Mycobacterium avium Subspecies *paratuberculosis* in Children with Early-Onset Crohn's Disease

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Background: *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the most enduring infectious candidate that may be associated with inflammatory bowel disease (IBD). It is possible that the inconsistencies in the prevalence studies of MAP in adults reflect clinical differences in adult patients studied, including duration of disease and treatment regimens, and also in lack of specificity of some of the assays used. The aim was to determine the presence of MAP in children with symptoms of Crohn's disease (CD) and ulcerative colitis (UC), using gut biopsy tissue and peripheral blood mononuclear cells (PBMC) collected at initial endoscopic examination prior to clinical treatment.

Methods: Mucosal biopsies and/or PBMC specimens were collected from a total of 142 children, comprising 62 with CD, 26 with UC, and 54 with non-IBD. MAP-specific IS*900* polymerase chain reaction (PCR) analysis was performed on all biopsies and PBMC specimens. Conventional MAP culture technique was performed on a subset of 10 CD, 2 UC, and 4 non-IBD patients to isolate MAP.

Results: MAP was identified by IS*900* PCR significantly more often in mucosal biopsies from CD 39% (22/56) than from non-IBD 15% (6/39) patients ($P < 0.05$), and in PBMC from CD 16% (8/50) than from non-IBD 0% (0/31) patients ($P < 0.05$). Viable MAP were cultured from mucosal biopsies from 4/10 CD, 0/2 UC, and 0/4 non-IBD patients, but were not cultured from PBMC specimens.

Conclusions: This unique study on the occurrence of MAP in gut tissue and blood from pediatric IBD patients suggests the possible involvement of MAP in the early stages of development of CD in children.

(Inflamm Bowel Dis 2009;000:000–000)

Received for publication March 12, 2009; Accepted March 20, 2009.

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DOI 10.1002/ibd.20967

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

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

Crohn's disease (CD) is a chronic relapsing inflammatory disease of the gastrointestinal tract.[1,2] Although CD has increased in prevalence over the last 40 years in many locations, including the United States and throughout many European countries, the disease characteristics have not changed.[3] Over the past 20 years, a 10-fold increase in incidence, especially in children, has been identified. Approximately 30 new cases of CD in children (age 2–15 years) are now diagnosed and treated at the Royal Children's Hospital (Melbourne, Australia) each year compared with ≈3 new cases reported annually in 1975.[4] This increase is not due to improved detection.

Several infectious agents, including *Mycobacterium avium* subspecies *paratuberculosis* (MAP), adherent invasive *E. coli*, *Yersinia*, and *Pseudomonas* have been implicated as triggering agents of CD.[5–9] Research has excluded many microorganism including *Salmonella, Campylobacter jejuni, Clostridium difficile*, adenoviruses, rotaviruses, and Mycoplasma as primary etiological agents, although some may be implicated in relapses of CD.[5] It is possible that more than 1 etiologic agent is capable of initiating CD. Etiological research conducted worldwide over many years has been more successful in excluding, than in implicating, putative agents. Research to date has examined the presence of infectious agents in tissue from adult patients, most with a history of long-standing disease and of variable treatment regimens that could have altered components of microbial flora present at onset. To date there are no published studies of children presenting (prior to treatment) with early onset of symptoms of inflammatory bowel disease (IBD) that are later differentiated into CD, ulcerative colitis (UC), or non-IBD.

MAP has been the most enduring infectious candidate to be proposed as a causative agent of CD,[10,11] although its role in the etiology of the disease has often been questioned. Prevalence studies of MAP in CD patients from many countries worldwide have reported widely divergent results ranging from 0%–100%.[10–12] The possible role of

TABLE 1. Clinical Characteristics of Crohn's Disease Patients

Patient No.	Age (yr)	Sex	Biopsy Location Collected	Granuloma Present in Histological Analyzed Biopsy	MAP in Biopsies	MAP in PBMC	MAP in Culture
CD1	11.2	m	N/T	yes	N/T	–	N/T
CD2	13.8	f	N/T	yes	N/T	–	N/T
CD3	12.4	m	N/T	no	N/T	–	N/T
CD4	13.6	f	N/T	no	N/T	–	N/T
CD5	14.7	m	I$^+$, Ca$^+$, Ca$^-$	no	–	+	N/T
CD6	10.2	f	Ca$^+$, Ca$^-$	yes	–	+	N/T
CD7	5.5	f	N/T	yes	N/T	–	N/T
CD8	10.9	m	I$^+$, Ca$^+$, Ca$^-$, S$^+$, R$^+$	yes	–	+	N/T
CD9	12.9	m	N/T	yes	N/T	–	N/T
CD10	7.9	m	I$^+$, Ca$^-$, Co$^-$	no	–	+	N/T
CD11	16.4	m	I$^+$, Co$^-$	no	–	+	N/T
CD12	11.5	m	**I**$^+$, Co$^+$, Co$^-$	no	+	–	N/T
CD13	9.4	m	I$^+$, Ca$^-$, R$^+$	yes	–	–	N/T
CD14	12	m	I$^+$, Ca$^+$, Co$^-$	no	–	N/T	N/T
CD15	17.6	f	I$^+$, I$^-$, Ca$^+$, Co$^-$	no	–	+	N/T
CD16	11.1	m	**I**$^-$, I$^+$, Ca$^-$, **R**$^-$, **R**$^+$	no	+	N/T	N/T
CD17	11.9	f	I$^+$, Ca$^+$	no	–	N/T	N/T
CD18	12.2	m	I$^-$, **Ca**$^+$, R$^+$	no	+	+	N/T
CD19	15.4	m	I$^+$, I$^-$	yes	–	–	N/T
CD20	10.1	f	I$^-$, Co$^+$	no	–	–	N/T
CD21	17	f	**I**$^+$, Ca$^-$	no	+	–	N/T
CD22	14.5	m	I$^+$, I$^-$	yes	–	–	N/T
CD23	14.8	f	I$^-$, Ca$^-$	yes	–	–	N/T
CD24	13	f	**Ca**$^+$, **S**$^-$	yes	+	N/T	N/T
CD25	12.6	m	**I**$^+$, Ca$^-$ R$^+$	no	+	–	N/T
CD26	10.5	f	Co$^-$, Co$^+$, **R**$^+$	no	+	–	N/T
CD27	8.2	f	S$^+$, R$^+$, R$^-$	yes	–	–	N/T
CD28	13.7	f	I$^+$, Ca$^-$, **Co**$^+$	no	+	–	N/T
CD29	12.8	f	I$^+$, Ca$^+$, Ca$^-$	yes	–	–	N/T
CD30	2.2	f	I$^+$, I$^-$	yes	–	N/T	N/T
CD31	11.4	m	I$^+$, Ca$^+$, Co$^+$	yes	–	–	N/T
CD32	10.5	m	I$^+$, I$^-$, Co$^+$, Co$^-$	yes	–	–	N/T
CD33	13.3	m	I$^+$, Ca$^+$, R$^+$	yes	–	–	N/T
CD34	15.4	m	**Ca**$^+$, Co$^-$	no	+	–	N/T
CD35	10.1	m	**I**$^-$, Ca$^+$, **R**$^+$	yes	+	–	N/T
CD36	10.3	f	**I**$^+$, Ca$^+$	yes	+	–	N/T
CD37	11	f	**I**$^+$	yes	+	N/T	+
CD38	12.2	m	Ca$^+$, **R**$^+$	yes	+	–	–
CD39	12.2	m	Ca$^+$, Co$^+$, S$^+$	yes	–	N/T	+
CD40	8.1	f	**Ca**$^+$, S$^+$	yes	+	N/T	+
CD41	6.5	m	I$^+$, Co$^+$, **Co**$^-$	no	+	N/T	+
CD42	14.4	m	I$^-$, Ca$^-$, **Co**$^+$	yes	+	–	–
CD43	13.4	m	I$^+$, S$^+$, R$^-$	yes	–	–	–
CD44	13	m	I$^-$, Co$^-$, Co$^+$	no	–	–	–
CD45	9.1	m	I$^-$, Co$^-$, **Co**$^+$	yes	+	–	–

TABLE 1. (*Continued*)

Patient No.	Age (yr)	Sex	Biopsy Location Collected	Granuloma Present in Histological Analyzed Biopsy	MAP in Biopsies	MAP in PBMC	MAP in Culture
CD46	11	f	I^{+}, Ca^{-}, Ca^{+}	no	–	–	–
CD47	5.5	m	I^{-}, $\mathbf{Ca}^{-}$	yes	+	–	N/T
CD48	11.5	m	I^{-}, Co^{+}, R^{+},	yes	–	–	N/T
CD49	8.2	f	I^{-}, Ca^{-}, Ca^{+}	no	–	–	N/T
CD50	13.6	m	I^{-}, Ca^{+}, R^{+}	yes	–	–	N/T
CD51	9.5	m	I^{+}, Ca^{-}	yes	–	–	N/T
CD52	13.3	f	I^{-}, I^{+}, Ca^{+}, Co^{+},	no	–	–	N/T
CD53	11.2	f	I^{+}, Ca^{-}, S^{+}	yes	–	–	N/T
CD54	13.7	f	$\mathbf{I}^{-}$, R^{-}	yes	+	–	N/T
CD55	11.5	m	I^{+}, I^{-}, Ca^{-}	no	–	N/T	N/T
CD56	14.5	m	$\mathbf{I}^{+}$, I^{-}, R^{+}	yes	+	–	N/T
CD57	9.5	m	I^{-}, Co^{+}, $\mathbf{R}^{+}$	yes	+	–	N/T
CD58	15.6	m	I^{+}, R^{+}	no	–	N/T	N/T
CD59	8	f	I^{+}, Co^{+}	yes	–	–	N/T
CD60	13.6	m	I^{+}, R^{-}	no	–	N/T	N/T
CD61	8.6	m	I^{-}, Ca^{+}	no	–	–	N/T
CD62	13.7	f	$\mathbf{I}^{+}$, $\mathbf{Ca}^{+}$, S^{+}	yes	+	+	N/T

CD, Crohn's disease; m, male; f, female; I, iIleum; Ca, cecum; Co, colon; S, sigmoid; R, rectum; N/T, not tested; +, visual inflamed tissue; –, visual non-inflamed tissue; bold, biopsy location MAP-positive.

MAP in CD has also been supported by the identification of MAP DNA using IS*900* polymerase chain reaction (PCR) analysis of media inoculated with peripheral blood mononuclear cells (PBMC) from patients.[13] A recent review based on a colloquium, sponsored by the American Academy of Microbiology, concluded that "the association of MAP and CD is no longer in question. However its role in causation of CD remains to be defined."[14]

Detection of the presence of MAP in tissue and PBMC is now based on molecular biological techniques. It has been suggested that the variable results reported in many prevalence studies could have been due to cross-contamination of specimens or to technical variations in restriction enzymes, labeling methods, and hybridization probes used in different laboratories. The specificity of IS*900*-PCR has also been questioned because of the presence of IS*900*-like sequences in other mycobacteria.[15] However, redesign of a nested IS*900* PCR specific for MAP, together with matching of the genetic sequence of nested PCR products with the known genetic sequence of MAP, has resolved this problem.[6]

In this study we describe a comprehensive investigation into the presence of MAP in intestinal tissue and PBMC from 142 children presenting with initial symptoms of IBD prior to treatment. The final diagnoses included CD (62 children), UC (26 children), and non-IBD (54 children). There was evidence of MAP infection in biopsy tissue and/or PBMC in a total of 45% of children with CD, 35% of children with UC, and 11% of non-IBD children. The presence of viable MAP in 4/10 CD patients was confirmed by isolation of MAP from biopsy specimens. These results support the hypothesis that MAP infection of intestinal tissue, perhaps associated with blood-borne spread, may be implicated in the pathogenesis of CD in a proportion of children.

MATERIALS AND METHODS

Patients and Sample Collection

Patients undergoing an initial endoscopy at the Royal Children's Hospital (Melbourne, Australia) as part of their clinical care for diagnosis of IBD were enrolled prospectively. None of the patients were being treated with anti-bacterial agents including anti-MAP medication and/or immunosuppressive medication at the time of presentation. The clinical diagnosis of CD was established using standard clinical, endoscopic, and histopathological criteria according to the Montreal Classification.[16] A total of 142 children were enrolled, comprising 62 with CD, 26 with UC, and 54 with non-IBD (Tables 1–3). The diagnosis of CD was confirmed after histological examination of gut tissue. Ethics approval for the study was obtained from the Human Ethics Committee of the Royal Children's Hospital

TABLE 2. Clinical Characteristics of Ulcerative Colitis Patients

Patient No.	Age (yr)	Sex	Biopsy Location Collected	Granuloma Present in Histological Analyzed Biopsy	MAP in Biopsies	MAP in PBMC	MAP in Culture
UC1	11.8	f	N/T	no	N/T	–	N/T
UC2	6.1	f	N/T	no	N/T	–	N/T
UC3	13.9	f	I$^-$, Ca$^-$, R$^-$	no	–	+	N/T
UC4	10.3	f	N/T	no	N/T	–	N/T
UC5	13.5	f	N/T	no	N/T	–	N/T
UC6	13.2	f	**Co**$^+$	no	+	–	N/T
UC7	8.4	m	**Co**$^+$	no	+	–	N/T
UC8	15.3	f	I$^-$, Ca$^-$	yes	–	–	N/T
UC9	10.8	m	I$^-$, **Ca**$^-$, Ca$^+$	no	+	–	N/T
UC10	1.5	m	I$^-$, **Co**$^-$, Co$^+$	no	+	N/T	N/T
UC11	15.7	f	I$^-$, Ca$^-$	no	–	–	N/T
UC12	14.2	m	I$^-$, Ca$^-$	no	–	–	N/T
UC13	9.3	f	**I**$^-$, **Ca**$^-$, R$^+$	no	+	–	N/T
UC14	6.2	f	I$^-$, Ca$^+$, R$^+$	no	–	–	N/T
UC15	10.7	f	I$^-$, Ca$^+$	no	–	–	N/T
UC16	4.1	f	I$^-$, Ca$^-$, R$^+$	no	–	–	N/T
UC17	14.8	f	I$^-$, Ca$^-$, R$^+$	no	–	–	N/T
UC18	13.4	f	**I**$^-$, **S**$^-$, R$^+$	no	+	–	–
UC19	4.7	m	I$^-$, Ca$^+$	no	–	+	–
UC20	12.2	m	I$^-$, Ca$^+$, S$^+$	no	–	–	N/T
UC21	12.6	f	I-, Co-, R-	no	–	–	N/T
UC22	13	m	I$^+$, Co$^+$	no	–	–	N/T
UC23	15.8	m	Ca$^+$, R$^+$	no	–	–	N/T
UC24	11.8	m	I$^-$, R$^+$	no	–	–	N/T
UC25	11.3	m	I$^-$, **Ca**$^-$	yes	+	–	N/T
UC26	14.2	f	I$^-$, R$^+$	no	–	–	N/T

UC, ulcerative colitis; m, male; f, female; I, ileum; Ca, cecum; Co, colon; S, sigmoid; R, rectum; N/T, not tested; +, visual inflamed tissue; –, visual non-inflamed tissue; bold biopsy location, MAP-positive.

(EHRC no. 23003). Informed consent was obtained from each individual parent or guardian.

From the CD group mucosal biopsy specimens were taken from the terminal ileum ($n = 57$), cecum ($n = 41$), colon ($n = 26$), sigmoid ($n = 8$), and rectum ($n = 20$) (Table 1). From the UC group, biopsies were taken from the terminal ileum ($n = 19$), cecum ($n = 15$), colon ($n = 6$), sigmoid ($n = 2$), and rectum ($n = 10$) (Table 2). In the non-IBD group biopsies were taken from the terminal ileum ($n = 34$), cecum ($n = 31$), and rectum ($n = 15$) (Table 3). For ethical reasons we could not collect biopsies from the colon and sigmoid from the non-IBD control group. Where possible, biopsy specimens were obtained from visually inflamed and noninflamed locations in each patient. All tissue specimens were removed from biopsy forceps with a sterile needle and placed immediately into a sterile screw-cap cryotube containing RNAlater (Ambion, Austin, TX). The samples were stored at −70°C until analyzed for MAP. Biopsy specimens used for MAP culture were stored immediately without buffer at –70°C. Samples were examined in the sequential order in which they were collected, ensuring that specimens from CD, UC, and non-IBD patients were examined in a random manner.

A single 8–10 mL whole blood sample was collected in sterile lithium-heparin tubes (Sarstedt, Germany) at the time of initial endoscopy from 50/62 patients with CD, 25/26 with UC, and 31/54 with non-IBD (Tables 1–3). PBMCs were isolated by density gradient using Ficoll Paque (GE Healthcare, Milwaukee, WI). An aliquot of ≈2 million cells (counted using a hemocytometer) was resuspended in 1 mL freeze mix media (1× DMEM [Gibco, Gaithersburg, MD] with 42.5% heat-inactivated fetal calf serum [In Vitro Technology] and 10% dimethyl sulfoxide [DMSO; Sigma

Chemical, St. Louis, MO]) prior to freezing in a vaporized liquid nitrogen storage facility.

Extraction of Total DNA from Intestinal Biopsies

DNA extraction from biopsy specimens was conducted as previously described.[6] Briefly, RNAlater stabilized tissue samples were thawed and transferred to a sterile tube. The sample was homogenized with an electric driven sterile minipestle in 200 μL of lysis buffer (20 mM Tris-HCl, pH 8.0, and 2 mM EDTA). The homogenate was transferred to a sterile screw-cap tube and subjected to 3 cycles of freeze/thaw. Lysozyme (20 mg/mL, Sigma) was added to each sample and incubated at 37°C for 30 minutes. A volume of 600 μL buffer AL (QIAamp DNA minikit, Qiagen, Chatsworth, CA) and glass beads (≈20%, diameter 150–212 μm, Sigma) were added to each sample. Tubes were chilled on ice for 5 minutes prior to mechanical disruption in a FastPrep (setting of 6.5 ms^{-2} for 45 sec), then immediately chilled again on ice for at least 5 minutes. Glass beads were pelleted (14,000*g* for 3 min), and the supernatant was harvested and recentrifuged (14,000*g* for 1 min). Supernatants were transferred to 2-mL screw tubes to which proteinase K (24 mU) was added. Samples were incubated at 56°C for 3 hours and vortexed briefly (2–3 times per hour). After the proteinase K treatment, DNA was extracted from all samples using the QIAamp DNA mini kit according to the manufacturer's instructions (Qiagen). DNA was eluted into 50 μL nuclease free water. All DNA extractions were conducted in biological safety cabinet class II. A mock homogenization and extraction (without sample material) was included in each extraction run to exclude possible contamination during the procedure.

Extraction of DNA from PBMC

DNA was extracted from $\approx 2 \times 10^6$ PBMCs. Briefly, frozen cells were washed and resuspended in 100 μL phosphate-buffered saline (PBS), then transferred to a sterile 2-mL screw cap tube containing 180 μL lysis buffer (see above). DNA was extracted using the same extraction procedure as described for the biopsy specimens, omitting mechanical disruption with minipestle.

IS900-Specic Nested PCR for the Detection of MAP

MAP was detected using the IS*900* nested PCR method modified from Bull et al.[6] The first-round PCR comprised 5 μL DNA in a final reaction volume of 50 μL, containing 2 μM of TJ1 forward primer (5′-GCTGATCG CCTTGCTCAT-3′) and TJ2 reverse primer (5′-CGGGAG TTTGGTAGCCAG-3′); 1× Expand HF reaction buffer with 1.5 mM $MgCl_2$ and 3.5 units Expand HF enzyme mix (Expand High Fidelity PCR system, Roche, Nutley, NJ); 10% DMSO and 200 μM of each dNTP. The PCR cycling conditions were: 94°C for 5 minutes, and 30 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 3 minutes, and a final extension of 72°C for 7 minutes. The nested PCR assay comprised 1 μL of first-round PCR product in a final reaction volume of 50 μL containing the same reagents as in the primary PCR reaction, but with 2 μM of TJ3 forward primer (5′-CAGCGGCTGCTTTATATTCC-3′) and 2 μM TJ4 reverse primer (5′-GGCACGGCTCTTGTTG TAGT-3′). The cycling conditions were the same as for the primary PCR except that 40 cycles were used instead of 30 cycles. All PCR products were separated on a 1.5% agarose gel and visualized under UV light using ethidium bromide. Nested PCR amplicons of the expected size (289 bp) were analyzed by sequence analysis at the Australian Genome Research Facility (Brisbane, Australia).

To prevent contamination during PCR preparation, all preparative steps were carried out in a biological safety cabinet class II in which no template DNA was used. Template DNA was added in a separate biological safety cabinet class II. All PCR assays included a negative control PCR in which template DNA was substituted with nuclease free water, together with a positive control PCR of 1 ng IS*900*-specific DNA.

Sequencing of Nested IS900-Specic DNA

Nucleotide sequence analysis was conducted on positive nested IS*900* PCR products. The 289 bp DNA fragment was purified by gel extraction using the Wizard SV gel and PCR clean-up system (Promega, Madison, WI) according to the manufacturer's instructions. Purified PCR products were sequenced in both directions using the TJ3 and TJ4 primers as sequencing primers, and the dideoxynucleotide chain termination method using an ABI terminator cycle sequencing kit (ABI, Foster City, CA) on an ABI 3730-96 capillary automated Sequencher at the Australian Genome Research Facility (Brisbane, Australia). The sequences were analyzed with the Sequencher program, v. 4.6, and aligned with reference sequence for MAP cattle (C) strain K10 (GenBank accession number NP_958968) using cluster function from BioManager available through the Australian National Genomic Information Service.[17]

Mycobacterial Culture

Mucosal biopsy tissue samples were cultured for the recovery of MAP using radiometric culture (BACTEC, Becton Dickinson, San Jose, CA) with BACTEC 12B vials (modified Middlebrook 7H9 broth) supplemented with the antibiotic mixture PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethroprim, azlocillin; Becton Dickinson), egg yolk, and mycobactin J (Allied Monitor, Fayette, MO). This procedure was carried out, following the initial processing of samples, by a modification of the double incubation method.[18,19] Briefly, 0.1 g of biopsy tissue was finely sliced and macerated for 2–5 minutes in 1 mL of a solution

TABLE 3. Clinical Characteristic of Noninflammatory Bowel Disease Control Patients

Patient No.	Age (yr)	Sex	Biopsy Location Collected	Granuloma Present in Histological Analyzed Biopsy	MAP in Biopsies	MAP in PBMC	MAP in Culture
N1	11.2	f	N/T	no	N/T	–	N/T
N2	11.7	m	N/T	no	N/T	–	N/T
N3	16.8	f	N/T	no	N/T	–	N/T
N4	13.3	f	N/T	no	N/T	–	N/T
N5	12.2	m	N/T	no	N/T	–	N/T
N6	12.5	f	N/T	no	N/T	–	N/T
N7	14.3	m	N/T	no	N/T	–	N/T
N8	14.8	f	N/T	no	N/T	–	N/T
N9	11.2	m	N/T	no	N/T	–	N/T
N10	9.6	m	N/T	no	N/T	–	N/T
N11	5.1	f	N/T	no	N/T	–	N/T
N12	10.7	m	N/T	no	N/T	–	N/T
N13	17.2	m	N/T	no	N/T	–	N/T
N14	5.1	m	N/T	no	N/T	–	N/T
N15	11.4	f	N/T	no	N/T	–	N/T
N16	5.9	f	Ca^{-}	no	–	–	N/T
N17	15.1	m	I^{-}, Ca^{-}	no	–	–	N/T
N18	17.8	f	I^{-}, Ca^{-}	no	–	–	N/T
N19	3.2	f	I^{-}, Ca^{-}	no	–	–	N/T
N20	16.8	f	I^{-}, Ca^{-}	no	–	–	N/T
N21	12.8	m	I^{-}	no	–	–	N/T
N22	11.2	f	I^{-}, Ca^{-}	no	–	N/T	N/T
N23	8.4	m	I^{-}, Ca^{-}	no	–	–	N/T
N24	16.1	f	I^{-}, Ca^{-}	no	–	–	N/T
N25	6.5	f	I^{-}, Ca^{-}	no	–	–	N/T
N26	10.8	f	I^{-}, Ca^{-}	no	–	–	N/T
N27	12.4	m	I^{-}, **R^{-}**	no	+	N/T	–
N28	6.6	m	I^{-}, Ca^{-}	no	–	N/T	–
N29	15.6	m	I^{-}, **Ca^{-}**	no	+	–	N/T
N30	8	m	**I^{-}**, Ca^{-}, R^{-}	no	+	–	N/T
N31	13.3	f	I^{-}, Ca^{-}	no	–	–	N/T
N32	10.5	m	I^{-}, Ca^{-}	no	–	–	–
N33	16.9	f	I^{-}, Ca^{-}	no	–	–	–
N34	12.2	m	**I^{-}**, Ca^{-}	no	+	–	N/T
N35	11	f	I-, Ca-, R-	no	–	N/T	N/T
N36	4.9	m	I^{-}, R^{-}	no	–	N/T	N/T
N37	14.4	f	I^{-}, R^{-}	no	–	N/T	N/T
N38	13.7	m	I^{-}, Ca^{-}, R^{-}	no	–	N/T	N/T
N39	7.6	m	I^{-}, Ca^{-}, R^{-}	no	–	N/T	N/T
N40	13.3	f	I^{-}, Ca^{-}, R^{-}	no	–	N/T	N/T
N41	11.3	f	I^{-}, Ca^{-}, R^{-}	no	–	N/T	N/T
N42	12.3	m	I^{-}, Ca^{-}	no	–	N/T	N/T
N43	14.1	f	**I^{-}**, Ca^{-}	no	+	N/T	N/T
N44	14.5	m	I^{-}, Ca^{-}	no	–	N/T	N/T
N45	14.5	f	I^{-}, Ca^{-}	no	–	N/T	N/T

TABLE 3. (*Continued*)

Patient No.	Age (yr)	Sex	Biopsy Location Collected	Granuloma Present in Histological Analyzed Biopsy	MAP in Biopsies	MAP in PBMC	MAP in Culture
N46	14.7	m	Ca^-, R^-	no	–	N/T	N/T
N47	11.9	m	Ca^-, **R-**	no	+	N/T	N/T
N48	9.8	m	Ca^-	no	–	N/T	N/T
N49	10.7	f	R^-	no	–	N/T	N/T
N50	15.7	f	I^-, R^-	no	–	N/T	N/T
N51	9.9	m	I^-, R^-	no	–	N/T	N/T
N52	11.3	f	I^-, R^-	no	–	N/T	N/T
N53	13.4	f	I^-, Ca^-	no	–	N/T	N/T
N54	9.3	m	I^-, Ca^-	no	–	N/T	N/T

N, noninflammatory bowel disease; m, male; f, female; I, ileum, Ca, cecum; R, rectum; N/T, not tested; +, visual inflamed tissue; –, visual noninflamed tissue; bold biopsy location, MAP-positive.

containing 1.85% brain heart infusion (BHI) and 0.75% hexadecylpyridinium (HPC; Sigma) with a Seward Stomacher 80. The cellular suspension was incubated at 37°C for 18 hours prior to centrifugation at 900*g* for 30 minutes. The pellet was resuspended in BHI broth and incubated at 37°C for a further 24 hours prior to the inoculation of 0.5 mL into the supplemented BACTEC12B culture vial. Selected biopsy samples were also cultured in fluorometric mycobacterium growth indicator tubes (BBL-MGIT) supplemented with oleic acid-albumin-dextrose-catalase (OADC) enrichment, PANTA, and mycobactin J. All cultures were incubated at 37°C for up to 1 year.

The growth index (GI) of the BACTEC 12B culture vials was determined weekly using the BACTEC460 instrument. Visible growth in all MGIT culture tubes was detected by fluorescence under a UV transilluminator. Aliquots were removed for Ziehl-Neelsen staining (to detect acid-fast bacilli) and subculture (to observe individual MAP colonies) from radiometric culture vials with a GI >100 and from MGIT tubes showing fluorescence. Subcultures were made to confirm typical MAP colony morphology on Herrold's egg yolk medium supplemented with sodium pyruvate, with and without mycobactin to test for mycobactin dependency.[20–24] PCR was used to confirm the presence of the IS*900* insertion element. Acid-fastness of isolates was confirmed microscopically by Ziehl-Neelsen staining.

Caprine and bovine ileal tissue from animals in mid and late stages of Johne's disease were used for comparative assessment of the culture systems. CLIJ623, an Australian wildtype cattle (C) isolate of MAP[21] was used as the standard positive control.

Statistical Analysis

Statistical analyses were performed using Stata statistical software v. 9 (College Station, TX). The chi-square test was used for the analysis of statistical difference in the detection rate of IS*900*-specific DNA in CD, UC, and non-IBD groups. In addition, the chi-square test was used to analyze differences between MAP-positive patients and granuloma positive/negative diagnosed patients, and between MAP-positive biopsies and inflammation status of biopsies. A chi-square test was also performed to test for an association between MAP and clinical phenotype. Fisher's exact test was used to compare IS*900* detection rate in relation to sex in the CD and UC groups. Student's *t*-test was used to compare IS*900*-specific DNA detection in relation to age in the different clinical groups (CD, UC, and non-IBD). All statistical analyses were conducted in the Clinical Epidemiology and Biostatistics Unit (CEBU) at the Murdoch Childrens Research Institute (Melbourne, Australia).

RESULTS

Clinical Characteristics of Patients

Clinical characteristics of CD, UC, and non-IBD patients are summarized in Tables 1–3. Overall, of the 142 patients, 62 had CD (37 male and 25 female), 26 had UC (10 male and 26 female), and 54 were non-IBD control patients (27 male and 27 female). The mean age of all patients was 11.6 years (1.5–17.8). No statistical difference was observed in ages of patients in each group.

Frequency of Detection of MAP DNA in Biopsies

Biopsies (n = 284) from a total of 117 patients were analyzed for MAP IS*900* DNA and included 152 biopsies from 56 CD patients, 52 biopsies from 22 UC patients, and 80 biopsies from 39 non-IBD patients (Tables 1–3). Overall, 94% (110/117) of patients had at least 2 biopsy specimens analyzed, including 2–5 biopsy specimens from CD patients, and 2–3 biopsy specimens from UC and non-IBD patients (Tables 1–3). The frequencies of MAP IS*900* DNA

TABLE 4. Frequency of MAP IS*900*-Positive Biopsies in Patients

	CD	UC	Non-IBD
	MAP + / Total Patients	MAP + / Total Patients	MAP + / Total Patients
Ileum[a]	10/48 (21%)	2/19 (11%)	3/34 (9%)
Cecum[b]	6/35 (17%)	3/14 (21%)	1/31 (3%)
Colon	4/20 (20%)	3/5 (60%)	No sample
Sigmoid	1/8 (13%)	1/2 (50%)	No sample
Rectum[c]	5/18 (28%)	0/10 (0%)	2/15 (13%)

[a]Comparison of CD versus non-IBD ($P > 0.05$, chi-square analysis).
[b,c]Comparison of CD versus non-IBD ($P < 0.1$, chi-square analysis).
Statistical analysis of MAP between CD and non-IBD, including only matched biopsy samples collected from ileum, cecum, and rectum only ($P < 0.1$, chi-square analysis).

at the different tissue sampling sites are summarized in Table 4.

MAP was detected in biopsies from 39% (22/56) of CD patients, 32% (7/22) of UC patients, and 15% (6/39) of non-IBD patients (Table 5). The difference in detection rates of MAP between CD and non-IBD controls was statistically significant when all biopsy locations were combined and analyzed ($P < 0.05$, chi-square test). Statistical comparison of results from matched biopsy samples from ileum, cecum, or rectum of CD patients compared with non-IBD patients showed a trend to significance ($P < 0.1$). The overall difference in detection rates between IBD (CD+UC) and non-IBD control patients was statistically significant ($P < 0.05$, chi-square test); however, the detection rate in UC and non-IBD was not significant.

In the CD group, 18 of 22 MAP-positive patients were positive at only 1 collection site, 3 of 22 were positive at 2 collection sites (CD24, CD35, and CD62 Table 1), and 1 patient was positive at 3 collection sites (CD16, Table 1). Overall, the MAP detection rate in CD patients (Table 4) was highest in the rectum, with 28% (5/18) positive for MAP, followed by the ileum with 21% (10/48), colon 20% (4/20), cecum 17% (6/35), and sigmoid 13% (1/8).

In the UC group, 5 of 7 patients were positive for MAP at a single collection site and 2 were positive at 2 collection sites (UC13 and UC18, Table 2). MAP was detected more frequently in the colon (3/5) and sigmoid (1/2) than in the cecum (3/14) and ileum (2/19). None of the rectal specimens from the 10 UC patients tested were positive for MAP (Table 4).

In the non-IBD group, MAP was detected in 13% (2/15) of rectal samples, followed by 9% (3/34) in ileal samples and 3% (1/31) in cecal samples. No colon or sigmoid samples were available for analysis from this patient group because of ethical limitations (Table 4).

All control samples were PCR-negative for MAP, thus eliminating the possibility of false-positive results due to contamination. In addition, all IS*900*-negative biopsy tissue and PBMC samples became positive after spiking with 200 ng of IS*900* DNA, indicating that none of the negative samples were inhibitory for PCR (data not shown).

Association of MAP Infection with Granulomas

All CD patients were analyzed for the presence of granulomas by histological analysis of biopsies. Thirty-six patients were granuloma-positive and 26 patients were granuloma-negative. In the positive granuloma patient group 42% (15/36), 15/36 patients were positive for MAP in biopsy samples and/or PBMC. In the granuloma-negative group 50% (13/26) of the patients were positive for MAP in either biopsy samples and/or PBMC. There was no statistical association between presence of granuloma and MAP infection in either biopsy samples or PBMC ($P > 0.05$, chi-square test).

Frequency of Detection of MAP DNA in PBMC

PBMCs from 105 patients were analyzed for MAP IS*900* DNA (Tables 1–3). MAP was detected in PBMC in 16% (8/50) of CD patients, 8% (2/25) of UC patients, and in none of the 31 non-IBD patients (Table 5). The difference in the detection rate of MAP DNA in CD patients versus non-IBD patients was statistically significant ($P < 0.05$, Fisher test), whereas the detection rate in PBMC between UC and non-IBD patients was not significantly different.

Comparison of MAP DNA Detection in Biopsy and PBMC from the Same Patients

MAP DNA detection in biopsy and PBMC specimens from the same patients could be compared in 44 patients with CD, 21 with UC, and 16 with non-IBD. In the CD group 2 showed MAP-positive results simultaneously in gut biopsies and PBMCs (CD18 and CD62, Table 1). No simultaneous detection of MAP was found in the UC and non-IBD group.

TABLE 5. Summary of MAP IS*900*-Positive Patients

	Biopsy		PBMC		Culture	
	No.	MAP +	No.	MAP +	No.	MAP +
CD	56	22 (39%)	50	8 (16%)	10	4 (40%)
UC	22	7 (32%)	25	2 (8%)	2	0
Non-IBD	39	6 (15%)	31	0 (0%)	4	0

TABLE 6. Clinical Phenotype of Crohn's Disease Patients and MAP Identification

Location of Disease	L1	L2	L3	L4	L1+L4	L2+L4	L3+L4
No. of MAP + patients	1	2	14	1	0	2	9
Location of MAP +	I+R 1/1	R 1/3 Ca+S 1/1	PBMC 3/8 I 3/10 Ca 3/4 Co 4/4 R 2/3 I+Ca 1/1	Ca 1/4		I+R 1/1	PBMC 5/8 I 4/10
No. of MAP – patients	1	5	13	0	2	0	12
Disease behavior	B1	B2	B3	B1P	B2P	B3P	
No. of MAP + patients	12	1	0	14	1	1	
Location of MAP +	PBMC 3/8 I 1/10 Ca 1/6 Co 2/4 R 1/3 I+R 2/2 Ca+S 1/1	PBMC 1/8		PBMC 4/8 I 4/10 Ca 3/6 Co 2/4 R 2/3 I+Ca 1/1	I 1/10	I 1/10	
No. of MAP – Patients	17	0	0	10	4	2	

The number of MAP+ biopsies, MAP+ PBMC, and MAP+ culture in each disease location and in each disease behavior type are outlined in each group. Numerator, total number of MAP+ samples in that particular specimens in all CD patients analyzed.
Disease phenotype classification abbreviations: L1, terminal ileum; L2, colonic; L3, ileocolonic; L4, upper GI; B1, nonstricturing, nonpenetrating; B2, stricturing; B3 penetrating; P, perianal disease; I, ileum; Ca, cecum; Co, colon; S, sigmoid; R, rectum; PBMC, peripheral blood mononuclear cells; MAP, *Mycobacterium avium paratuberculosis*.

MAP Association with Clinical Phenotype of CD

Patients were categorized according to the Montreal Classification (Table 6). All patients were age class A1 (16 years or less) except 2 CD patients. In 26/61 patients with CD the location of disease was the ileocolonic region (L3, 16 MAP-positive and 10 MAP-negative). In 21 of these patients the upper GI was also involved (L3+L4, 9 MAP-positive and 12 MAP-negative). The disease behavior was nonstricturing (B1) in 29 patients (14 MAP-positive and 15 MAP-negative) and nonstricturing with perianal disease (B1P) in 23 patients (13 MAP-positive and 10 MAP-negative). There was no statistical association between MAP infection in either biopsy samples and/or PBMC and disease location/or disease behavior ($P > 0.05$, Fisher test). Multivariate analysis of the specific MAP location and clinical phenotype was not possible due to the low numbers in each group.

Association of MAP with Patient Age and Sex

The occurrence of MAP was not influenced by the age or sex of patients. The age-ranges of MAP-positive CD and UC patients (biopsy and PBMC combined) were 6.5–17.6 years and 1.5–13.9 years, respectively. Statistical analysis (*t*-test) showed no significant age difference in detection of MAP IS*900* fragment between CD, UC, and non-IBD groups. The MAP-positive detection rate (biopsy and PBMC combined) between female (11/25) and male (17/37) patients in the CD group and between female (4/16) and male (5/10) patients in the UC group was not statistically significant ($P > 0.05$, Fisher test).

Confirmation of MAP IS900 by Sequence Analysis

All 44 IS*900* PCR products were sequenced to confirm the identity and to investigate the genetic diversity of the MAP-positive isolates. The 253 bp sequence fragment, after removal of the TJ3 and TJ4 primer regions, were analyzed using NCBI nucleotide blast search and were aligned against each other and the IS*900* sequence of the MAP cattle (C) strain K-10 (Fig. 1). All sequences were confirmed as MAP IS*900*. Thirty-six of the 44 samples (Fig. 1, sequence 2) exhibited 100% nucleotide identity with the published IS*900* elements of the genome sequence of MAP K-10 strain (Fig. 1, sequence 1) as well as to a sequence obtained from the intestinal and lymph node tissue of water buffaloes (accession number AY 660658).[25] Three

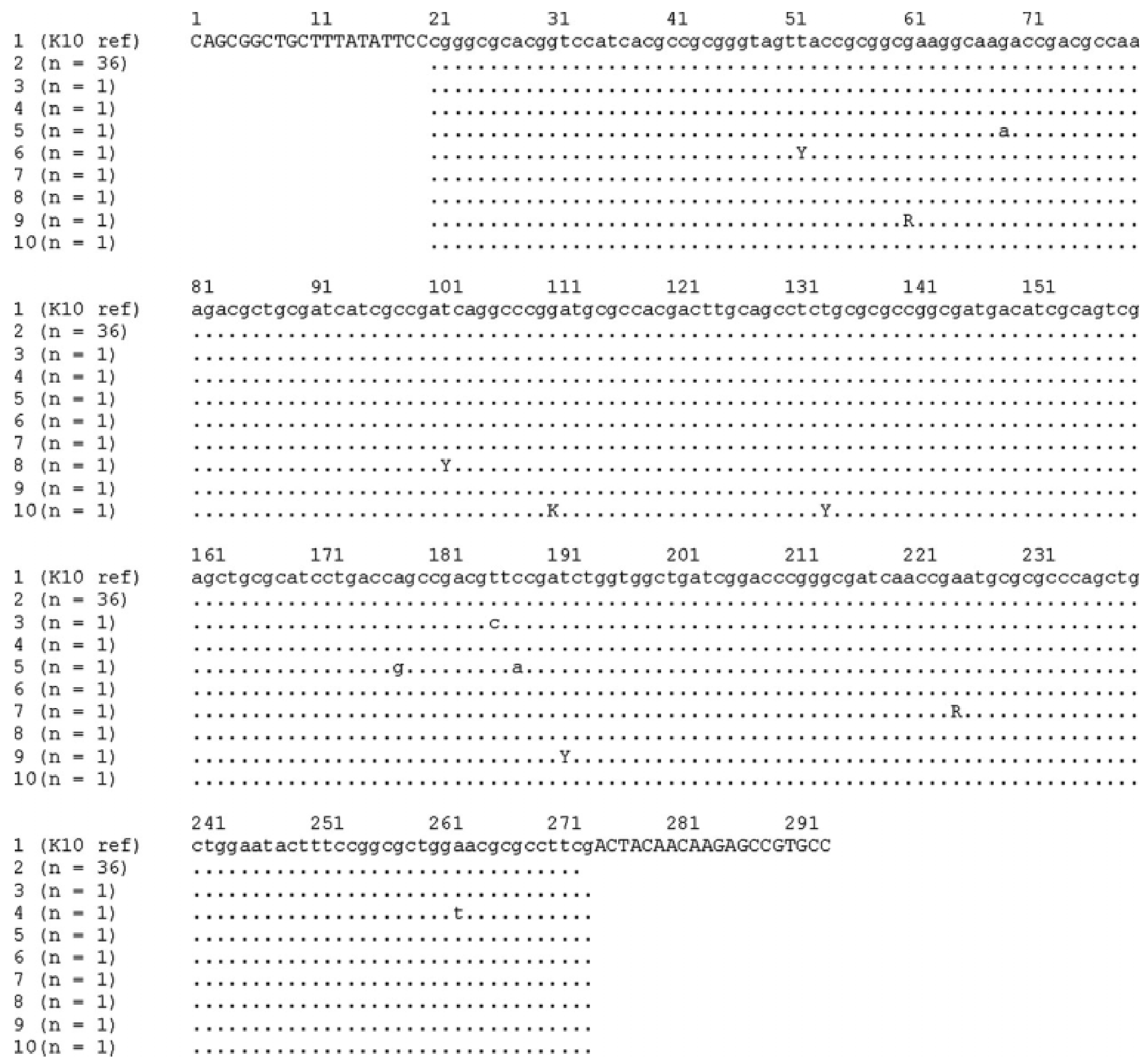

FIGURE 1. Alignment of patient MAP IS900 TJ3/TJ4 PCR products. Nucleotide sequence 1 corresponds to MAP strain K10 (AE016958). Capital letters are TJ3 (1-20) and TJ4 (272-291) primers, respectively. Dots represent the same base. Nucleotide sequence 2 corresponds to 36 patient TJ3/TJ4 PCR products (31 biopsy DNA and 5 PBMC DNA) with 254/254 nucleotide identity to strain K10. Nucleotide sequences 3, 4, and 5 (all PBMC DNA from CD patients) showed nucleotide polymorphism, T>C transition at position 186 in sequence 3, A>T transition at position 263 in sequence 4 and an A>G and a C>A transition at position 178 and 188, respectively, in sequence 5. Ambiguity sequences were present in 5 patients, Y at position 52 in sequence 6 (PBMC DNA), R at position 225 in sequence 7 (biopsy DNA), Y at position 102 in sequence 8 (biopsy DNA), R and Y at position 61 and 192, respectively, in sequence 9 (biopsy DNA), and K and Y at positions 111 and 134, respectively, in sequence 10 (biopsy DNA).

sequences exhibited nucleotide polymorphism and 5 showed ambiguity sequences (Fig. 1).

Frequency of MAP in Patients Analyzed by Bacterial Culture

Mycobacterial culture was performed on a total of 35 biopsies (a random collection of lower and upper bowel specimens) from 16 patients (10 CD, 2 UC, and 4 non-IBD patients) (Table 7). Biopsies from individual patients were pooled using a minimum of 2 specimens per patient, except for 1 patient in whom 2 single large biopsies were cultured. Viable MAP were detected in 40% (4/10) of CD patients. No viable MAP was detected in UC (0/2) or non-IBD (0/4) patients (Table 5). The average incubation time for the initial detection of MAP-positive cultures was 24 weeks (9–27 weeks) in radiometric and/or fluorometric culture

TABLE 7. Recovery of Viable MAP from Patient's Biopsy

Patient No.	MAP+ by Culture	Biopsy Used
CD36	+	Ileum + Ileum*
CD 37	–	Cecum + Rectum
CD38	+	Cecum + Colon + Sigmoid
CD39	+	Cecum + Sigmoid
CD40	+	Ileum + Colon + Colon
CD41	–	Ileum + Colon
CD42	–	Ileum + Sigmoid
CD43	–	Ileum + Colon
CD44	–	Ileum + Colon
CD45	–	Ileum + Cecum
UC18	–	Ileum + Sigmoid + Rectum
UC19	–	Ileum + Cecum
N26	–	Ileum + Rectum
N27	–	Ileum + Cecum
N30	–	Ileum + Cecum
N31	–	Ileum + Cecum

CD, Crohn's disease; UC, ulcerative colitis; N, noninflammatory bowel disease.
*Biopsies were not pooled for culture.

systems. Typical individual MAP colonies were observed on subsequent subcultures onto HEYM slopes after 7–8 weeks incubation. The identity of each MAP isolate was confirmed by the characteristic acid-fast microscopic morphology following ZN staining, typical colonial morphology on Herrold's egg yolk medium supplemented with mycobactin J (hence confirming mycobactin dependency), and the presence of the MAP IS*900* insertion element detected by PCR. Cultures were considered negative if growth of acid-fast mycobactin-dependent MAP were not observed in BACTEC 12B liquid medium or MGIT tubes following 52 weeks.

DISCUSSION

Much conjecture surrounds the involvement of MAP in the etiopathology of CD. However, a recent review and meta-analysis of 28 case-control studies of the occurrence of MAP in adult patients concludes that the results support a specific association between CD and the presence of MAP in affected intestinal tissue.[11] A recent review from the American Academy of Microbiology also concludes that: "the association of MAP and CD is no longer in question. However the role of MAP in causation of CD remains to be defined."[14] The study described here is the first to investigate the occurrence of MAP in children who were undergoing initial endoscopy for the diagnosis of IBD. All investigations were performed prior to treatment. MAP was identified significantly more often in gut biopsy specimens (22/56; 39%) from patients with early onset CD, than from non-IBD control patients (6/39; 15%) ($P < 0.05$). When analysis was limited to comparison of results of the occurrence of MAP in biopsy specimens from 3 matched locations (ileum, cecum, and rectum) statistical analysis showed a trend to significance, with the difference being statistically significant at the 90% interval. MAP was also identified in high numbers of biopsies in UC patients (32%).

The specificity of MAP-positive IS*900* results was confirmed by sequence analysis of the PCR products. Cross-contamination of specimens was excluded by inclusion of negative control specimens, and by performance of assay steps in separate biological safety cabinets. In this study the IS*900* sequence obtained for the human strains exhibited similarity to that of the cattle strain K10. However, further studies are needed to fully characterize the genome of human MAP strains.

In addition to detection of MAP by IS*900* PCR, viable MAP were cultured from biopsies from 4/10 CD patients and identified by growth and staining characteristics. In some patients, biopsy tissues were positive for MAP IS*900* DNA by PCR but no viable MAP could be cultured. Differences in the total number of viable bacteria present and the phase at which the organism exists (e.g., dormant, cell wall impaired) could have affected its culturability. It is also possible that the sequence detected by PCR in the patient's tissue represents the molecular footprint (IS*900*) of a dead microorganism. In some patients viable MAP was isolated from biopsies negative for MAP IS*900* DNA by PCR, perhaps because identical biopsies were not always used for PCR and bacteriological culture. These results are likely to be due to heterogeneous distribution of low number of MAP organisms throughout the gut epithelium.

In addition to detection of MAP in biopsy tissue, MAP-positive DNA was detected in PBMC from 8/50 (16%) patients with CD, 2/25 (8%) patients with UC. No MAP-positive DNA was detected in PBMC from 0/31 non-IBD control patients. The identification of MAP in PBMC has been previously reported by Naser et al,[13] although the prevalence of MAP in CD patients in our study (16%) was much less than was found in this previous study (50%). There was no association between MAP detected in tissue and in PBMC samples in our study. This lack of association could be explained by collection of specimens from patients at different stages after onset of disease, or because the samples of gut biopsy analyzed did not contain foci of infection. It is also possible that MAP may exist as either a local infection restricted to gut tissue, or may have a systemic stage with limited tissue invasion. A recent study showed that MAP isolated from CD patients can evade phagocytosis in both human polymorphonuclear cells and macrophage cells, thus permitting survival of a viable

and possibly virulent organism.[26] A possible mechanism was recently proposed where microbial agents were able to suppress the mucosal phagocyte function, thus inhibiting bacteria killing and permitting persistence.[27] The occasional presence of viable MAP in circulating PBMC, if it occurs, could partly explain the puzzling "skip lesions" described in CD. The skip lesion hypothesis can be explained where viable MAP circulating in blood could periodically lodge in small peripheral blood vessels and multiply, resulting in local infection.

The variation in MAP detection rates across different studies reported in the literature[11] could be due to differences in sample handling and processing, extraction methods, or PCR assays. Our study focused on rapid storage of tissue after biopsy and utilized a redesigned second-round primer pair specific for MAP IS*900*.[6] Our MAP PCR findings have been confirmed, in part, by the ability to culture viable MAP from some of the biopsy specimens, suggesting that the MAP present in gut tissue is infectious. Its role is unclear. A recent clinical trial of antimycobacterial antibiotic therapy failed to result in long-term disease remission.[28] However, in that study the MAP status of patients undergoing treatment was unknown.

One proposed model of CD suggests that the disease is the consequence of an inability to eliminate specific microbes, or to control cytokine expression elevated in the attempt to remove these microbes. The existence of genetic mutations that result in dysregulation of the innate immune system are crucial to this hypothesis. Apart from *CARD15*, several other mutations of the immune regulatory genes have been suggested to be associated with CD, including mutations in *TLR4* and *IL23R*. All these genetic mutations could provide conditions that permit survival and growth of microbial agents, including MAP.[29] The similar rate of MAP identified in children with early-onset CD and UC suggests a role for MAP in initiation of both forms of disease. The clinical differences apparent between CD and UC could then be a consequence of differences in genetic makeup that alter mucosal and immunological responses.

The identification of MAP in a high proportion of children with CD at an early stage after onset of symptoms, and prior to treatment, strengthens the hypothesis that it is implicated in the development of IBD. The results cannot be explained by the long-standing presence of disease symptoms, followed by extensive clinical treatment.[6,13,27] The results of this study represent the earliest identification of MAP in CD. Nonetheless, the ability to determine whether MAP was present at onset, and hence involved in initiation of disease symptoms, remains a challenge.

ACKNOWLEDGMENT

We thank the children and their families for their participation in this study. We thank Associate Professor Susan Donath (Clinical Epidemiology and Biostatistics Unit, Murdoch Childrens Research Institute, Victoria, Australia) for assistance with statistical analysis. This project was supported by research grants from the Murdoch Childrens Research Institute, The CASS Foundation, and The Lynne Quayle Charitable Trust, Equity Trustees LTD. Dr J Wagner is supported by an Murdoch Childrens Research Institute Fellowship and Dr C Kirkwood is supported by an NHMRC RD Wright Fellowship (ID 334364).

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