Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's disease

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See Comment

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Summary

Background **Crohn's disease, a form of inflammatory bowel disease, resembles some aspects of tuberculosis, leprosy, and paratuberculosis. The role of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in Crohn's disease is controversial.**

Methods **We tested for MAP by PCR and culture in buffy coat preparations from 28 individuals with Crohn's disease, nine with ulcerative colitis, and 15 without inflammatory bowel disease.**

Findings **MAP DNA in uncultured buffy coats was identified by PCR in 13 (46%) individuals with Crohn's disease, four (45%) with ulcerative colitis, and three (20%) without inflammatory bowel disease. Viable MAP was cultured from the blood of 14 (50%) patients with Crohn's disease, two (22%) with ulcerative colitis, and none of the individuals without inflammatory bowel disease. Current use of immunosuppressive medication did not correlate with a positive MAP culture. Sequencing of PCR products from MAP cultures confirmed the presence of the MAP-specific IS900 fragment. Among 11 MAP isolates assessed, we identified nine strains that were not identical.**

Interpretation **We detected viable MAP in peripheral blood in a higher proportion of individuals with Crohn's disease than in controls. These data contribute to the evidence that MAP might be a cause of Crohn's disease.**

Introduction

In 1913, Dalziel[1] reported the histopathological and clinical similarities of animal paratuberculosis, intestinal tuberculosis, and human chronic granulomatous enteritis that later became known as Crohn's disease.[2] Paratuberculosis, also known as Johne's disease, is a chronic enteritis that affects cattle and other species and is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP).[3] Because of the similarities with Johne's disease, a mycobacterial cause of Crohn's disease has been sought for many years.[4] Early studies did not detect MAP in tissues from patients with Crohn's disease by conventional staining and culture techniques. Chiodini and colleagues[5] isolated cell-wall-deficient cells, also known as spheroplasts, from tissue samples from patients with Crohn's disease after several months of incubation. The spheroplasts were subcultured and later developed a cell wall that stained positively with Ziehl-Neelsen staining; they were classified as a *Mycobacterium*-like organism. These isolates were confirmed as MAP by DNA hybridisation,[6] leading to renewed interest in a mycobacterial origin of Crohn's disease.[7,8] The unusual nature of cell-wall-deficient MAP in patients with Crohn's disease, and the challenges in cultivating MAP with its fastidious and slow-growing characteristics, led investigators to rely heavily on the use of the DNA insertion element IS900 for accurate identification of MAP.[9-11] However, results have been inconsistent, probably because of differences in methodology; PCR cannot differentiate between viable MAP and MAP DNA. Mishina and colleagues[12] used RT-PCR to detect IS900 RNA and found viable MAP in all of eight patients with Crohn's disease and two of four controls; the two controls with MAP were patients with ulcerative colitis. We cultured MAP in Mycobacterial Growth Indicator Tube (MGIT, Becton Dickinson, Franklin Lake, NJ, USA) culture media after 10–12 weeks of incubation from seven of eight tissue samples from patients with Crohn's disease and none from three controls.[13] By use of the same protocol, MAP was isolated from breastmilk samples from two lactating mothers with Crohn's disease and not from five without the disease.[14] During the early weeks of culture in MGIT, MAP isolated from human milk contained cell-wall-deficient cells, which later reverted to bacillary form and stained positively with Ziehl-Neelsen staining.[13] The association between *CARD15* (formerly *NOD2*) mutations and risk of Crohn's disease provides evidence for a pathogen-host interaction.[15–17] Most recently, viable MAP from MGIT culture of biopsy samples has been reported in 92% of patients with Crohn's disease and 26% of controls.[18] In this study, we investigated the presence of viable MAP in the peripheral blood of patients with Crohn's disease.

Methods

Patients and samples

52 participants were included in the study: 28 with Crohn's disease, nine with ulcerative colitis, and 15 without inflammatory bowel disease (two with colon cancer, one with diverticulitis, one with gastro-oesophageal reflux, and 11 healthy individuals). Informed consent was obtained in accordance with institutional review board regulations at the University of Florida, Gainesville Veterans Affairs Medical Center, and the University of Central Florida. Of the patients with Crohn's disease, 22 of 28 (78%) and all the patients with

ulcerative colitis were recruited from the inflammatory bowel disease clinic at the University of Florida, a tertiary referral centre. The diagnosis of Crohn's disease or ulcerative colitis was established on standard clinical, endoscopic, histological, and radiographic criteria.[19] Disease activity was assessed by use of the Harvey-Bradshaw index[20] or the clinical findings of the treating physician. Only one of the patients with Crohn's disease, and none of those with ulcerative colitis, were newly diagnosed. Patients with indeterminate colitis were excluded.

Two 4-mL whole blood samples drawn into sterile K_2-EDTA Vacutainer tubes were obtained from every participant. All samples were coded to conceal the patient's identity and the diagnosis. The samples were immediately processed in a class II biosafety cabinet. The buffy coat layer from each tube was transferred into a separate sterile tube that was used for either genomic DNA extraction or culture. A medication history was completed for each participant (table 1). Use of prednisone, budesonide, azathioprine, mercaptopurine, methotrexate, or infliximab at any dose was judged to be immunosuppressive. Products containing mesalamine were not considered to be immunosuppressive.

Procedures

For culture, each buffy coat sample was resuspended in 1 mL of sterile phosphate buffer saline, pH 6·8. Samples (0·5 mL) of the suspension were used to inoculate one MGIT and one 12B* BACTEC bottle (Beckton Dickinson, Franklin Lake, NJ, USA), each containing 4 mL of modified Middlebrook 7H9 broth base media (Becton Dickinson) with supplements as previously described.[13] All inoculated culture media were incubated at 37°C in a 5% carbon dioxide incubator. The BACTEC bottles were assessed every week with the BACTEC 460 TB Analyzer (Becton Dickinson) and the MGIT were checked every 2 weeks for visible turbidity and fluorescence-quenching activity with a 365 nm UV-illuminator. The BACTEC bottles with a growth index reading of 50 or more, and all MGIT cultures incubated for 8–12 weeks, were assessed for the presence of MAP by staining and nested PCR. MAP cells were inactivated by heating the cell pellet from a 0·5 mL sample of the culture in 500 µL TE buffer (10 mM Tris and 1 mM EDTA, pH 8·0 HCl) at 80ºC for 10 min. The sample was centrifuged at 10 000 rpm (12 800 g) for 5 min and the cell pellet was suspended in 150 µL of sterile water. 50 µL was used to prepare smears for Ziehl-Neelsen staining, acridine orange staining, or immunostaining with adsorbed rabbit-anti-MAP polyclonal IgG antibodies as described previously.[21] The remaining volume was used for extraction of genomic DNA for PCR analysis.

Genomic DNA was extracted from uncultured buffy coat cells and cell culture pellets in a separate class II biosafety cabinet. The cells were re-suspended in 100 µL sterile TE buffer and incubated in a dry heat bath at 100°C for 30 min, then placed on ice for 15 min. They were then centrifuged at 12 000 rpm (18 500 g) and 4°C for 10 min. The supernatant was transferred to 2·0 mL phase-lock gel tubes (PLG, Eppendorf, Westbury, NY, USA). 200 µL of phenol/chloroform/isoamyl alcohol (1:1:24 v/v; Acros Organics Morris Plains, NJ, USA) was added, mixed, and centrifuged at 12 000 rpm (18 500 g) and 4°C for 5 min. The nucleic acid was precipitated, washed, dried, and dissolved in 50 µL of sterile water.

Primers

P90 5′-GTTCGGGGCCGTCGCTTAGG-3′
P91 5′-GAGGTCGATCGCCCACGTGA-3′
AV1 5′-ATGTGGTTGCTGTGTTGGATGG-3′
AV2 5′-CCGCCGCAATCAACTCCAG-3′

Both primary and secondary rounds of the nested PCR were done in a class II biosafety cabinet used only for PCR. Oligonucleotide primers were derived from the DNA insertion sequence IS900, which is unique to MAP. The primers P90 and P91 (panel) were used for the first round to amplify a unique 398 bp fragment of the IS900 gene. The sensitivity and specificity for the amplified MAP DNA fragment were achieved by use of AV1 and AV2 oligonucleotide primers (panel) in the second round to re-amplify an 298 bp internal nucleotide sequence of the 398 bp template. The PCR reaction mixture consisted of 40 µL PCR buffer: 5—mM $MgCl_2$, 0·2 mM dNTP, 6% DMSO or 0·5 M Betaine, 2 µM primers, and 2.5 U Platinum Taq polymerase (Invitrogen, Carlsbad, CA, USA) or 1 U TFL DNA polymerase (Promega, Madison, WI, USA)—and 10 µl of DNA template. The PCR reaction mixture in the second round of the nested PCR was the same, except that 5 µL of PCR product from the first round was used as a template and the AV1 and AV2 oligonucleotide primers were substituted. The PCR cycling conditions were: 95°C for 5 min, 34 cycles of 95°C for 1 min, 58°C for 1·5 min, 72°C for 1·5 min, and a final extension of 10 min at 72°C. The amplification product size was assessed on 2% agarose gel. Negative controls for PCR consisted of sterile TE buffer or sterile water used in place of the DNA template, and were used in parallel with every round of PCR preparation. Positive MAP DNA from strain ATCC 43015 was prepared independently and added to PCR tubes at a different facility using separate supplies.

Nucleotide sequencing verified the specificity of the amplified IS900 fragment. PCR products from the second round of the nested PCR from positive samples were purified and sequenced at the Biomolecular Science Center DNA Sequencing Core facility at the University of Central Florida. BLAST and alignment sequence analyses were also done.

Group and sex	Age (years)	Disease duration (years)	Disease location	Type	Disease activity	Immunosuppression	PCR	Culture
Crohn's disease								
M	48	21	SB/colon	Penetrating	Inactive	6MP/infliximab	+	–
M	77	2	SB/colon	Inflammatory	Inactive	None	–	+
M	25	12	SB/colon	Stricturing	Inactive	6MP/infliximab	+	+
F	23	8	Colon	Inflammatory	Inactive	None	–	–
M	72	8	SB/colon	Inflammatory	Inactive	Aza	–	–
F	21	6	SB/colon	Penetrating	Inactive	6MP	–	–
F	20	3	No data	Penetrating	Active	None	+	+
M	20	No data	No data	No data	No data	No data	+	+
F	20	2	SB	Stricturing	Inactive	Steroids	–	–
F	33	7	Colon	Penetrating	Inactive	6MP	–	+
F	24	4	SB/colon	Penetrating	Inactive	None	–	–
F	46	21	Colon	Penetrating	Inactive	Aza	–	+
F	18	4	SB/colon	Inflammatory	Active	6MP	–	–
M	27	12	SB/colon	Penetrating	Inactive	Steroids/mtx	–	–
M	26	7	Colon	Inflammatory	Active	Aza/infliximab	–	+
F	43	20	Colon	Penetrating	Active	Infliximab	+	+
F	60	6	SB/colon	Inflammatory	Active	None	–	–
F	25	3	SB/colon	Inflammatory	Active	Aza	+	+
M	25	7	SB/colon	Penetrating	Inactive	6MP	+	–
F	36	22	SB/colon	Stricturing	Inactive	Steriods/inflixmab	+	+
M	64	<1	SB/colon	Inflammatory	Active	None	–	–
F	No data	No data	No data	No data	No data	No data	–	–
F	20	3	SB	Stricturing	Active	None	+	+
M	36	10	Colon	Stricturing	Inactive	None	+	–
M	21	9	SB/colon	Inflammatory	Inactive	None	+	+
F	40	2	SB	Stricturing	Active	None	–	–
F	50	10	SB	Penetrating	Active	6MP	+	+
F	39	No data	SB	Inflammatory	Active	Steroids	+	+
Ulcerative colitis								
M	34	8	Pancolitis	..	Active	None	–	–
M	24	6	Pancolitis	..	Inactive	None	–	–
M	76	25	Left sided	..	Inactive	None	+	–
M	64	8	Left sided	..	Inactive	6mp	–	–
F	40	18	Pancolitis	..	Active	None	–	–
F	22	2	Left sided	..	Inactive	6MP	+	–
M	30	10	Pancolitis	..	Inactive	6MP	+	+
M	37	5	Left sided	..	Inactive	None	+	–
M	44	5	Pancolitis	..	Inactive	Steroids	–	+
No inflammatory bowel disease								
F*	41	..	..	..	..	None	–	–
M†	72	..	..	..	..	None	–	–
M‡	57	..	..	..	..	None	+	–
F†	68	..	..	..	..	None	+	–
M	37	..	..	..	..	None	+	–
F	41	..	..	..	..	None	–	–
F	28	..	..	..	..	None	–	–
F	31	..	..	..	..	None	–	–
M	40	..	..	..	..	None	–	–
M	60	..	..	..	..	None	–	–
F	29	..	..	..	..	None	–	–
F	31	..	..	..	..	None	–	–
M	31	..	..	..	..	None	–	–
M	26	..	..	..	..	None	–	–
M	32	..	..	..	..	None	–	–

M=male. F=female. SB=small bowel. Aza=azathioprine. 6MP=6-mercaptopurine. mtx=methotrexate **[A: ok?]**. Results of PCR for MAP DNA and culture shown as positive (+) or negative (–). Controls with no inflammatory bowel disease: *had reflux, †had colon cancer, ‡had diverticulitis; the others were healthy.

Table: **Clinical data and MAP test results of participants**

Statistical analysis

Groups were compared with 2×2 contingency tables and Fishers exact test. A p value of less than 0·05 was judged to be significant.

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

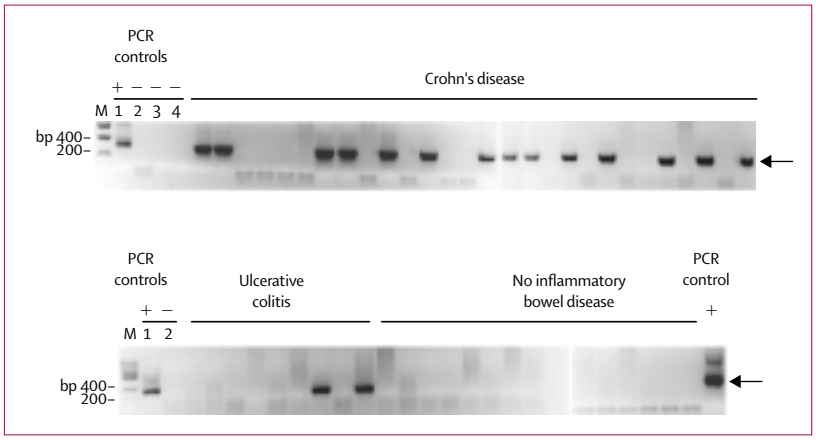

***Figure 1*: Nested PCR detection of MAP DNA from cultured peripheral blood samples**
Positive result indicated by a bright 298 bp band (arrow) on 2% agarose gel electrophoresis. M=molecular weight marker. Lane 1 represents DNA from MAP strain ATCC 43015 as a positive control (+); lanes A2 and B2 represent negative controls for second round of nested PCR, lanes A3 and A4 represent negative controls for first round of nested PCR and for DNA extraction steps, respectively. Positive and negative controls were run along with each sample analysis; for this composite image, only representative positive and negative controls are shown.

Results

The code for concealment of participants' identities and diagnoses was broken and data were tabulated after the conclusion of all experiments (table). Participants were aged 18–77 years. Overall, 50% were female; 17 (61%) of those with Crohn's disease, 2 (22%) of those with ulcerative colitis, and 7 (47%) of those without inflammatory bowel disease. 16 (62%) of the 26 patients with Crohn's disease for whom data were available were known to be on immunosuppressive medications. Four (44%) patients with ulcerative colitis and none of those without inflammatory bowel disease were on such medications.

Samples that were positive for the MAP-specific IS900 element clearly showed a single bright 298 bp band on 2% agarose gel. MAP DNA was detected in 20 of 52 (38%) of all blood samples (table); 13 (46%) patients with Crohn's disease, four (44%) with ulcerative colitis, and three (20%) without inflammatory bowel disease. None of the PCR internal controls, including one for genomic DNA extraction and one in each round of the nested PCR, were positive for MAP DNA, indicating no laboratory contamination. Nucleotide sequencing of purified MAP DNA fragments from representative samples that were positive in the second round of the nested PCR confirmed the amplification of IS900 nucleotide sequence.

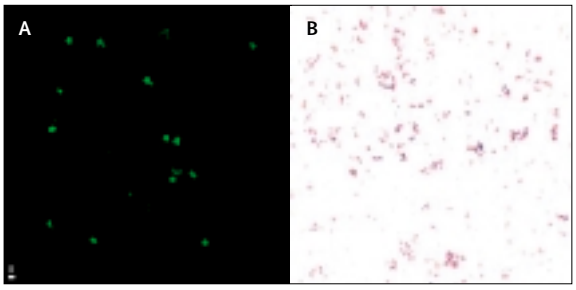

***Figure 2*: Microscopic examination of MAP cultures isolated from blood**
(A) Cell-wall-deficient MAP from 5-week-old MGIT culture from patient with Crohn's disease, viewed with confocal scanning laser microscopy after immunostaining. (B) Representative acid-fast positive MAP culture from patient with Crohn's disease, showing pink short rods after 12 weeks of incubation. Images represent ×100 oil magnification.

After 12 weeks of incubation, possible mycobacterial growth was detected in three of 52 BACTEC cultures. Detection of possible mycobacterial growth in MGIT cultures inoculated with blood-derived buffy coat based on the observation of visible turbidity and increase in fluorescence intensity is unreliable. All 52 MGIT cultures and the three BACTEC cultures in which a possible mycobacterial growth was detected were assessed for the presence of MAP. IS900-nested PCR identified MAP in all three suspected BACTEC cultures and in 14 of 52 MGIT cultures. Only one patient was positive for MAP by both culture methods. MAP was isolated from culture in 14 (50%) patients with Crohn's disease, two (22%) of those with ulcerative colitis, and none of those without inflammatory bowel disease ($p=0\cdot0005$ for group with Crohn's disease *vs* without inflammatory bowel disease; figure 1).

11 of the 13 patients with Crohn's disease who were positive for MAP DNA in blood by PCR were also positive by culture, and ten of the 14 subsequently found to be positive by culture had also tested positive by PCR. Of the four PCR-positive patients with ulcerative colitis, one was positive by culture; the other culture-positive ulcerative colitis patient was PCR negative. None of the three PCR-positive individuals without inflammatory bowel disease was positive by culture. Among the 26 Crohn's disease patients for whom data on the use of immunosuppressive medications were available, current use of immunosuppressive medication did not correlate with a positive culture result ($p=0\cdot23$; table). The sample size was too small to make any distinction about specific immunosuppressive medications, age of onset, disease duration, or clinical phenotype.

MAP-positive cultures of buffy coat were negative by acid-fast Ziehl-Neelsen staining during the early weeks of culture incubation, but were positive by acridine orange or immunostaining with rabbit anti-MAP IgG polyclonal antibodies (figure 2A). After 12 weeks of incubation, all 16 MAP-positive cultures contained acid-fast bacilli, with the occasional presence of deformed cells containing remnant cell wall components, also known as pre-spheroplasts (figure 2B).

To investigate the diversity of the MAP isolates, we sequenced the 298 bp amplified IS900 fragment from 11 representative MAP-positive cultures. The nucleotide sequences were assessed against each other, the sequence from our laboratory MAP strain (positive control), and several IS900 loci available in the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov). All sequences were confirmed

as IS900. Nine of the 11 MAP isolates were different from each other because of nucleotide polymorphisms including insertions, deletions, and substitutions. All 11 MAP isolates differed from the MAP-positive control used in our laboratory. Of 11 IS900 loci published in the database, ten (accession numbers AF416985, AJ250023, AJ250022, AJ250015, AJ250016, AJ250017, AJ250018, AJ250019, AJ250020, AJ250021) showed two consecutive base substitutions (CG instead of GC located at 114–115 of the 1451 bp encoding sequence of the IS900 locus) in seven MAP clinical isolates. Only one IS900 locus (accession number X16293) showed no CG switching.

Discussion

We detected viable MAP in the peripheral blood in a substantial proportion of individuals with Crohn's disease, adding to the evidence for a role of the organism in the aetiology of this disease. A possible microbial role in the pathogenesis of Crohn's disease was suggested by the discovery that the illness was associated with mutations in *CARD15*.[15,16] *CARD15* mutations are observed in 15–22% of white patients with Crohn's disease, but are not noted in Korean, Chinese, or Japanese patients with the disease.[22] Although previously suspected,[1] MAP was identified clinically as a possible human pathogen when a specimen from the cervical lymph nodes of a 7-year-old boy with scrofula was positive for the MAP-specific DNA element IS900 and the patient was subsequently diagnosed with Crohn's disease.[23] We judge MAP to be a leading candidate pathogen in Crohn's disease on the basis of many reports, including the findings that MAP could be cultured from intestinal and lymph nodes,[13] breastmilk,[14] and now from peripheral blood, from patients with Crohn's disease.

Identification of MAP in blood from patients with Crohn's disease should not be surprising if MAP has a role in the pathogenesis of the disease. In animals with Johne's disease, MAP infection is systemic.[24] MAP is secreted into milk,[25] and infiltrates the extraintestinal tissue and the bloodstream.[24] Evidence from the UK suggests that people are exposed to MAP through retail milk supplies[26] and that this route might be a source of zoonotic disease.[27]

Our findings confirm that MAP infection in human beings can be systemic, as suggested in our earlier report,[14] in which MAP was identified in human milk from two mothers with Crohn's disease. The finding of MAP DNA in the blood of patients with and without inflammatory bowel disease suggests that MAP is common in the environment, with exposure most likely to happen through the food and water supply. The observation that MAP could be cultured from most of the PCR-positive patients with Crohn's disease but from none of the PCR-positive individuals without inflammatory bowel disease would be consistent with an active infection in association with Crohn's disease and a state of colonisation or dormancy, which is well recognised in other mycobacteria,[28] in the absence of Crohn's disease. Alternatively, people without inflammatory bowel disease might more effectively kill MAP after exposure. Host susceptibility—either genetic, immunological, or both—might be essential for MAP to survive phagocytosis and oxidative bursts. The discovery of the *CARD15* genetic defect lends support to the concept of genetic susceptibility associated with failure to respond to the intracellular pathogen. The identification of viable MAP in two patients with of ulcerative colitis in this study is consistent with an earlier report,[12] in which two tissue specimens from patients with ulcerative colitis were MAP-positive by RT-PCR. These cases might represent undiagnosed Crohn's disease presenting as ulcerative colitis, or mixed disease.[29] An alternative hypothesis is that Crohn's disease and ulcerative colitis might share a common environmental trigger or be part of a spectrum of a single disease.

A positive MAP culture did not correlate with the use of immunosuppressive medications, but we cannot exclude a type II error. If MAP is a cause of Crohn's disease, then our results are consistent with the known occurrence of bacteraemia observed in many infectious diseases, including infections with mycobacterial pathogens.[30] A potential weakness of our finding is that we might be underestimating the magnitude of the number of patients with MAP bacteraemia. We used 4 mL of whole blood for each culture; the likelihood of a sampling error might be reduced if a larger volume of blood was used for culture. Genomic DNA extraction was done by a TE-boiling method as described. However, mechanical disruption of MAP cells might increase the DNA extraction yield.[18] A strength of our study was the sequencing of the IS900 element from most of the positive MAP cultures, which showed genetic heterogeneity and distinction from our positive control laboratory strain, indicating that laboratory contamination did not affect our results. This diversity among strains might affect the assessment of the epidemiological distribution of the organism, and the source of MAP in cases of environmental faecal contamination or MAP in the milk or blood of animals infected with Johne's disease. Ultimately, a blood-culture technique specific for MAP might aid in the diagnosis of Crohn's disease patients with MAP involvement, and might lend support to the choice of a therapeutic approach, such as the use of macrolide antibiotics that have shown some success in treatment of the disease in open label trials.[23,31]

Although our findings do not prove that MAP is a cause of Crohn's disease, they suggest that a larger scale investigation is needed to ascertain the role of the organism in this illness.

Contributors

S A Naser contributed to conception and design of the study, supervised MAP culture and PCR assays, and participated in data interpretation and writing of the manuscript. J F Valentine contributed to conception and design of the study, obtained blood samples from most of the patients with Crohn's disease and all of those with ulcerative colitis, supervised blood MAP DNA assays, and participated in data interpretation and writing of the manuscript. G Ghobrial was responsible for preparation of cultures and assessment of samples by PCR. C Romero was responsible for blood processing and data collection, and assisted with PCR analyses.

Conflict of interest statement

We declare that we have no conflict of interest.

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