	Not stated	No	Not stated	Not stated	Not stated	Not stated	(10/19)	(1/6)	0.36 (–0.01–0.73)	Houston, US
McFadden 1992[66]	DNA IS900 pMB22	Not stated	No	Not stated	Not stated	Not stated	Not stated	(2/82)	(0/26)	0.02 (–0.04–0.09)	Surrey, UK
Hulten 2001[67]	insitu IS900	No	No	Yes	Yes	Not stated	Yes	(7/37)	(0/22)	0.43 (0.26–0.60)	Houston, US
Sechi 2001[72]	In situ IS900 p89 & p92	Not stated	Not stated	Yes	Yes	Not stated	Not stated	(27/33)	(0/20)	0.82 (0.67–0.97)	Sassari, Italy
Sechi 2004[20]	In situ IS900 p89 & p92	Not stated	Not stated	Not stated	Yes	Not stated	Not stated	(73/82)	(0/20)	0.89 (0.80–0.98)	Sassari, Italy
Pooled risk difference										0.43 (0.01–0.84)	

CSLM, confocal scanning laser microdissection; RT, reverse transcriptase; OFG, orofacial granulomatosis.

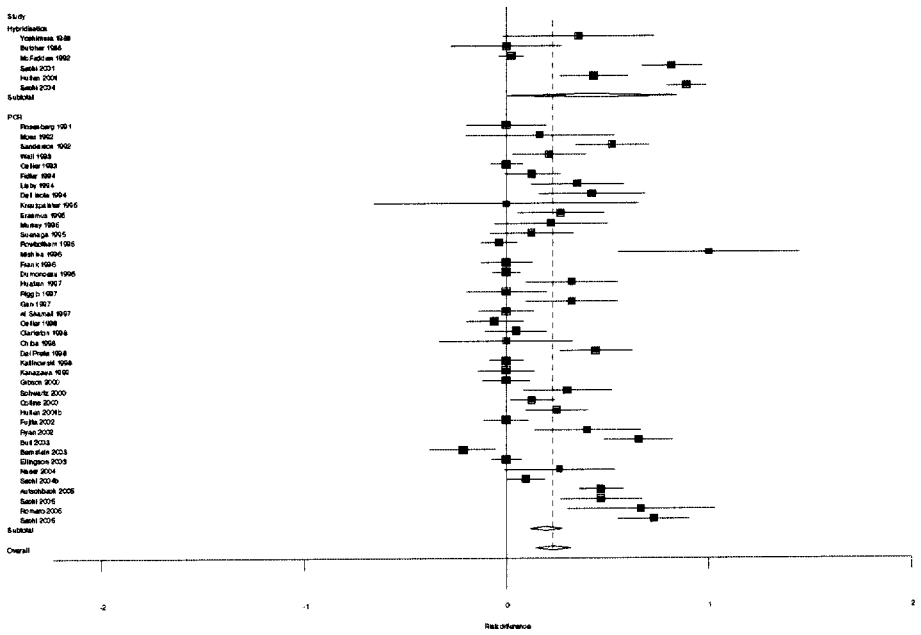

FIGURE 2. Forest plot for risk difference in the detection of *Mycobacterium paratuberculosis* in CD patients compared with non-IBD controls using PCR and DNA hybridization assays. Each solid box represents the risk difference estimate from a study with error bars indicating 95% confidence intervals. The dotted vertical line is the random effects summary estimate of risk difference and 95% confidence intervals for overall effect is represented by the diamond box.

level of detection by PCR assays in CD patients compared with controls.[16,20,27,28,29]

Interpreting the Findings

The pathogenic role of MAP in CD is uncertain. Our analysis reveals a high strength of association with a reasonable level of consistency among recently conducted studies. However, for any agent to be considered a cause of a disease, evidence that the infection precedes the pathology is essential. The studies in this review do not provide evidence of temporality in the association.

The strength of the association and consistent detection of MAP DNA does not prove causation, and may arise from prior ingestion of nonviable organisms in water or milk. However, the detection of MAP DNA in the blood of CD patients[8] may suggest that a viable form of the organism is present in CD. This study by Naser et al did not report the detection of MAP in intestinal tissue, nor explore the possi-

TABLE 2. Meta-regression of the Relationship of Risk Difference and Study Characteristics in 49 Studies Comparing the Detection of *M. paratuberculosis* in Crohn's Disease and Control Patients.

Variable	Yes	No	Missing	Coefficient	*P*-value
Inclusion of children	9	21	19	0.22	0.045
PCR versus hybridization	43	6	0	0.24	0.040
Year				0.02	0.027
Granuloma	26	22	1	–0.01	0.877
Blinding	12	10	27	0.07	0.485
Immunosuppression	13	0	36	–0.02	0.118
CSLM	2	47		0.30	0.164
Mechanical disruption	6	29	14	0.21	0.056
Nested PCR	13	36		–0.50	0.598
Controls	40	9		0.19	0.099

CSLM, confocal scanning laser microdissection; PCR, polymerase chain reaction.

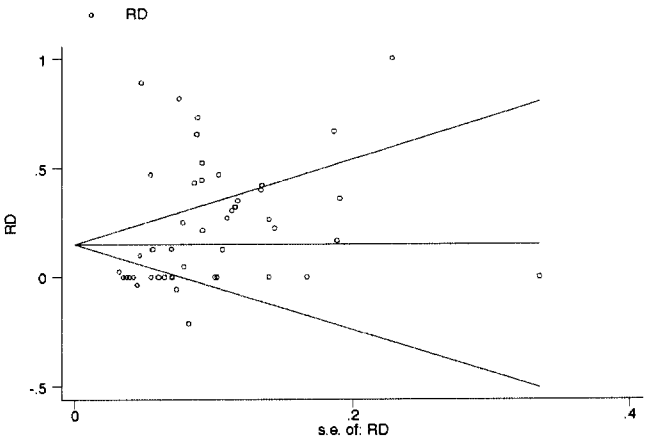

FIGURE 3. Funnel plot for the assessment of publication bias in all studies. Each circle represents a study in the meta-analysis. The central line represents the random effects summary risk difference. The presence of studies outside the funnel-shaped lines diverging from the summary effect is suggestive of publication bias. This is supported by the statistically significant results of the Begg's test. RD, risk difference; SE, standard error.

bility that the results are a secondary phenomenon due to increased gut permeability or the inability of macrophages in CD to kill MAP. The increased gut leakiness hypothesis is supported by the more frequent detection of other organisms in CD patients compared with controls[30,31] and suggest a lack of specificity of MAP detection. For instance, the risk difference for *Bacteriodes* and *Escherichia coli* from the Fujita et al[31] study was 0.5 and 0.4, respectively. In contrast, the frequent detection of MAP in CD compared with UC argues against increased gut permeability as the sole explanation.

Subgroup analyses were undertaken since IS900-based ISH has been discredited as a nonspecific tool.[32] Therefore, the results of PCR assays were considered more reliable. Similarly, the analysis was restricted to those studies investigating intestinal tissue. This did not alter the findings.

It has been suggested that most of the positive results have been reported by a limited number of laboratories.[33] However, data from this review indicates that positive results have been observed by several laboratories in different parts of the world. Low assay sensitivity used by certain laboratories may contribute to this observation, whereby only groups that have developed sufficiently sensitive tests are successful. Conversely, many studies detect MAP DNA in the control patients. This may be because MAP is potentially common in the environment. Alternatively, this may suggest that it is not pathogenic to all exposed individuals.

Strengths and Weaknesses

A comprehensive search strategy was used in this review. However, despite this, significant evidence of publication bias was found, suggesting that not all studies were identified.

In addition to presenting summary measures alone, meta-regression analysis was used to explore heterogeneity. Factors included in the meta-regression model were agreed a priori. However, limited availability of data hampered the exploration of sources of heterogeneity, for example, the effect of factors such as contamination in the meta-regression analysis. Nevertheless, this review investigated important factors related to the variation in the results of published studies.

This review does not systematically identify and critique other studies that may be important in the understanding of the role of MAP in CD such as the use of antimycobacterial therapy. This is the subject of a Cochrane review[34] that showed that antibiotics may have a role in maintaining remission. The results of the largest randomized controlled trial to date does not support the use of antibiotics in CD.[35]

Exploring Heterogeneity

The aim of a review should not be limited to producing a summary estimate of divergent results but also to explore the reasons for the observed differences. Heterogeneity is inevitable in a meta-analysis of observational studies. Furthermore, the studies were carried out over a long time period during which laboratory methods evolved. A significant positive association with year of study with more recent studies more likely to have positive results was identified. This is very likely a proxy measure of changes over time in the assay techniques. The sensitivity of assays improved over the last 2 decades due to a combination of better extraction methods and enhanced detection limits. A significant positive association was observed when studies including children were compared with those with an adult population. It has been suggested that if the initial MAP infection occurs in childhood, its detection in studies among children[25] would be more likely. This implies that earlier in the course of the disease, MAP is present. However, others have found no difference in the detection of MAP based on age using PCR.[36] Other factors considered a priori to be potential causes of heterogeneity were not statistically significant.

Due to the hypothesized paucibacillary nature of MAP infection in CD, it is possible that there are very low numbers of infecting organisms, below the detection limits of the assay. Limiting the studies in the meta-analysis to those that have detected MAP in at least 1 sample either from a patient with CD, UC, or a control did not alter the results.

The exclusion of the study with a different target sequence[23] did not alter the results. A number of studies included immunosuppressed patients. The results of this review showed no association with reported use of immunosuppressive agents in the study population; however, the majority of studies did not explicitly look for an association. Authors reporting negatives results have argued that contamination is a major factor in the detection of MAP.[24] Unfortunately, due

to variation in techniques for reducing contamination and limitations of the data available in published articles, this factor was not assessed. The use of appropriate controls and the meticulous steps taken by most of the studies suggest that this is unlikely to be the explanation for the detection of MAP.

There are other factors that may be associated with differences in the ability of the studies to detect MAP that we did not investigate. These include limitations of sampling techniques, failure to process fresh tissue samples (fixing results in fragmentation of DNA), and the role of concomitant use of antimicrobial drugs. Tissues from operative procedures provide more quantity and reduce sampling errors, but it is unclear whether patients with early lesions are more likely to have MAP compared with those that have developed complications requiring surgery.[37] Sampling error due either to low abundance of MAP in tissues or lower numbers in nongranulomatous tissue has been suggested as an explanation for studies failing to detect MAP. Laser capture microdissection techniques have been used to specifically isolate granulomas that were subsequently assayed, resulting in the detection of MAP more frequently in CD patients compared to controls.[38] However, the authors of the same report detected the DNA of other bacteria more frequently in CD tissue compared to controls using the same technique.[39] Furthermore, other studies[24,36] that include intestinal granulomas and lymph nodes failed to detect MAP, while some detected the organism in various sites including noninflamed tissue.[41] This meta-analysis did not show that investigating granulomas is a significant source of heterogeneity. Considerable heterogeneity remained after controlling for the effect of all the factors investigated.

It is unlikely that similar studies using PCR assays are going to change the current state of evidence. Our analysis demonstrates that there is an association between MAP and CD, across many sites, by many investigators, and controlling for a number of factors. While the summary effect measures from this study show an association between CD and MAP, this association remains controversial and inconclusive. Future studies should determine whether there is a pathogenic role. Such studies should also assess the evidence of temporality and specificity of the association.

REFERENCES

1. Crohn BB, Ginzburg K, Oppenheimer GD. Regional ileitis: a pathological and clinical entity. *JAMA*. 1932;99:1323–1328.
2. Dalziel TK. Chronic Intestinal Enteritis. BMJ. 1913. 1068–1070.
3. Johne HA, Frothingham J. Ein eigenthuemlicher fallvon tuberculose beim rind. *Dtsch Z Tiermed Pathol.* 1895;21:438–454.
4. Shanahan F. Crohn's disease. *Lancet.* 2002;359:62–69.
5. Hermon-Taylor J, El-Zaatari FAK. The *Mycobacterium avium* subspecies *paratuberculosis* problem and its relation to the causation of Crohn disease. In: Bartram J, Dufour JC, Rees G, Pedley S, eds. Pathogenic Mycobacteria in Water: A Guide to Public Health Consequences, Monitoring and Management. London: IWA Publishing; 2004:74–94.
6. Grant IR, Kirk RB, Hitchings E, et al. Comparative evaluation of the MGIT and BACTEC culture systems for the recovery of Mycobacterium avium subsp. paratuberculosis from milk. *J Appl Microbiol.* 2003;95: 196–201.
7. Selby WS. Mycobacterium avium subspecies paratuberculosis bacteraemia in patients with inflammatory bowel disease. *Lancet.* 2004;364: 1013–1014.
8. Naser SA, Ghobrial G, Romero C, et al. Culture of Mycobacterium avium subspecies paratuberculosis from the blood of patients with Crohn's disease. *Lancet.* 2004;364:1039–1044.
9. Frank TS, Cook SM. Analysis of paraffin sections of Crohn's disease for Mycobacterium paratuberculosis using polymerase chain reaction. *Mod Pathol.* 1996;9:32–35.
10. Kanazawa K, Haga Y, Funakoshi O, et al. Absence of Mycobacterium paratuberculosis DNA in intestinal tissues from Crohn's disease by nested polymerase chain reaction. *J Gastroenterol.* 1999;34:200–206.
11. Cousins DV, Whittington R, Marsh I, et al. Mycobacteria distinct from Mycobacterium avium subsp. paratuberculosis isolated from the faeces of ruminants possess IS900-like sequences detectable IS900 polymerase chain reaction: implications for diagnosis. *Mol Cell Probes.* 1999;13: 431–442.
12. Englund S, Bolske G, Johansson KE. An IS900-like sequence found in a Mycobacterium sp. other than Mycobacterium avium subsp. paratuberculosis. *FEMS Microbiol Lett.* 2002;209:267–271.
13. Bartos M, Hlozek P, Svastova P, et al. Identification of members of Mycobacterium avium species by Accu-Probes, serotyping, and single IS900, IS901, IS1245 and IS901-flanking region PCR with internal standards. *J Microbiol Methods.* 2006;64:333–345.
14. Naser S, Shafran I, El Zaatari F. Mycobacterium avium subsp. paratuberculosis in Crohn's disease is serologically positive. *Clin Diagn Lab Immunol.* 1999;6:282.
15. Bull TJ, Hermon-Taylor J, Pavlik I, et al. Characterization of IS900 loci in Mycobacterium avium subsp. paratuberculosis and development of multiplex PCR typing. *Microbiology.* 2000;146(Pt 12):3285.
16. Bull TJ, McMinn EJ, Sidi-Boumedine K, et al. Detection and verification of Mycobacterium avium subsp. paratuberculosis in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. *J Clin Microbiol.* 2003;41:2915–2923.
17. Scientific Committee on Animal Health and Animal Welfare. Possible links between Crohn's disease and paratuberculosis. SANCO/B3/R16/ 2000, 1-76. 21-3-2000. European Commission.
18. Dear K, Begg C. An approach to assessing publication bias prior to performing a meta-analysis. *Stat Sci.* 1992;7:237–245.
19. Hulten K, El Zimaity HM, Karttunen TJ, et al. Detection of Mycobacterium avium subspecies paratuberculosis in Crohn's diseased tissues by in situ hybridization. *Am J Gastroenterol.* 2001;96:1529–1535.
20. Sechi LA, Mura M, Tanda E, et al. Mycobacterium avium sub. paratuberculosis in tissue samples of Crohn's disease patients. *New Microbiol.* 2004;27:75–77.
21. Quirke P, Dockey D, Taylor GR, et al. Detection of Mycobacterium paratuberculosis in inflammatory bowel disease. *Gut.* 1991;32:A572.
22. Chiba M, Kono M, Hoshina S, et al. Presence of bacterial 16S ribosomal RNA gene segments in human intestinal lymph follicles. *Scand J Gastroenterol.* 2000;35:824–831.
23. Kreuzpaintner G, Kirschner P, Wallner A, et al. Mycobacteria of Runyon groups I, II and IV do not play an aetiological role in Crohn's disease. *Eur J Gastroenterol Hepatol.* 1995;7:1177–1182.
24. Cellier C, De Beenhouwer H, Berger A, et al. Mycobacterium paratuberculosis and Mycobacterium avium subsp. silvaticum DNA cannot be detected by PCR in Crohn's disease tissue. *Gastroenterol Clin Biol.* 1998;22:675–678.
25. Dell'Isola B, Poyart C, Goulet O, et al. Detection of Mycobacterium paratuberculosis by polymerase chain reaction in children with Crohn's disease. *J Infect Dis.* 1994;169:449–451.
26. Autschbach F, Eisold S, Hinz U, et al. High prevalence of Mycobacterium avium subspecies paratuberculosis IS900 DNA in gut tissues from individuals with Crohn's disease. *Gut.* 2005;54:944–949.
27. Sechi LA, Scanu AM, Molicotti P, et al. Detection and isolation of Mycobacterium avium subspecies paratuberculosis from intestinal mucosal biopsies of patients with and without Crohn's disease in Sardinia. *Am J Gastroenterol.* 2005;100:1529–1536.
28. Sechi LA, Gazouli M, Ikonomopoulos J, et al. Mycobacterium avium

subsp. paratuberculosis, genetic susceptibility to Crohn's disease, and Sardinians: the way ahead. *J Clin Microbiol.* 2005;43:5275–5277.
29. Autschbach F, Eisold S, Hinz U, et al. High prevalence of Mycobacterium avium subspecies paratuberculosis IS900 DNA in gut tissues from individuals with Crohn's disease. *Gut.* 2005;54:944–949.
30. Liu Y, Van Kruiningen HJ, West AB, et al. Immunocytochemical evidence of Listeria, Escherichia coli, and Streptococcus antigens in Crohn's disease. *Gastroenterology.* 1995;108:1396–1404.
31. Fujita H, Eishi Y, Ishige I, et al. Quantitative analysis of bacterial DNA from Mycobacteria spp., Bacteroides vulgatus, and Escherichia coli in tissue samples from patients with inflammatory bowel diseases. *J Gastroenterol.* 2002;37:509–516.
32. Jeyanathan M, Alexander DC, Turenne CY, et al. Evaluation of in situ methods used to detect Mycobacterium avium subsp. paratuberculosis in samples from patients with Crohn's disease. *J Clin Microbiol.* 2006;44: 2942–2950.
33. Quirke P. Antagonist. Mycobacterium avium subspecies paratuberculosis is a cause of Crohn's disease. *Gut.* 2001;49:757–760.
34. Borgaonkar M, MacIntosh D, Fardy J, et al. Anti-tuberculous therapy for maintaining remission of Crohn's disease. *Cochrane Database Syst Rev.* 2000;CD000299.
35. Selby WS, Crotty B, Florin T, et al. Antimycobacterial therapy: Past it or present? Results of the 2004 Australian Trial — Balanced Overview. Birmingham. British Society of Gastroenterology.
36. Rowbotham DS, Mapstone NP, Trejdosiewicz LK, et al. Mycobacterium paratuberculosis DNA not detected in Crohn's disease tissue by fluorescent polymerase chain reaction. *Gut.* 1995;37:660–667.
37. Murray A, Oliaro J, Schlup MM, et al. Mycobacterium paratuberculosis and inflammatory bowel disease: frequency distribution in serial colonoscopic biopsies using the polymerase chain reaction. *Microbios.* 1995; 83:217–228.
38. Ryan P, Bennett MW, Aarons S, et al. PCR detection of Mycobacterium paratuberculosis in Crohn's disease granulomas isolated by laser capture microdissection. *Gut.* 2002;51:665–670.
39. Ryan P, Kelly RG, Lee G, et al. Bacterial DNA within granulomas of patients with Crohn's disease—detection by laser capture microdissection and PCR. *Am J Gastroenterol.* 2004;99:1539–1543.
40. Rosenberg WMC, Bell I, Jewell DP. Mycobacterium paratuberculosis DNA cannot be detected in Crohn's disease tissues. *Gastroenterology.* 1991;100:A611.
41. Sanderson JD, Moss MT, Tizard ML, et al. Mycobacterium paratuberculosis DNA in Crohn's disease tissue. *Gut.* 1992;33:890–896.
42. Moss MT, Sanderson JD, Tizard ML, et al. Polymerase chain reaction detection of Mycobacterium paratuberculosis and Mycobacterium avium subsp silvaticum in long term cultures from Crohn's disease and control tissues. *Gut.* 1992;33:1209–1213.
43. Wall S, Kunze ZM, Saboor S, et al. Identification of spheroplast-like agents isolated from tissues of patients with Crohn's disease and control tissues by polymerase chain reaction. *J Clin Microbiol.* 1993;31:1241–1245.
44. Cellier C, Chaussade S, Noel LH. Perinuclear antineutrophil cytoplasm antibodies: frequency in ulcerative colitis, Crohn' disease and others GI tract disorders [Abstract]. *Gastroenterology.* 1993:104.
45. Lisby G, Andersen J, Engbaek K, et al. Mycobacterium paratuberculosis in intestinal tissue from patients with Crohn's disease demonstrated by a nested primer polymerase chain reaction. *Scand J Gastroenterol.* 1994;29:923–929.
46. Fidler HM, Thurrell W, Johnson NM, et al. Specific detection of Mycobacterium paratuberculosis DNA associated with granulomatous tissue in Crohn's disease. *Gut.* 1994;35:506–510.
47. Suenaga K, Yokoyama Y, Okazaki K, et al. Mycobacteria in the intestine of Japanese patients with inflammatory bowel disease. *Am J Gastroenterol.* 1995;90:76–80.
48. Erasmus DL, Victor TC, van Eeden PJ, et al. Mycobacterium paratuberculosis and Crohn's disease. *Gut.* 1995;36:942.
49. Mishina D, Katsel P, Brown ST, et al. On the etiology of Crohn disease. *Proc Natl Acad Sci U S A.* 1996;93:9816–9820.
50. Dumonceau JM, Van Gossum A, Adler M, et al. No Mycobacterium paratuberculosis found in Crohn's disease using polymerase chain reaction. *Dig Dis Sci.* 1996;41:421–426.
51. Gan H, Ouyang Q, Bu H. [Mycobacterium paratuberculosis in the intestine of patients with Crohn's disease]. *Zhonghua Nei Ke Za Zhi.* 1997;36:228–230.
52. Al Shamali M, Khan I, Al Nakib B, et al. A multiplex polymerase chain reaction assay for the detection of Mycobacterium paratuberculosis DNA in Crohn's disease tissue. *Scand J Gastroenterol.* 1997;32:819–823.
53. Huatian G, Qin O, Hong B. Mycobacterium paratuberculosis in the intestine of patients with Crohn's disease. *Chin J Intern Med.* 1997;36: 228–230.
54. Riggio MP, Gibson J, Lennon A, et al. Search for Mycobacterium paratuberculosis DNA in orofacial granulomatosis and oral Crohn's disease tissue by polymerase chain reaction. *Gut.* 1997;41:646–650.
55. Clarkston WK, Presti ME, Petersen PF, et al. Role of Mycobacterium paratuberculosis in Crohn's disease: a prospective, controlled study using polymerase chain reaction. *Dis Colon Rectum.* 1998;41:195–199.
56. Kallinowski F, Wassmer A, Hofmann MA, et al. Prevalence of enteropathogenic bacteria in surgically treated chronic inflammatory bowel disease. *Hepatogastroenterology.* 1998;45:1552–1558.
57. Del Prete R, Quaranta A, Lippolis B, et al. Detection of *Mycobacterium paratuberculosis* in stool samples of patients with inflammatory bowel disease by IS900-based PCR and colorimetric detection of amplified DNA. *J Microbiol Methods.* 1998;33:105–114.
58. Chiba M, Fukushima T, Horie Y, et al. No Mycobacterium paratuberculosis detected in intestinal tissue, including Peyer's patches and lymph follicles, of Crohn's disease. *J Gastroenterol.* 1998;33:482–487.
59. Collins MT, Lisby G, Moser C, et al. Results of multiple diagnostic tests for Mycobacterium avium subsp. paratuberculosis in patients with inflammatory bowel disease and in controls. *J Clin Microbiol.* 2000;38: 4373–4381.
60. Gibson J, Riggio M, McCreary C, et al. Looking for Mycobacterium paratuberculosis DNA by polymerase chain reaction (PCR) in orofacial granulomatosis (OFG) and oral Crohn's disease tissue in an Irish population. *Ir Med J.* 2000;93:218.
61. Ellingson JL, Cheville JC, Brees D, et al. Absence of Mycobacterium avium subspecies paratuberculosis components from Crohn's disease intestinal biopsy tissues. *Clin Med Res.* 2003;1:217–226.
62. Bernstein CN, Nayar G, Hamel A, et al. Study of animal-borne infections in the mucosas of patients with inflammatory bowel disease and population-based controls. *J Clin Microbiol.* 2003;41:4986–4990.
63. Romero C, Hamdi A, Valentine JF, et al. Evaluation of surgical tissue from patients with Crohn's disease for the presence of Mycobacterium avium subspecies paratuberculosis DNA by in situ hybridization and nested polymerase chain reaction. *Inflamm Bowel Dis.* 2005;11:116–125.
64. Butcher PD, McFadden JJ, Hermon-Taylor J. Investigation of mycobacteria in Crohn's disease tissue by Southern blotting and DNA hybridization with cloned mycobacterial genomic DNA probes from a Crohn's disease isolated mycobacteria. *Gut.* 1988;29:1222–1228.
65. Yoshimura HH, Graham DY, Estes MK, et al. Investigation of association of mycobacteria with inflammatory bowel disease by nucleic acid hybridization. *J Clin Microbiol.* 1987;25:45–51.
66. McFadden J, Collins J, Beaman B, et al. Mycobacteria in Crohn's disease: DNA probes identify the wood pigeon strain of Mycobacterium avium and Mycobacterium paratuberculosis from human tissue. *J Clin Microbiol.* 1992;30:3070–3073.
67. Sechi LA, Mura M, Tanda F, et al. Identification of Mycobacterium avium subsp. paratuberculosis in biopsy specimens from patients with Crohn's disease identified by in situ hybridization. *J Clin Microbiol.* 2001;39:4514–4517.