

High prevalence of viable *Mycobacterium avium* subspecies *paratuberculosis* in Crohn's disease

Juan L Mendoza, Amparo San-Pedro, Esther Culebras, Raquel Cies, Carlos Taxonera, Raquel Lana, Elena Urcelay, Fernando de la Torre, Juan J Picazo, Manuel Díaz-Rubio

Juan L Mendoza, Carlos Taxonera, Manuel Díaz-Rubio, Department of Gastroenterology, Hospital Clinico San Carlos, 28040 Madrid, Spain

Amparo San-Pedro, Esther Culebras, Raquel Cies, Fernando de la Torre, Juan J Picazo, Department of Clinical Microbiology, Hospital Clinico San Carlos, 28040 Madrid, Spain

Raquel Lana, Department of Internal Medicine, Hospital Clinico San Carlos, 28040 Madrid, Spain

Elena Urcelay, Department of Immunology, Hospital Clinico San Carlos, 28040 Madrid, Spain

Author contributions: San-Pedro A, Culebras E, Cies R, de la Torre F and Urcelay E performed the majority of experiments; Mendoza JL and Lana R provided the collection of human material for this work; Mendoza JL, Taxonera C, Culebras E, Picazo JJ and Díaz-Rubio M designed the study and wrote the manuscript; Díaz-Rubio M provided financial support for this work.

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Correspondence to: Juan L Mendoza, MD, PhD, Department of Gastroenterology, Hospital Clinico San Carlos, Martin Lagos s/n, 28040 Madrid, Spain. jmendozah@meditex.es

Telephone: +34-91-3303693 Fax: +34-91-3303757

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Abstract

AIM: To examine the detection rate of viable *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in patients with inflammatory bowel disease [Crohn's disease (CD) and ulcerative colitis (UC)].

METHODS: Thirty patients with CD (15 with at least one *NOD2/CARD15* mutation), 29 with UC, and 10 with no inflammatory bowel disease (IBD). were tested for MAP by polymerase chain reaction (specific IS900 fragment) and blood culture.

RESULTS: MAP DNA was detected in all original blood samples and 8-wk blood cultures (CD, UC and non-

IBD). Positive MAP DNA status was confirmed by dot blot assays. All 69 cultures were negative by acid-fast Ziehl-Neelsen staining. Viable MAP, in spheroplast form, was isolated from the 18-mo blood cultures of all 30 CD patients, one UC patient, and none of the non-IBD controls. No association was found between positive MAP cultures and use of immunosuppressive drugs or CD-associated single nucleotide polymorphisms.

CONCLUSION: MAP is widely present in our area and MAP DNA can be recovered from the blood of CD, UC and non-IBD patients. However, MAP spheroplasts were only found in CD patients.

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Key words: *Mycobacterium avium* subspecies *paratuberculosis*; Crohn's disease; Ulcerative colitis; Inflammatory bowel disease; Polymerase chain reaction; Genetic susceptibility

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are two

related chronic remitting and relapsing inflammatory diseases of the gastrointestinal tract, commonly known as inflammatory bowel disease (IBD). Although the causes of IBD are unknown, it is thought that inflammation results from inappropriate chronic activation of the innate and adaptive mucosal immune systems in a genetically susceptible host, and that enteric microflora play a central role in initiation and maintenance of disease^[1]. Some investigators have postulated that some mycobacterial infections are involved in development of CD, based on the similarities between CD and intestinal tuberculosis^[2]. *Mycobacterium avium* subspecies *paratuberculosis* (MAP) has the specific ability to cause chronic bowel inflammation of a number of histopathological types in many animals, including primates^[3,4]. Systematic review and meta-analysis of research from many laboratories have shown a significant and specific association between MAP infection and chronic bowel inflammation of the CD type in humans^[5,6]. Recently, Naser *et al.*^[7] have found viable MAP in the peripheral blood from 50% of CD patients.

Several findings suggest that the presence of certain bacteria in the face of permissive *NOD2/CARD15* mutations is necessary for development of CD and provides evidence for a pathogen-host interaction^[8-10]. A recent study has demonstrated an association in IBD with a coding variant of *ATG16L*, *IL-23R*^[11] and *IRGM*^[12] genes, thereby implicating the autophagy pathway that is crucial in inhibiting *Mycobacterium tuberculosis* survival in infected macrophages^[13].

The aim of this study was to examine, after stratifying CD patients based on the presence or absence of the well-established *NOD2/CARD15* mutations, the culture detection rate of viable MAP in peripheral blood from patients with IBD (CD and UC) and healthy controls (non-IBD).

MATERIALS AND METHODS

Study population

Sixty-nine subjects were recruited into the study: 30 CD patients in clinical remission (of whom 15 carried at least one *NOD2/CARD15* mutant; these patients were matched one to one with patients with no *NOD2/CARD15* mutants based on the following criteria: time since IBD diagnosis, age at diagnosis, disease location, behavior and prior surgery); 29 UC patients in clinical remission; and 10 healthy controls (non-IBD), members of the staff of the Department of Clinical Microbiology, Hospital Clinico San Carlos de Madrid, Spain. Diagnosis of CD and UC was based on standard clinical, radiographic, endoscopic, and histological criteria^[14], and all patients were recruited at an IBD unit of a single referral center in Madrid, Spain. Phenotypic details were obtained by review of clinical records and personal interview with the patients.

The protocol was approved by the Ethics Committee of Hospital Clinico San Carlos, Madrid, and all patients were recruited into the study after giving informed consent.

Samples and cultures

Two venous blood samples (10 mL) were taken from all

patients and controls and drawn into sterile K2-EDTA vacutainer tubes.

Genomic DNA extraction and blood cultures were done using culture methods previously reported by Naser *et al.*^[7], except that 10 mL of whole blood was used for each culture. Cultures were incubated at 37°C for 8 wk (bottle I) or 18 mo (bottle II).

DNA extraction and sequencing

Total DNA was prepared by two different methods. DNA was obtained from original samples and 8-wk cultures using the QIAamp DNA Blood Kit (Qiagen). Total DNA from 18-mo cultures was obtained using an EasyMag magnetic extractor (bioMerieux) according to the manufacturer's recommendations.

Polymerase chain reaction assay

Amplification of IS900 was conducted basically as described by Naser *et al.*^[7] with minor alterations: after the first round of nested polymerase chain reaction (PCR), amplicons were purified using the QIAquick PCR Purification Kit (Qiagen) and then diluted 1:100 with sterile water.

Dot blot assays

To confirm PCR results, DNA that had been extracted from original samples and 8-wk cultures was analyzed by dot blot hybridization using the DIG System (Roche Molecular Biochemicals). *M. avium* subs. *paratuberculosis* ATCC 43544 was used as a positive control. One *Mycobacterium fortuitum* and one *M. avium* subs. *avium* were used as negative controls in dot blot assays.

Staining methods for microscopy

Smears were prepared by placing one drop from 18-mo cultures on a microscope slide. Smears were heat-fixed and stained using the Ziehl-Neelsen and phenolic acridine orange techniques to detect mycobacterial bacilli and/or spheroplasts^[15].

Genotyping

Genotyping of rs2241880 (*ATG16L1*), rs4958847 (*IRGM*), rs7517847 (*IL23R*) and *NOD2/CARD15* polymorphisms was performed as previously described^[16-18].

Statistical analysis

This was a case-control study. Numerical variables were summarized by the mean, median, and range. Nominal variables were summarized based on their frequency distribution.

RESULTS

The group of 30 CD patients consisted of 11 men and 19 women. Median age at diagnosis was 27 years (mean: 31, range: 14-48 years). Median follow-up duration was 7 years (mean: 8, range: 6-9 years). Table 1 shows the characteristics of the 30 CD and 29 UC patients enrolled into this study.

Table 1 Characteristics of patients with crohn's disease and ulcerative colitis *n* (%)

Phenotypic characteristics	CD <i>CARD15/NOD2</i> (+) (<i>n</i> = 15)	CD patients <i>CARD15/NOD2</i> (-) (<i>n</i> = 15)	UC patients (<i>n</i> = 29)
Male/female	5 (33.4)/10 (66.7)	6 (49.1)/9 (50.9)	15 (51.7)/14 (47.3)
Median age at diagnosis (range, yr)	27 (14-48)	27 (14-48)	
A1, < 40	14 (93.4)	14 (93.4)	
A2, ≥ 40	1 (6.6)	1 (6.6)	
Family history	4 (26.7)	3 (21.4)	1 (3.4)
Disease behavior			
Nonstricturing, nonpenetrating (B1)	6 (40)	6 (40)	
Stricturing (B2)	3 (20)	3 (20)	
Penetrating (B3)	6 (40)	6 (40)	
Perianal disease	7 (46.6)	7 (46.6)	
Disease location			
Terminal ileum (L1)	6 (40)	6 (40)	
Colon (L2)	3 (20)	3 (20)	
Ileocolon (L3)	5 (33.4)	5 (33.4)	
Upper gastrointestinal (L4)	1 (6.6)	1 (6.6)	
Pancolitis			15 (51.7)
Left side			14 (48.3)
Treatment			
Surgery	6 (40)	6 (40)	1 (3.4)
Infliximab	8 (53.3)	6 (40)	0 (0)
Immunosuppressants	7 (46.6)	7 (46.6)	10 (34.5)
No immunosuppressive therapy	5 (33.4)	5 (33.4)	19 (65.5)
Genotype characteristics			
At least one <i>CARD15/NOD2</i> mutation	15 (100)	0 (0)	4 (13.8)
<i>ATG16L1</i> (rs2241880) AA/GA/GG (<i>n</i>)	0/9/5	1/9/4	2/11/13
<i>IRGM</i> (rs4958847) AA/GA/GG (<i>n</i>)	0/5/8	1/4/9	0/7/15
<i>IL23R</i> (rs7517847) GG/TG/TT (<i>n</i>)	2/6/3	2/5/7	3/10/13

CD: Crohn's disease; UC: Ulcerative colitis.

Table 2 Results of samples and cultures in patients and controls (positive) %

	Original blood samples MAP DNA	BACTEC culture 8 wk of incubation (Bottle I)		BACTEC culture 18 mo of incubation (Bottle II)		
		BACTEC MGIT ¹	Dot-blot MAP	BACTEC MGIT ¹	Acid-fast Ziehl-Neelsen staining	Spheroplasts
CD patients (<i>n</i> = 30)	100	0	100	0	0	100
UC patients (<i>n</i> = 29)	100	0	100	0	0	3.4
Healthy controls (<i>n</i> = 10)	100	0	100	0	0	0

¹Automatically detected; CD: Crohn's disease; UC: Ulcerative colitis; MAP: *Mycobacterium avium* subspecies *paratuberculosis*.

MAP DNA was detected in all original blood samples. No PCR internal control was positive for MAP DNA, which indicated no laboratory contamination. Nucleotide sequencing of purified MAP DNA fragments was also positive in the second round of nested PCR, which confirmed amplification of the IS900 nucleotide sequence (Table 2).

After 8 wk incubation (bottle I), no mycobacterial growth was automatically detected in the 69 BACTEC MGIT cultures.

Dot blot assays confirmed the positive MAP status of all original blood samples and 8-wk cultures.

After 18 mo incubation (bottle II), no mycobacterial growth was automatically detected in the 69 BACTEC MGIT cultures. All 69 buffy coat cultures were negative by acid-fast Ziehl-Neelsen staining. However, all of the 30 18-mo cultures from CD patients were positive by phenolic acridine orange staining, which suggested the presence

of wall-deficient cells or spheroplasts (Figure 1).

Thus, 18-mo blood cultures were MAP-positive in all CD patients. No association could be found between positive cultures and use of tumor necrosis factor (TNF)- α antibodies and thiopurine drugs. No correlation was seen between MAP-positive blood cultures and *CARD15/NOD2*, *ATG16L1*, *IRGM* or *IL-23R* CD-associated single nucleotide polymorphisms (SNPs).

DISCUSSION

In this study, nested IS900-specific PCR showed that MAP DNA is widespread in our environment. Original blood samples and 8-wk cultures from all CD and UC patients and non-IBD controls were PCR-positive. However, viable MAP spheroplasts (cell-wall-deficient forms) were only found in the 18-mo blood cultures from all CD and one UC patient, but in none of the non-IBD

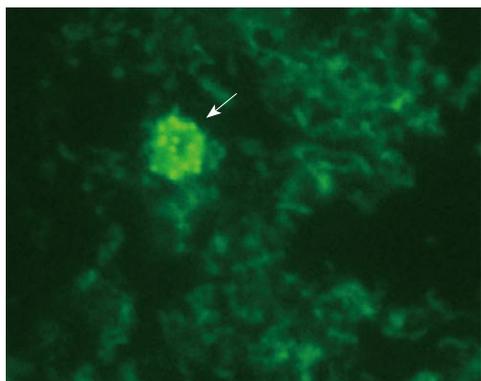


Figure 1 Microscopic examination of *Mycobacterium avium* subspecies *paratuberculosis* cultures isolated from blood of patients with Crohn's disease. Phenolic-acridine orange techniques to detect cell-wall-deficient *Mycobacterium avium* subspecies *paratuberculosis* [spheroplasts (arrow)].

controls. The observation that MAP could be cultured from CD patients was not correlated with use of immunosuppressive drugs (TNF- α antibodies and thiopurine drugs), *NOD2/CARD15* mutations, or other studied genes, which implicated the autophagy pathway of the innate immune system (*ATG 16L1*, *IRGM*, *IL-23R*).

The equal circulation of MAP DNA in patients with and without IBD lends support to the contention that environmental exposure to MAP is widespread, possibly from water, milk, or other sources^[2]. Our group of subjects with no IBD was recruited from the staff of the Department of Microbiology, Hospital Clinico San Carlos de Madrid, Spain. These healthy subjects were most likely colonized by MAP, but none of them showed detection of viable MAP in 18-mo blood cultures. This result supported the findings in the study by Naser *et al*^[7], where no viable MAP was subsequently cultured from any of the IS900-positive samples from healthy controls. The same occurred with the blood cultures from UC patients, as viable MAP was detected in only one of them. Thus, in our area, MAP is an ubiquitous environmental organism that does not usually cause disease unless the host is predisposed to infection, as occurs with other members of the *M. avium* complex.

Previous studies have shown that reliable and reproducible detection of *MAP* by PCR tests applied directly to DNA extracted from human tissue and other samples is extremely difficult. Use of suboptimal sample processing procedures results in false-negative results^[19]. Results of MAP detection using nucleic-acid-based techniques have recently been reported in two meta-analyses that have suggested that there is adequate evidence for the presence of MAP in the bowel of CD patients, regardless of whether these patients are compared to subjects with no IBD or those with UC^[5,20]. However, this association remains controversial and inconclusive. PCR data can be criticized on the grounds that the procedure assesses DNA that might come from live bacteria or merely be scattered debris from killed organisms, and therefore of questionable biological consequence^[21].

The gold standard for detection of MAP is based on

isolation of the organism using culture methods. However, this method is time-consuming because of the fastidious nature and slow growth, and pleomorphic, variably acid-fast and spheroplast-like organisms. Chiodini *et al*^[15] have demonstrated that MAP strains isolated from CD tissue first appeared as cell-wall-deficient forms (spheroplasts), and have suggested that MAP is present in CD tissue in a spheroplast-like form^[15,22]. We could not detect viable MAP by acid-fast Ziehl-Neelsen staining, but all cultures from CD patients were shown to contain spheroplasts. Naser *et al*^[7] have reported that the MAP-positive cultures of the buffy coat were negative by acid-fast Ziehl-Neelsen staining during the early weeks of culture incubation but were positive by acridine orange (spheroplast), but this observation has not been confirmed by other studies^[23-25]. Therefore, MAP spheroplasts might play a role in development of CD, as well as in paucibacillary forms of Johne's disease in other species. It has not been determined whether the presence of MAP in CD is related to infection, colonization, or a defect in the intestinal barrier/microbial killing. We did not study MAP in intestinal tissue, nor explore the possibility of increased bowel permeability. However, detection of viable MAP in the blood of CD patients could be due to the inability of macrophages in CD to kill MAP^[26].

No association was found between a positive MAP culture from the blood of CD patients and *CARD15/NOD2*, *ATG16L1*, *IRGM* or *IL-23R* CD-associated SNPs, but our sample was small and a type II error cannot be excluded.

The most irrefutable evidence that a microbial agent causes a disease is long-term remission of clinical manifestations and a change in the natural history of disease after clearance of infection. Recently, a large, well-designed, randomized, placebo-controlled trial of clarithromycin, rifabutin, and ethambutol failed to show a sustained response in CD patients, although a short-term benefit of antibiotics at 16 wk, additional to the effect of corticosteroid therapy, was reported^[27]. A recent study^[28] has shown that antimycobacterial and thiopurine drugs used in concert might have an interactive effect. The apparently bacteriostatic effects of 6-mercaptopurine on *M. paratuberculosis* renders the organism less susceptible to the bactericidal effects of antibiotics.

An argument against a role of MAP in CD is that if CD were a chronic mycobacterial infection, immunosuppressive therapies would be associated with increased rates and severity of mycobacterial disease, rather than with improvement^[29]. We were not able to show any association between occurrence of MAP bacteremia and use of immunosuppressive drugs, because all CD patients showed positive MAP cultures. Viable MAP could not be cultured from UC patients who received immunosuppressive therapy. This might indicate that use of immunosuppressive therapy has no influence on the presence of viable MAP in blood.

In conclusion, MAP is widely present in our area and MAP DNA can be recovered from the blood of CD and UC and healthy controls. Spheroplasts were only found in

the blood cultures from CD patients. However, the pathogenetic role of MAP remains controversial and inconclusive. However, even if MAP is not causally related to CD, the presence of viable MAP in the blood might have secondary clinical implications.

COMMENTS

Background

The hypothesis postulating that *Mycobacterium avium paratuberculosis* (MAP) is the cause of Crohn's disease (CD) has been circulating for many years. Advances in molecular techniques, such as PCR and culture methods, have allowed researchers to demonstrate an association between MAP and CD.

Research frontiers

MAP is a recurrent candidate as the cause of CD for several reasons: MAP induces epidemic chronic colitis in cattle and other species, including primates; it is reportedly detectable in the intestinal tissues and blood of many CD patients; MAP antibodies are often associated to the disease; and in some cases, antimycobacterial drugs improve the disease. In this study, authors demonstrated that MAP spheroplasts were cultured from the peripheral blood of CD patients only, but not from patients with ulcerative colitis (UC) or normal controls.

Innovations and breakthroughs

MAP is widely present in Spain, and MAP DNA may be recovered from the blood of CD patients, UC patients, and healthy controls, but in this study MAP spheroplasts were only found in the 18-mo blood cultures from all CD patients. The observation that MAP could be cultured from CD patients was not correlated with the use of immunosuppressive drugs or mutations associated with CD patients.

Applications

The status of viable MAP spheroplasts might play a role in development of CD, as well as in paucibacillary forms of Johne's disease in other species.

Terminology

CD is a chronic remitting and relapsing inflammatory disease of the gastrointestinal tract. MAP is a bacterium that is a member of the *M. avium* complex. *M. avium* strains are widely distributed in the environment and also occur in normal animal and human intestines. Spheroplasts are cell-wall-deficient forms of MAP.

Peer review

This is a small case-control study that attempted to correlate the presence of MAP with inflammatory bowel disease. Although this is not an entirely original study and the number of patients is relatively small, the authors report some interesting findings.

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