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

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Abstract: Crohn's disease (CD) is a chronic inflammatory bowel disease (IBD) with tissue granuloma and histopathological alteration that resembles aspects in tuberculosis, leprosy, and paratuberculosis. *Mycobacterium avium* subsp *paratuberculosis* (MAP) is the causative agent of paratuberculosis, with a suspected role in the etiology of CD. We investigated the presence of MAP DNA in 31 surgical tissue samples from 20 subjects using fluorescence in situ hybridization (FISH) with the aid of confocal scanning laser microscopy and nested polymerase chain reaction (PCR) using the IS900 sequence unique to MAP. MAP DNA was detected by PCR in tissue from 10 of 12 (83%) patients with CD: 7/12 (58%) in inflamed, 6/11 (55%) in noninflamed and in 10 (83%) of either tissue and by FISH in 8 of 12 (67%) patients with CD: 7 of 12 (58%) in inflamed, 4 of 11 (36%) in noninflamed, and in 8(67%) of either tissue. In non-IBD subjects, MAP DNA was detected in the tissue of only 1 of 6 patients (17%) by PCR and 0 of 6 patients (0%) by FISH. MAP DNA was identified by PCR in inflamed tissue from 2 of 2 patients with ulcerative colitis. The detection of MAP DNA by either technique in tissue from subjects with CD is significant compared with non-IBD subjects ($P < 0.005$). Identification of MAP DNA in both inflamed and noninflamed tissue by both techniques suggests that MAP infection in patients with CD may be systemic. The data add more evidence toward a possible association of MAP in the pathogenesis of CD.

Key Words: Crohn's disease, fluorescence in situ hybridization, *Mycobacterium avium* subsp *paratuberculosis*, PCR

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Crohn's disease (CD), as described first by Dalziel, is a debilitating chronic inflammatory bowel disease (IBD) that affects all parts of the gastrointestinal tract, with the inflammation extending through all layers of the gut wall to involve adjacent lymph nodes.[1–3] The inflammatory process is frequently discontinuous, with normal bowel separating portions of diseased bowel. Patients with CD tend to have abnormalities in the immune system, but it is not clear whether these abnormalities are cause or result of the disease. There are a variety of cellular processes and proinflammatory mediators that influence the pathogenesis of the disease. It is believed that there is a continuous immune response in the gut in response to an environmental stimulus.

Some pathologic aspects in CD are similar to those present in animals diagnosed with paratuberculosis (also known as Johne's disease; JD) as first described by Dalziel[1] and later on by Chiodini.[4] JD is a chronic inflammatory disease of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), a slow grower, fastidious, and intracellular pathogen.[5] Chiodini et al[6] isolated cell wall-deficient organisms, also known as spheroplast, from tissue of 6 of 28 (21.4%) patients with CD after long-term incubation culture. The spheroplast cultures then reverted to acid-fast *Mycobacterium*-like organism after a single in vitro passage.[6,7] They were confirmed as MAP culture using IS900 probe.[8] This was followed by several studies, which resulted in mixed results in which MAP or MAP DNA presence ranged from 0 to 100%.[9–17] In 2001, the first susceptibility gene for CD, Nod2 (nucleotide-binding oligomerization domain), was identified.[18,19] Mutations in *Nod2* gene have been shown to be associated with CD, suggesting a role for intracellular pathogen-host interactions in the etiology of CD. Detection of anti-MAP IgG antibodies in sera of more CD patients compared with controls added more evidence that supported a possible role for MAP in CD etiology.[20–22]

Although the culture methodology remains the most sensitive and convincing approach for confirming or excluding a mycobacterial role in CD etiology, this approach faces many obstacles related to the fastidious characteristics and lack of

cell wall of the MAP bacterium in human CD tissue. There is a need for additional methodologies in which MAP may be identified directly in tissue specimens. As reported in the literature, regions in the 1.451-kb IS900 sequence remain to be unique to MAP.[14] Ultimately, these IS900-derived nucleotide sequences may be used as markers for verification of MAP involvement in tissue[23,24] and in milk from patients with CD.[25]

In situ detection of nucleic acid sequences provides a direct visualization of the spatial location of specific sequences that is important for diagnosis of bacterial and viral infections.[26] Confocal scanning laser microscopy (CSLM) is a technique in which the specimen is illuminated pointwise by a focused beam of light. An image is recorded by scanning the beam of light over the specimen, and the reflected or fluorescent light from the specimen is focused onto a small detector aperture. This combination of point illumination and point detection results in a unique "optical sectioning" capability. This optical sectioning makes it possible to record images of thin layers within the specimen without cutting it into slices. By collecting a "stack" of such images from different depths, it is possible to display and quantify the 3-dimensional structure of a specimen.[27] In this study, we have used both fluorescence in situ hybridization using a specific MAP DNA probe and CSLM and 2 rounds of nested polymerase chain reaction (PCR) in an attempt to investigate the presence or absence of MAP DNA in surgical tissue specimens from patients with CD, ulcerative colitis (UC), or non-IBD.

MATERIALS AND METHODS

Tissue Collection

A total of 31 surgical resectioned full-thickness tissue specimens, including 23 tissues from 12 CD subjects (consisting of 12 inflamed and 11 noninflamed tissue), 2 inflamed tissue from 2 patients with UC, and 6 cancerous tissue samples from 6 patients with colon cancer, were included in this study. Coded tissue specimens were obtained at the time of surgery from consented participating subjects following institutional review board regulation. The diagnosis of CD or UC was established following standard clinical, endoscopic, histologic, and radiographic criteria.[28] Disease activity was determined by use of the Harvey–Bradshaw index[29] and/or the clinical impression of the treating physician. The tissue specimens were kindly provided either by Dr. I. Shafran (Florida Hospital, Orlando, Fla.) or by Dr. J. Valentine (University of Florida and Gainesville Veterans Affairs Medical Center, Gainesville, Fla.). Samples representing inflamed and noninflamed areas of the bowel from CD patients were sought. Colonic specimens from UC and other non-IBD patients that are undergoing colectomy or partial colectomy were also sought and used as controls. The tissue samples were examined macroscopically upon arrival, removed from the transport tube, flash frozen, and stored in liquid nitrogen. Table 1 summarizes the demographic data related to subjects including disease history, use of immunosuppressive, and the type of tissue samples used in the study.

Tissue Sectioning and Processing

Tissue specimens were removed from liquid nitrogen, placed in the cryostat, left for 30 minutes to equilibrate at –20°C, placed onto cryostat tissue holders, and embedded in mounting medium (OCT embedding medium; RA Lamb, Apex, NC). A total of 20 serial 20-μm sections from each tissue specimen were cut, placed on 20 microscope slides, and allowed to air dry. All slides containing tissue sections were fixed in 4% PFA (Sigma, St. Louis, Mo.) for 30 minutes at room temperature. Under the same conditions, slides were then washed 3 times in phosphate-buffered saline (PBS; pH 6.8). Tissue sections were permeabilized with 100 μL (20 μg/mL) of Proteinase K (Sigma) and 1% of SDS for 30 minutes at 55°C. Proteinase K was then inactivated with 200 μL of 0.2% glycine (Fisher Scientific, Pittsburgh, Pa.) for 3 minutes, and slides were then washed 3 times in PBS (pH 6.8). Tissue sections were dehydrated in 1 minute washes of 70%, 90%, and 100% ethanol. Sections were then washed for 1 minute with 100% xylene (Fisher Scientific) to remove excess lipids. Tissue sections were rehydrated with 1-minute washes of 100%, 90%, and 70% ethanol and incubated in PBS (pH 6.8) at room temperature for 1 hour. Finally, sections were incubated in 25 mL of prehybridization solution (2× SSC, 20% dextran sulfate, and 50% formamide) for 5 minutes at 50 °C immediately before hybridization. MAP cultured in our laboratory from CD tissue was used as a positive control to optimize the protocol conditions. MAP was grown in mycobacterial growth indicator tube media as described previously.[17] Growth was observed as nonhomogenous turbidity in approximately 8 weeks. A volume of 0.05 mL was aseptically removed from the culture to a sterile 1.5-mL microcentrifuge tube and centrifuged (12,800g) at 10,000 rpm for 10 minutes at 4 °C. The cell pellet was washed 3 times in PBS (pH 6.8) and then resuspended in 0.10 mL of PBS, with 20 μL aliquoted to each slide. Several slides containing thin smear were prepared and then allowed to air dry in a Biosafety class II cabinet for 1 hour. Slides were then heat fixed at 70 °C for 1 hour.

Quality Controls

The fluorescence in situ hybridization (FISH) protocol was optimized earlier in the study by testing sections from normal tissue of colon cancer patients that were spiked with MAP ATCC strain 43015 and tissue sections without the spiking with MAP. The specificity of the IS900-labeled probe was tested using slides containing no bacterial smear, *Escherichia coli*, *Mycobacterium smegmatis*, *Mycobacterium avium* subspecies *avium*, *Staphylococcus aureus*, and MAP.

TABLE 1. Demographic Information and Type of Specimens for Subjects Used in This Study

Subject			Disease					Tissue Specimen	
Sex	Age	Diagnosis	Site	Duration	Type	Surgeries	Immunosuppression	Inflamed	Noninflamed
F	25	CD	Colon	4 yrs	F	2	6MP	X	X*
M	26	CD	Small bowel	9 yrs	I	1	Steroids	X	X
F	68	CD	Bowel–colon	42 yrs	F+S	3	None	X	X
F	56	CD	Bowel–colon	11 yrs	S	1	6MP	X	X
NA	NA	CD	Small bowel	20 yrs	S	1	Steroids	X	X
M	60	CD	Colon	5 yrs	I	0	Steroids	X	X
M	43	CD	Small bowel	14 yrs	I+F+S	1	Steroids	X	X
F	39	CD	NA	19 yrs	I+F+S	3	Infliximab, steroids	X	X
F	54	CD	Bowel–colon	4 yrs	S	2	Steroids, 6MP	X	X
M	21	CD	Small bowel	12 yrs	S	1	Steroids	X	X
M	25	CD	Bowel–colon	8 yrs	F+S	2	Steroids, 6MP	X	X
F	40	CD	Bowel–colon	1 yr	S	1	Infliximab, steroids	X	NA
M	54	UC	NA	NA	NA	1	None	X	NA
M	42	UC	NA	NA	NA	1	None	X	NA
F	79	Non-IBD	NA	NA	NA	1	None	NA	X
NA	NA	Non-IBD	NA	NA	NA	1	None	NA	X
M	64	Non-IBD	NA	NA	NA	1	None	NA	X
M	73	Non-IBD	NA	NA	NA	2	None	NA	X
F	26	Non-IBD	NA	NA	NA	1	None	NA	X
M	71	Non-IBD	NA	NA	NA	1	None	NA	X

*X, tissue specimen available.

CD indicates Crohn's disease; UC, ulcerative colitis; non-IBD, non-inflammatory bowel disease subjects; M, male; F, female; 6MP, 6-mercaptopurine; NA, not available; S, stricturing state; F, fistulizing state; I, inflammatory state.

Probe Preparation and Hybridization

A 20-mer MAP specific oligonucleotide probe (5′-CTTCGGGGCCGTCGCTTAGG-3′) derived from the IS900 sequence was labeled at the 5′-end with fluorescein (Invitrogen, Carlsbad, CA). Aliquots of 1 µg/uL were stored at –20 °C. One hundred nanograms of fresh probe were mixed with 20 µL of hybridization solution [1% Triton X-100, 2X SSC, denatured sperm DNA (500 µg/mL), 10% dextran sulfate, and 50% deionized formamide], boiled for 10 minutes, and placed on ice for 10 more minutes. The hybridization mixture was added to the tissue sections and placed inside a hybridization chamber (Corning Inc., Acton, Mass.) overnight at 37 °C. After 24 hours, tissue samples were removed from the chamber and extensively washed. The first wash was with 2× SSC for 15 minutes at room temperature, followed by another wash with 1× SSC under the same conditions. The last 2 washes were performed with 0.3× SSC, 1 for 15 minutes at 40 °C, and the other 1 for 15 minutes at room temperature. Slides were then incubated in 40 mL of counterstain solution containing 40 ng of propidium iodide (Sigma) for 15 minutes at room temperature in the dark. Slides were then washed 3 times with sterile water each time for 5 minutes at room temperature and air-dried in the dark. Slides were then mounted with antifade solution (Molecular Probes, Eugene, Ore.) before storage to preserve the fluorescence.

CSLM Analysis

A Carl Zeiss LSM 510 system was used in this study (Carl Zeiss Inc., New York, NY). Slides were examined to localize the targets on the slide by using transmitted-light. Once the target was found, 2 simultaneous confocal channels were used for reflection and fluorescence. The detector on the microscope was set to include light from the negative control and was kept constant for all test samples. All variables, such as photometric gain, photomultipler setting, pixel size, objective size, and number of scans were recorded for each scan and were used comparatively for sample slides. Using the Zeiss image analysis software, a threshold was applied to eliminate pixels with no fluorescence (black) and residual noise from the detector. Images with relevant data were saved for documentation.

Statistical Analysis

Groups were compared in using 2 × 2 contingency tables and the Fisher exact test. Significant data had a P value < 0.05.

Genomic DNA Extraction and Nested PCR Analysis

Approximately 500 mg of fresh tissue was diced into fine pieces using sterile disposable scalpels. The tissue sample was resuspended in 4.0 mL of lysis buffer (2 mM ethylenediaminetetraacetic acid; 400 mM NaCl; 10 mM TrisHCl, pH 8; 100 μg/mL Proteinase K; and 0.6% sodium dodecyl sulfate) and homogenized by an Omni Tip disposable generator probe (Fisher Scientific) for 1 minute at 30,000 rpm. The tissue was then incubated with shaking (200 rpm) at 37°C for 3 hours, transferred to disposable FastPrep lysing matrix B blue cap tube (Qbiogene, Carlsbad, Calif.), and ribolysed at 6.5 m/s^{-2} for 45 seconds by using a FastPrep ribolyser (Qbiogene). The lysates were placed on ice immediately for 15 minutes. The DNA was then isolated by phenol/chloroform/isoamyl alcohol and ethanol precipitation. DNA pellet was dissolved in 250 μL of TE buffer. The MAP-specific IS900 DNA element was evaluated in triplicate using a nested primer PCR technique. Briefly, oligonucleotide primers P90 (5′-GTT-CGG-GGC-CGT-CGC-TTA-GG-3′) and P91 (5′-GAG-GTC-GAT-CGC-CCA-CGT-GA-3′) were selected to amplify a unique 398-bp fragment of 5′ region of IS900. To reliably detect MAP, AV1 (5′-ATG-TGG-TTG-CTG TGT-TGG-ATG-G-3′) and AV2 (5′-CCG-CCG-CAA-TCA-ACT-CCA-G-3′) were used for the nested PCR in the second round using the amplified fragment as a template to amplify a 298-bp internal sequence. The PCR mixture of 50 μL consisted of 1× PCR buffer, 5 mM $MgCl_2$, 0.2 mM dNTP, 6% DMSO or 0.5 M Betaine, 2 μM primers, and 2.5 μm of Platinum Taq polymerase (Invitrogen) or 1 μm of TFL DNA polymerase (Promega, Inc., Madison, Wis.) and 10 μL of DNA template. Five microliters of DNA template was used in first around PCR in 50 μL, and 1 μL of PCR product from first round was used in second round PCR in 50 μL. The PCR cycling conditions were as follows: 95 °C for 5 minutes, 40 cycles of 95 °C for 1 minute, 58 °C for 1 minute, 72 °C for 3 minutes; and a final extension of 5 minutes at 72 °C. The amplification product size was determined on an agarose gel. Some of the nested PCR products were gel purified and sequenced by the University of Florida DNA Sequencing Core facility confirming that the product was identical to the MAP IS900 sequence.

RESULTS

A total of 20 patients who underwent for surgery for resection of bowel tissue were enrolled in this study between July 2002 and January 2004. Twelve patients received diagnoses of CD, 2 of UC, and 6 of colon cancer (non-IBD). The median age was 41 years for CD patients and 52 years for non-CD patients equally distributed between male and female patients. The data in Table 1 show that 11 of 12 patients (92%) with CD were receiving immunosuppressive medications; neither of the 2 patients with UC nor the 6 non-IBD control subjects was on immunosuppressive medications.

Specificity of the IS900-Based FISH

Using slides containing cultures representing MAP, *M. smegmatis*, *M. avium* subspecies *avium*, *S. aureus*, and *E. coli* were used to test the specificity of the FITC-labeled IS900-derived DNA probe. As shown in Figure 1, the presence of MAP was detected as a yellow color indicating the colocalization of the FITC-green fluorescence labeled-IS900 probe hybridized with corresponding MAP DNA present in the bacterial samples (E). Slides containing *E. coli* (A), *S. aureus* (B) and *M. smegmatis* (C), and *M. avium* subspecies *avium* (D) cultures were negative against the FITC-labeled IS900 probe. Hybridization signals with MAP DNA were detected mostly in clumps distribution, a typical characteristic of mycobacterial morphology after microscopic staining. Slides without any bacterial smear were blank (data not shown). All the variables mentioned, including various dilutions of bacterial cells and probes, were repeated several times with duplicate slides in every attempt. The results remained consistent at all times. After several attempts for adjustment of variables, parameters were identified and then employed during this study.

MAP was absent in slides containing sections from normal tissue of a colon cancer patient when analyzed using the optimized protocol described earlier (Fig. 2A). However, MAP in clumps was clearly visualized in slides with tissue sections spiked with MAP culture (Fig. 2B).

Detection of MAP DNA in Tissue by FISH and CSLM

Slides containing CD and non-CD tissue sections were used, and the presence of MAP was detected as a yellow color indicating the specific hybridization of the green fluorescent MAP probe with the MAP DNA present in the red-stained human tissue samples. For the isotypic control slides, PBS was used instead of MAP probe to establish the threshold to eliminate residual noise during image analysis. In 12 CD patients tissue, MAP DNA was detected in 7/12 (58%) of inflamed, 4/11 (36%) of noninflamed, and in 8/12 (67%) of either tissue type (Table 2). Figure 3 illustrates representative CSLM images of tissue sections from CD patients after hybridization with the IS900 probe; MAP DNA was detected only in inflamed tissue (A:I), only in noninflamed tissue (B:N), and in both inflamed and noninflamed tissue (C:I and C:N). MAP infiltration was observed in 3 patients with CD since both tissue types contained MAP DNA. On the contrary, MAP DNA was absent in both Tissue types in 2 patients with CD. As shown in Figure 4, MAP DNA was not detected in tissue sections from all 6 non-IBD patients (A to F). There was no tissue available from patients with UC for this analysis. Detection of MAP DNA by FISH in 67% of CD tissue compared with zero in non-CD tissue is significant ($P < 0.005$).

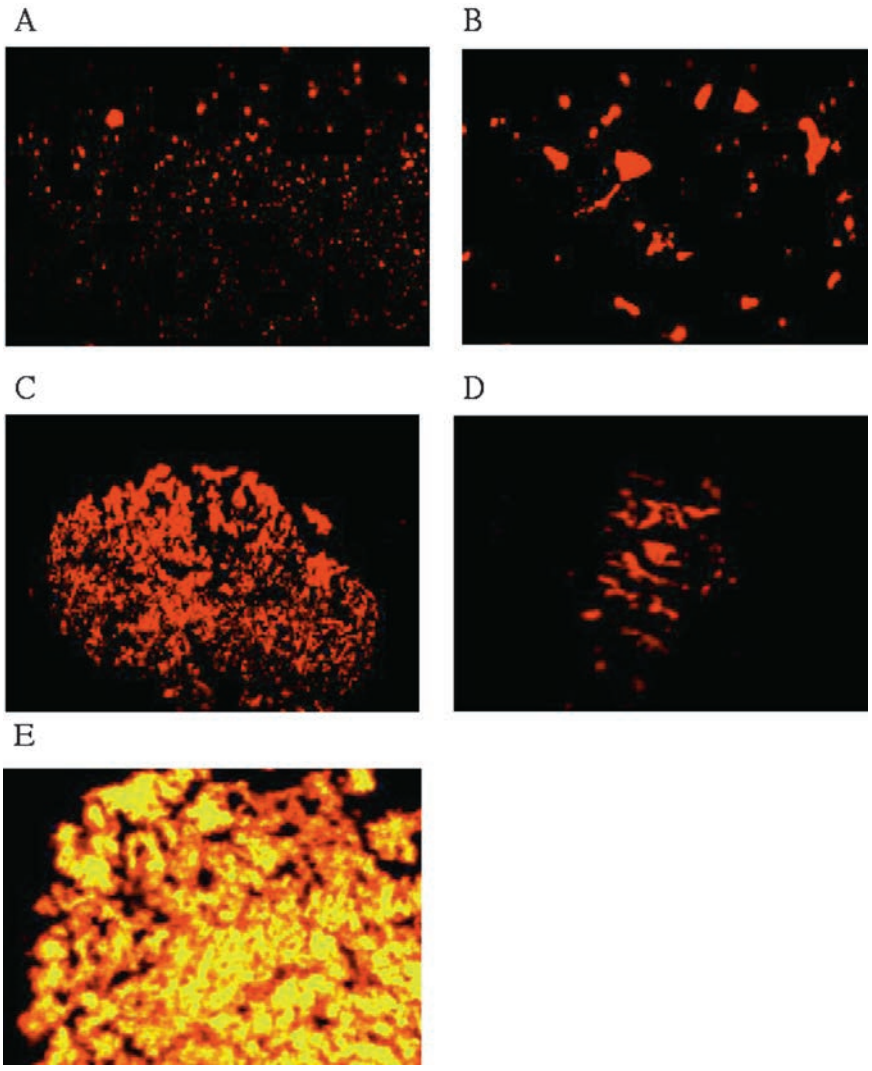

FIGURE 1. Specificity of IS900-derived FITC-labeled DNA probe. The specificity of the DNA probe was evaluated against culture smears from *E. coli* (A), *S.* (B), *M. smegmatis* (C), *M. avium* subspecies *avium* (D), and MAP (E). The presence of MAP was detected as a yellow color, indicating the colocalization of the green FITC-labeled IS900 probe specific for MAP DNA hybridized with corresponding genomic MAP DNA present in the bacterial samples using CSLM. Slides containing non-MAP bacterial culture stained with the propidium iodide (red color) indicating negative colocalization with the fluorescent IS900 probe.

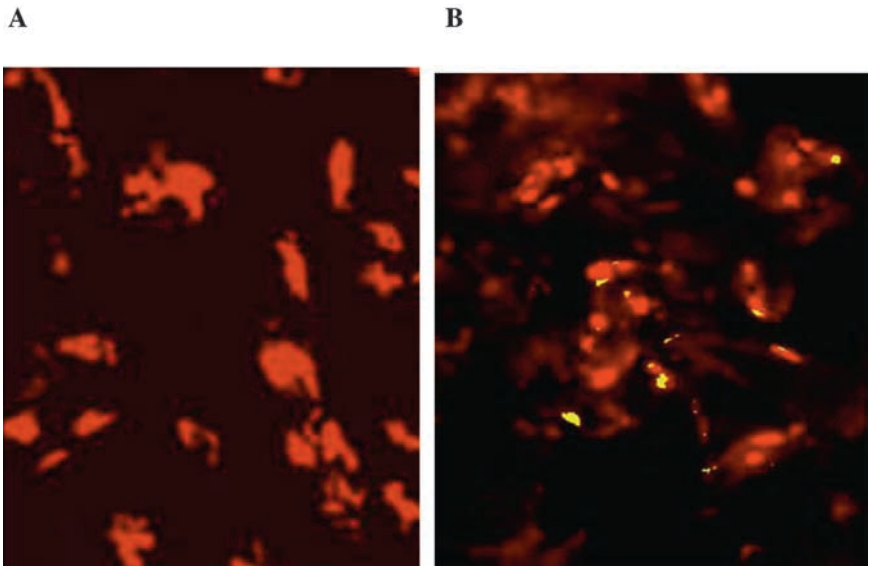

FIGURE 2. Detection of MAP in spiked tissue by in situ hybridization. The optimized in situ hybridization technique was evaluated after the analysis of slides with normal tissue sections from a colon cancer patient (A) and slides with same tissue spiked with MAP culture (B) as visualized by CSLM. The presence of MAP was detected as a yellow color, indicating the colocalization of the green FITC-labeled IS900 probe with MAP DNA if present. The red staining observed corresponds to tissue stained with the propidium iodide.

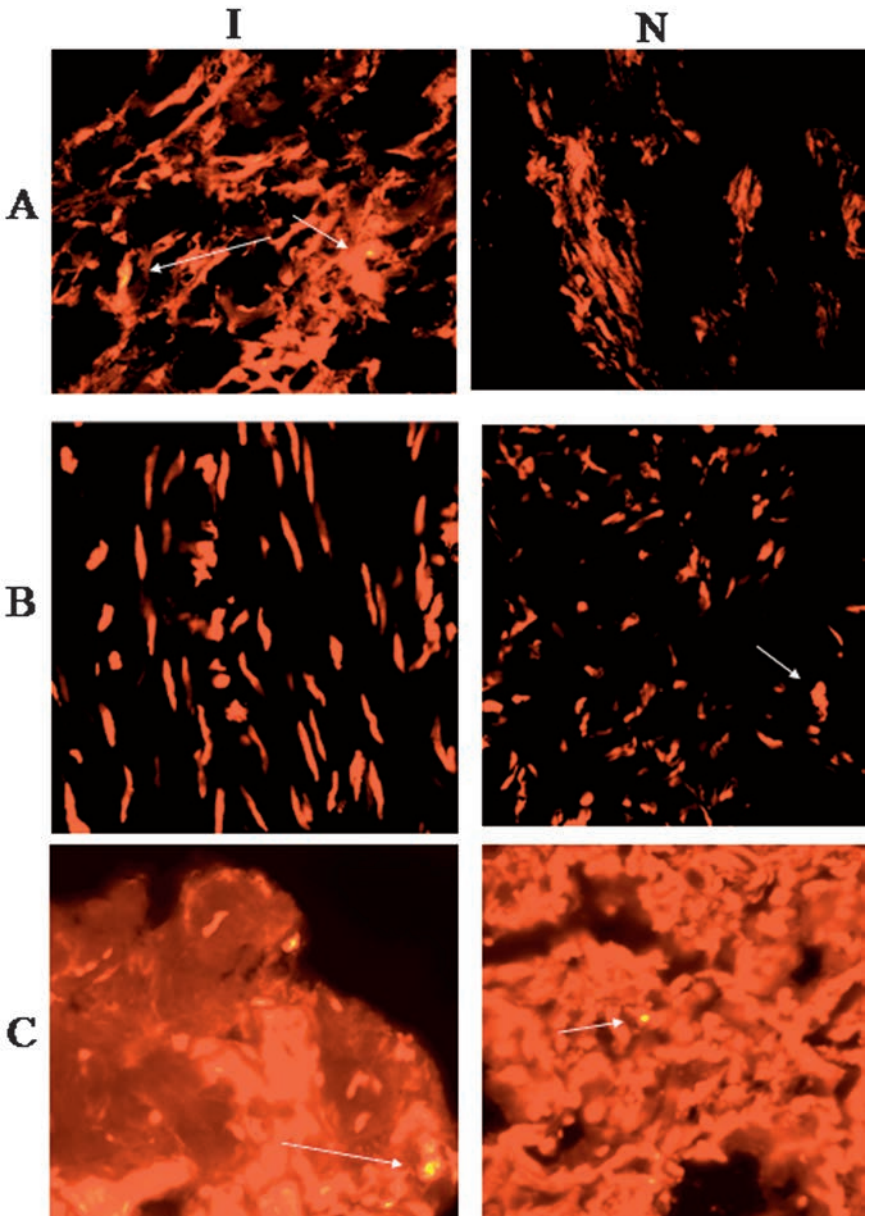

FIGURE 3. In situ identification of MAP DNA in CD tissue samples. CSLM images illustrate the presence or absence of MAP in tissue from 3 patients with CD (A, B, and C). I corresponds to inflamed tissue and N corresponds to noninflamed tissue. The presence of MAP was detected as a yellow color, indicating the colocalization of the green FITC-labeled IS900 probe with MAP DNA if present using CSLM. The red staining observed corresponds to tissue stained with the propidium iodide.

Detection of MAP DNA in Tissue by Nested PCR

For low levels of MAP DNA to be specifically detected, the second round of the nested PCR technology was used in this study, which amplifies an internal 298-bp fragment from a template of 398-bp fragment amplified in the first round of the PCR. An intense bright band has been observed in all positive results. The amplified fragment was confirmed to be IS900 by nucleotide sequencing. In this study, a total of 31 DNA extracts isolated directly from homogenized 31 tissue samples were analyzed. As shown in Table 2, MAP DNA was detected in tissue from 10 of 12 patients (83%) with CD, 2/2 (100%) inflamed tissue from 2 patients with UC compared with 1/6 (17%) of non-IBD subjects ($P < 0.005$). Interestingly, MAP DNA in CD tissue was detected in 7 inflamed samples (Figs. 5A, lanes 4, 8, 10, and 14, and 5B, lanes 4, 6, 8) and in 6 noninflamed samples (Fig. 5A, lanes 3, 5, 11, 13, and 15, and 4B, lane 3). MAP DNA in non-IBD tissue was detected in two tissue samples (Fig. 5B, lanes 9 and 10).

DISCUSSION

Investigating the association of MAP or other agents in CD etiology remains a source of a major debate among investigators. The data reported by those supporting the association of MAP with CD etiology is credible, and their argument has merit. However, it is also responsible to be objective regarding

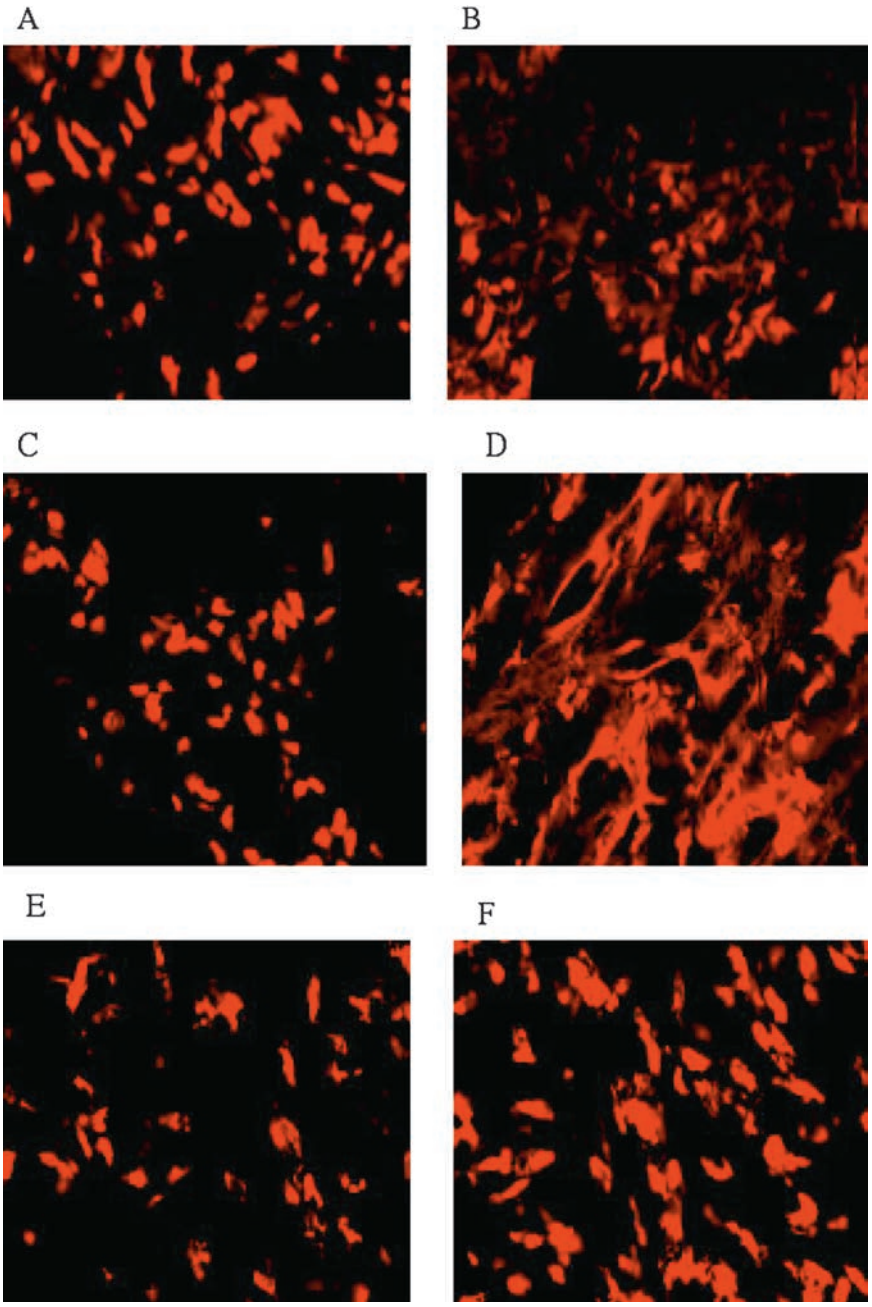

FIGURE 4. In situ identification of MAP DNA in non-IBD tissue samples. CSLM Images illustrate the absence of MAP DNA in tissue from all 6 non-IBD patients (A–F). MAP DNA, if present, is visualized as a yellow color, indicating the colocalization of the green FITC-labeled IS900 probe with MAP genome using CSLM. The red staining observed corresponds to tissue stained with the propidium iodide.

other studies, which reported the possible involvement of other factors in the etiology of CD. Variation in the selection and use of methodology is the underlining cause for the mixed outcome in these studies. Looking carefully at the data reported in the present study, we conclude that there is more evidence of the role of MAP in CD etiology. The debate may vary with regard to the percentage of CD cases associated with MAP. Attempts to culture MAP from tissue samples from CD patients have been tried in the past. The outcome depended significantly on the technology used and the specimen selection and processing. In our laboratory, we reported previously the isolation of MAP from full-thickness tissue samples obtained from patients with CD after short-term incubation in mycobacterial growth indicator tube culture media.[17] Using the same culture media, we isolated MAP from breast milk from 2 CD lactating mothers.[25] In both reports IS900-based PCR was used to verify MAP presence in the culture. This was recently confirmed in Hermon-Taylor laboratory, in which 92% of intestinal biopsy specimens were positive for MAP compared with 26% in control subjects.[24] The nature of MAP or the pro-

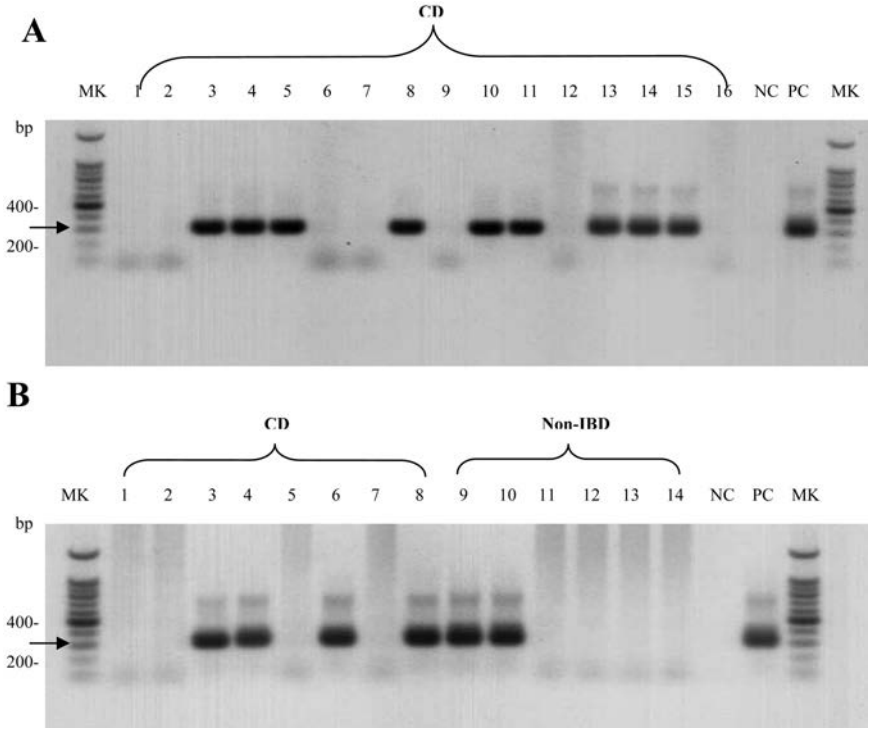

FIGURE 5. PCR detection of MAP DNA in surgical tissue samples. PCR product from the second round of the nested PCR was analyzed on 2% agarose gel. An amplified 298-bp fragment indicates positive sample for MAP DNA. CD represents tissue samples from CD patients whereas non-IBD represents tissue from non-inflammatory bowel disease (colon cancer patients). NC, negative control; PC, positive control; MK, molecular weight marker. Lanes with odd numbers represent DNA from Inflamed tissue and even numbers represent those from noninflamed tissue.

posed virulence form of MAP (a cell wall-deficient form of cell called spheroplast) in CD tissue is significantly different from those in animals with JD. Therefore, using technology designed for isolation of the bacillary form of MAP as in JD sources may not be applicable for use in humans with CD. That alone will play a major factor in the outcome of the study. This led us to use in this study a direct analysis, which investigates the presence or absence of MAP DNA in tissue. We used 2 sequential rounds of nested PCR, on DNA preparations extracted directly from tissue samples, to substitutes for Southern hybridization. The use of FISH with the aid of CSLM may provide physical evidence of the MAP bacterium in human tissue and thus may become essential in investigating any link between MAP and CD. The FISH methodology used in this study has been carefully optimized to provide significant sensitivity and specificity to the MAP bacterium. As shown in this study, spiking of normal tissue with MAP culture was clearly evident in the slides after our protocol (Fig. 2B). When coded tissue sections were investigated using FISH, MAP presence was easily reported. The data shown in Figure 3 illustrate that MAP may be present in inflamed tissue, in neighboring noninflamed tissue, or in both. Although we accept that a possible sampling error during tissue collection and coding may have occurred, we believe that the presence of MAP in inflamed tissue only in patients with CD may reflect on MAP involvement with the stages of the disease. The detection of MAP DNA in noninflamed tissue but not in the inflamed tissue samples may be explained by several scenarios, including sampling error, poor inflamed tissue quality, or low number and diversity in the distribution of MAP in the inflamed tissue. The detection of MAP in both the inflamed and noninflamed tissue samples suggests that MAP infiltrates host tissue as reported in animals with JD.[30] The finding that MAP DNA was identified in tissue from more patients with CD than non-IBD subjects would be consistent with an active infection in association with CD and a state of dormancy, well-recognized in other mycobacteria or colonization in the absence of CD disease. Alternatively, non-IBD subjects may more effectively kill MAP after exposure. Host susceptibility, either genetic, immunologic, or both may be essential for MAP to survive phagocytosis and oxidative bursts. This finding also supports a very recent report, in which viable MAP was cultured from peripheral blood in 50% of patients with CD.[31] On the contrary, MAP may be absent in some patients with CD, as we reported in this study where MAP DNA was not detected in tissue by either technique. It is also of significant importance to report that MAP may be present in some patients with UC, as reported in a previous study in which pleomorphic variable acid-fast organisms were isolated from more than 50% of patients with UC.[32] Furthermore, Mishina et al[16] reported that 2 of 4 UC tissues were MAP positive by real-time PCR. In this study, 2 inflamed UC tissues had MAP DNA detected by nested PCR. We regret that FISH was not performed on the UC tissues samples because of the small size of tissue samples. MAP in tissue from patients with UC may be explained as the result of (1) IBD misdiagnosis, which is well acknowledged in

TABLE 2. Detection of MAP DNA in Tissue by FISH Using CSLM and Nested PCR

	Inflamed Tissue		Noninflamed Tissue		Either Tissue	
Diagnosis	**FISH**	**PCR**	**FISH**	**PCR**	**FISH**	**PCR**
CD	Positive	Negative	Positive	Negative	Positive	Negative
CD	Positive	Positive	Positive	Positive	Positive	Positive
CD	Positive	Positive	Positive	Negative	Positive	Positive
CD	Negative	Negative	Positive	Positive	Positive	Positive
CD	Positive	Negative	Negative	Positive	Positive	Positive
CD	Negative	Positive	Negative	Negative	Negative	Positive
CD	Negative	Positive	Negative	Positive	Negative	Positive
CD	Positive	Positive	Negative	Negative	Positive	Positive
CD	Negative	Negative	Negative	Negative	Negative	Negative
CD	Positive	Positive	Negative	Positive	Positive	Positive
CD	Negative	Negative	Negative	Positive	Negative	Positive
CD	Positive	Positive	NA	NA	Positive	Positive
Total = 12	7/12 (58%)	7/12 (58%)	4/11 (36%)	6/11 (55%)	8 (67%)	10 (83%)
UC	NA	Positive	NA	NA	NA	NA
UC	NA	Positive	NA	NA	NA	NA
Total = 2		2 (100%)	NA	NA	NA	2 (100%)
Non-IBD	NA	NA	Negative	Positive	Negative	Positive
Non-IBD	NA	NA	Negative	Negative	Negative	Negative
Non-IBD	NA	NA	Negative	Negative	Negative	Negative
Non-IBD	NA	NA	Negative	Negative	Negative	Negative
Non-IBD	NA	NA	Negative	Negative	Negative	Negative
Non-IBD	NA	NA	Negative	Negative	Negative	Negative
Total = 6			0 (0%)	1 (17%)	0 (0%)	1 (17%)

CD indicates Crohn's disease; UC, ulcerative colitis; NA, not available; Non-IBD, noninflammatory bowel disease subjects.

the gastroenterology community, (2) a possible mixed infection as reported recently,[33] or (3) a possible need for the reclassification of IBD as 1 disease with 2 forms, as seen in tuberculosis and leprosy.[3] With regard to tissue from non-IBD patients, MAP was absent in tissue using FISH.

Despite the need for visible number of MAP in the tissue to be visualized by CSLM, the majority of CD tissue contained MAP DNA compared with none in the non-IBD controls. Because of this concern related to the strength of MAP signal detected by FISH and recorded by CSLM in tissue, we used 2 rounds of nested PCR on DNA extracts purified directly from same tissue samples. The PCR data clearly confirmed the data obtained by FISH. Furthermore, nested PCR detected MAP DNA in those samples with a weaker MAP presence (83% of CD tissue contained MAP DNA by PCR compared with 67% by FISH). The distribution of MAP in intestinal tissue is not homogenous, which may explain the results in which some CD tissue samples were positive by FISH and not by PCR or were positive in the noninflamed tissue sections and not in the inflamed tissue sections despite our effort in performing multiple PCR analysis on different sections of the tissue and by extensive scanning of multiple slides by CSLM when sample size allow. This is a clear example of why some reports concluded a lack of MAP DNA presence in tissue from CD patients whereas others confirmed the opposite.

Finally, these data provide further evidence of a possible role of MAP in CD etiology. This study provides further choices of the type of methodology to be used in investigating the link between CD and MAP. Moreover, there is a possibility that exposure to MAP could occur through environmental fecal contamination or from the milk or blood from JD-infected animals.[34,35] Because of the relatively small size of subjects population used in this study, we could not determine any association between use of immunosuppressant medication and the presence of MAP DNA in CD tissue. Regarding the viability of MAP in CD tissue, studies in our laboratory are underway to address such concerns. Sections of the tissue samples used in this study have been homogenized, decontaminated, and inoculated into mycobacterial culture media. The outcome of the microbiology study is pending due to the fastidious nature of MAP and the need for long-term incubation. Additionally, we are in the process of using real-time PCR analysis on

these tissues to overcome the challenges associated with semi-unculturable nature of MAP in hope that may substitute for future cultivation need. The outcome of the study clearly suggests that a larger scale investigation is needed. This includes analyses of tissue samples from different onset disease patients, including those with CD, UC, and non-IBD who are going under different course of drug therapy.

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