ORIGINAL ARTICLE

Use of short-term culture for identification of *Mycobacterium avium* subsp. *paratuberculosis* in tissue from Crohn's disease patients

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Objective To investigate the role of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in Crohn's disease (CD), using short-term mycobacterial culture media.

Methods Sixty-three tissue specimens from 27 CD patients and 36 controls were processed and inoculated into a modified 7H9 broth base medium and incubated at 37 °C and 5% CO_2 for up to 1 year. Acid-fast staining, determination of mycobactin dependency, PCR analysis using two IS900-derived oligonucleotides and hybridization with an internal probe were performed.

Results MAP was present in six of seven (86%) surgically resected tissue samples and in four of 20 (20%) biopsies, with an overall 37% from CD patients, as compared to two of 36 (5.6%) of control specimens. The presence of MAP in Mycobacterial Growth Indicator Tube (MGIT) cultures was detected within 10–12 weeks for surgically resected tissue and after 40 weeks for biopsy specimens, with no MAP growth detected in 12B* Bactec cultures.

Conclusions Because MAP was present in 86% of resected tissue compared to 20% of biopsy specimens from CD patients, we speculate that MAP resides in the submucosal layer closer to the active part of the ulcer rather than on the surface of the mucosal cells. Thus, surgically resected tissue cultured in MGIT medium is a favorable protocol for rapid cultivation of MAP and for investigating its role in CD pathogenesis. The data support the mycobacterial role in CD pathogenesis.

Keywords Crohn's disease, *Mycobacterium*, PCR, culture, milk

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INTRODUCTION

Crohn's disease (CD) is a gastrointestinal disorder associated with progressive thickening of the bowel wall caused by fibrosis and narrowing of the lumen. Proliferative CD leads to changes in the mucous membrane, giving it a cobblestone appearance and causing varying degrees of ulceration [1, 2]. Histologically, there are granulomas and extensive lymphocytic infiltrates in the submucosa associated with microfistula formation [3]. The disease emerged perceptibly in Western Europe and North America in the late 1940s and early 1950s. The incidence then increased progressively in both continents to a level which, in some areas such as northeast Scotland (11.6/100 000 per year), now approaches that of an epidemic [4]. Granulomas and lymph node alterations in CD patients resemble those of tuberculosis, leprosy, sarcoidosis and bovine paratuberculosis [5–8]. Bovine paratuberculosis (also known as Johne's disease (JD) is a gastrointestinal disease in ruminants and primates caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The anatomic and pathologic distribution as well as the signs and symptoms of both CD and JD are largely shared [9]. Most recently, MAP has been identified in commercial pasteurized milk, raising speculation that CD may be associated with consumption of contaminated cattle milk [10].

The association of MAP with CD has been investigated more intensively in recent years. Several studies reported that a cell-wall-deficient form of MAP had been isolated from tissues of some CD patients instead of intact bacilli as in JD [11, 12]. These studies also reported that MAP was present in tissues of CD patients to a much greater extent than in controls. Similarly, anti-MAP antibodies have been reported to be pre-

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sent in sera from CD patients at a higher level than in controls. The serology studies were done using recombinant antigens and cytoplasmic antigens from MAP [13–15]. In contrast, some studies reported that MAP was absent or present in some tissues of CD patients at levels similar to those in controls [16]. Consequently, MAP's role in CD pathogenesis became controversial.

Cultivation of MAP from tissue specimens, even from JD animals, is usually associated with many obstacles. This, in part, is because MAP is a fastidious and mycobactin-dependent bacillus with a generation time of over 22 h. The spheroplastic form of MAP as reported in tissues from CD patients would present more obstacles to its isolation in a culture medium. In fact, cultivation of MAP spheroplasts would require special media supplemented with additives that support the difference in the osmotic pressure between the intra- and extracellular environments. Despite that, many studies have reported the isolation of MAP from tissue specimens of CD patients, but with variable percentages [17, 18]. The insertion element IS900 has been the standard tool for genotyping the cultured isolates either by PCR or hybridization. IS900 is an insertion element of 1451 bp that is present in multiple copies within the genomic DNA of MAP [19]. Additionally, the IS900-based PCR assay has also been used to investigate uncultured tissue from CD patients for the presence of MAP [20]. This technique has been criticized for failing to differentiate between viable and metabolically inactive MAP cells. Thus, cultivation of MAP using special culture media from tissue, followed by microscopic, mycobactin dependency and PCR and hybridization analyses, would be the appropriate approach to investigate the role of MAP in the pathogenesis of CD.

The objectives of this study are to identify a culture medium that supports the growth of the spheroplastic form of MAP and to investigate the presence of MAP in tissue from CD patients.

PATIENTS AND METHODS

Specimens and growth conditions

In total, 63 tissue specimens, from 27 CD patients (seven surgically resected tissue samples and 20 microscopic biopsies) and 36 controls (three surgically resected tissue samples and 33 microscopic biopsies from normal (N), non-specific colitis (NSC) and ulcerative colitis (UC) patients) were used in this study (Table 1). All tissue specimens designated by a code were collected at Florida Hospital (Orlando, Florida) and kindly provided by Dr Ira Shafran (Florida Hospital, Orlando, Florida). Tissue specimens preserved in sterile saline were transported in a NovaChamber cooling system (NovaSense, Inc., Orlando, Florida, USA) to the University of Central Florida.

Table 1 Summary of MAP presence in tissue specimens used in this study

Source	Number and percentage of MAP in tissue		
	Biopsy	Surgically resected	Overall
Crohn's disease	4/20 (20%)	6/7 (86%)	37%
Controls	2/33 (6%)	0/3 (0%)	5.6%
Normal	1/13 (7.7%)	0/2 (0%)	–
Non-specific colitis	1/7 (14%)	0/0 (0%)	–
Ulcerative colitis	0/13 (0%)	0/1 (0%)	–
Total	53	10	63

They were processed immediately in a Biosafety Cabinet class II for culture analysis. One gram from surgically resected tissue specimens or the entire biopsy was transferred to sterile 50-mL centrifuge tubes; this was followed by grinding with a disposable 50-mL tube grinder (Fisher Scientific, Pittsburgh, PA, USA) for a few minutes. A volume of 5.0 mL of NACL solution (NaOH–*N*-acetyl-L-cysteine) was added to the ground tissue in the tube, as described by the manufacturer. The tube was vortexed for 1 min and then left to stand at room temperature for 15–20 min with occasional swirling. Phosphate buffer solution (PBS, pH 6.8) was then added to the 50-mL mark. After thorough mixing, the tube was centrifuged at 3000 rev/min for 20 min. The supernatant was decanted and the sediment was washed twice in 1.0 mL of PBS at 10 000 rev/min and 4 °C. The washed pellet was dissolved in 1.0 mL of PBS. Volumes of 0.5 mL were used to inoculate one Mycobacterial Growth Indicator Tube (MGIT) and one 12B★ Bactec bottle (Becton Dickinson, Pittsburgh, PA, USA). Both culture media were supplemented with OADC (oleic acid, bovine albumin, dextrose, catalase) enrichment, mycobactin J (2 mg/L, Allied Monitor, Fayette, MO, USA) and PANTA antibiotics mixture (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) as described by the manufacturer (Becton Dickinson). Although both culture media are derivatives of 7H9-broth base, MGIT medium contains, in addition, L-asparagine, pyroxidine, trace elements, biotin and glycerol. All inoculated media were incubated at 37 °C and 5% CO_2. The Bactec bottles were read using the Bactec 460 TB analyzer, and the MGIT tubes were read using a 365-nm UV-illuminator, as described by the manufacturer. All negative cultures were incubated for 1 year.

Identification of MAP

Microscopic staining and DNA extraction

An aliquot of 1.0 mL of culture suspension was transferred to a sterile 1.5-mL centrifuge tube. Following centrifugation at

10 000 rev/min for 5 min, the supernatant was discarded and the pellet was washed three times with PBS (pH 6.8). Each pellet was suspended in 500 μL of TE buffer (10 mM Tris; 0.1 mM EDTA, pH 8.0) and then heated at 80 °C for 10 min to deactivate the cells. The sample was centrifuged at 10 000 rev/min for 5 min and the supernatant was discarded. The cell pellet was suspended in 150 μL of sterile distilled water. A volume of 50 μL was used to prepare a microscopic slide smear for staining with Kinyoun acid-fast stain [21]. The remaining volume was used for extraction of genomic DNA for PCR analysis as described previously [22]. The cell suspension was adjusted to 200 μL with Perkin-Elmer DNA Extraction Reagent as described by the manufacturer. The tube was heated at 56 °C for 30 min, mixed again, and then heated at 100 °C for 10 min. The cell lysate was mixed, centrifuged at 15 000 rev/min and 4 °C for 3 min, and then transferred to a new tube and used immediately for PCR or stored at −20 °C until used.

PCR detection and southern hybridization

Each DNA extract was subjected to PCR analysis for the presence of the IS900 amplified fragment, as described previously [22]. The two oligonucleotide primers used for PCR amplification of a unique 400-bp region of IS900 (position 22–421) were P90 (5′GTTCGGGGCCGTCGCTTAGG-3′) and P91 (5′-GAGGTCGATCGCCCACGTGA-3′). The PCR reaction consisted of 50 μL, containing 25 μL of DNA template and 25 μL of the PCR reaction mixture (20 mM Tris, 100 mM KCl (pH 8.3), 0.6 mM each dNTP, 2 ng of each primer, 5 mM $MgCl_2$, and 2.5 units of *Taq* polymerase). The reaction mixtures were then subjected to PCR amplification using a Robocycler Gradient 96 temp Cycler (Stratagen, La Jolla, CA, USA). The program used included 5 min of denaturation at 95 °C, followed by 35 cycles of amplification (2 min at 94 °C, 2 min at 55 °C and 3 min at 72 °C), followed by a 10-min extension at 72 °C and a 4 °C indefinite hold until retrieved. Appropriate controls were used in parallel with each PCR experiment, including a DNA template from MAP strain ATCC 43015 as positive control. PCR products were analyzed on 2.0% agarose gel following standard procedures [23]. The gel was photographed, and the amplified fragments were then transferred overnight onto nylon membranes (Hybond-N+, Amersham Life Science Inc., Arlington, IL, USA) by the alkaline transfer method using 0.4 M sodium hydroxide as described previously [22]. Hybridization was performed using a specific MAP probe consisting of a 251-bp DNA fragment isolated and kindly provided by Professor Hermon-Taylor (Department of Microbiology, University of Surrey, Guildford, UK) through Dr Fouad El-Zaatari (Baylor College of Medicine, Houston, Texas, USA). This probe, located at the 5′-region of the insertion element IS900 at position 163–414 [22], was labeled by the hexanucleotide priming technique with digoxigenin-11-dUTP, using the Genius labeling kit and following the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN, USA).

RESULTS

Although microbial growth was observed in some cultures of both media, only MGIT cultures were positive for the presence of MAP. All Bactec cultures were negative for the presence of MAP during the course of this study. Overall, MAP was present and confirmed in 10 of 27 (37%) CD tissue specimens (6/7 (86%) surgically resected tissue specimens and 4/20 (20%) of the biopsies), compared to two of 36 (5.6%) controls (2/33 (6%) biopsies and none in the three resected tissue specimens), as summarized in Table 1. Positive cultures for MAP were confirmed to be acid-fast positive and mycobactin dependent when subcultured in the presence and absence of mycobactin J. Figure 1 shows one of the acid-fast-positive cultures from early cultivation of CD resected tissue specimens. Interestingly, two forms of MAP were observed: the prespheroplast form with a partial cell wall compartment (Figure 1A) and the bacillus intact cell form (Figure 1B). Additionally, and as shown in Figure 2, a 400-bp amplified PCR fragment from IS900 analyzed on 2% agarose gel was present in six of seven resected tissue specimens from CD patients (lanes 3–9) and not in resected

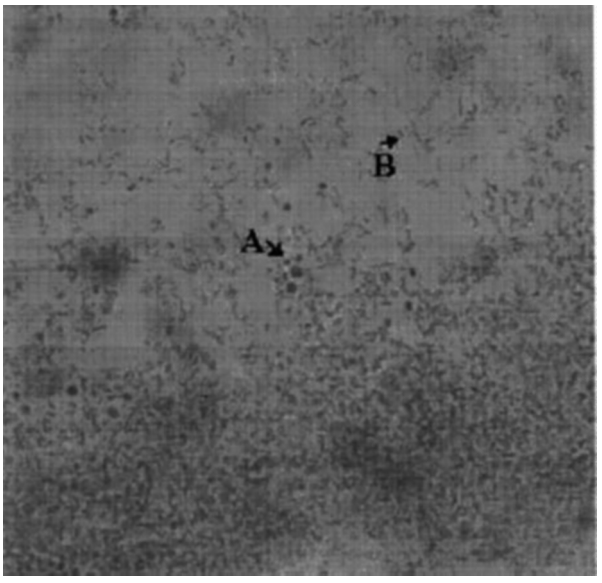

Figure 1 Microscopic analysis of short-term culture of a resected tissue specimen from a CD patient. Acid-fast stain was performed on a culture sample obtained following short-term incubation of processed CD tissue. (A) Corresponds to the prespheroplastic form of MAP with partial cell wall materials. (B) Corresponds to the intact bacillus form of MAP.

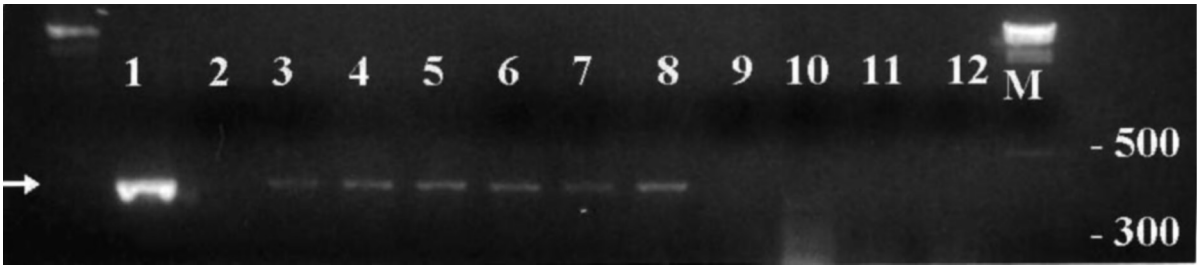

Figure 2 IS900–PCR detection of MAP from surgically resected tissue cultured in MGIT media. PCR products were analyzed on 2.0% agarose gel. Lane 1 represents DNA from MAP strain 43015. Lane 2 has no DNA. Lanes 3–9 represent resected tissue from CD patients. Lanes 10–12 represent tissue from controls. M is the molecular weight marker in base pairs (bp).

tissue from the controls (lanes 10–12). The specificity of the fragment was confirmed by hybridization using the 251-bp internal probe (data not shown). The presence of MAP in positive cultures was reconfirmed independently by Dr William Safranek at Wousheoff Hospital Rocklege, Florida using the p90 and p91 oligonucleotide primers.

DISCUSSION

Most published reports related to proving or disproving the association of MAP with CD have been based on identification of MAP in tissue specimens or anti-MAP antibodies in sera from CD patients. MAP isolated from tissues of CD patients has been reported following long-term incubation of cultured tissue. In some studies, 2 years of incubation was needed before cultures became positive for MAP. The outcomes of experiments that require 2 years of incubation may be altered by factors such as dehydration of media, malfunction of instrumentation and poor follow-up by the investigator. Consequently, false-negative results will be reported. Despite that, MAP was isolated from and confirmed to be present in tissues of some CD patients. Specifically, the positive cultures were reported as being from cultured surgically resected tissue. The study described in this paper aimed to identify culture media for the rapid isolation of MAP and to investigate surgically resected tissue versus microscopic biopsies for the determination of the presence of MAP in CD patients. Ultimately, is MAP present in tissues of CD patients? Bactec 12B* and MGIT culture media have been reported to be successful in primary isolation of *Mycobacterium avium* complex (MAC) from clinical specimens. The data as summarized in Table 1 indicate that MAP is present in tissue specimens from CD patients (37%) at higher levels than in controls (5.6%). This alone confirms previous data that support the role of MAP in CD pathogenesis. Interestingly, the data illustrated in Figure 2 demonstrate that MAP is present in 86% of surgically resected tissue compared to 20% of biopsy specimens of CD patients compared to 0% of controls. These data suggest that MAP may be residing in the submucosal layer of ulcerated tissue in CD patients rather than in biopsies obtained from the surface of the mucosal layer. The fact that MAP was cultured in several weeks from resected tissue of CD patients, rather than several months as seen in some positive biopsies, may indicate that MAP in deep tissue is virulent and metabolically active. On the other hand, MAP on the surface of the mucosal layer may be avirulent with inactive metabolic status. This may explain why some controls may be positive for MAP after long-term incubation. Consequently, we propose that using surgically resected tissue may be more appropriate for investigating the association of MAP with CD etiology. We suggest that the MGIT medium is more appropriate for cultivation of MAP from clinical specimens. This may be related to the extra additives in MGIT medium. In fact, these additives are known to be more supportive of cell differentiation and division.

The two positive control biopsies were from a normal healthy individual and from a patient with non-specific colitis. The presence of MAP in the two positive control samples was confirmed as those from CD samples. These two MAP isolates will be analyzed in a future study for epidemiologic and virulence markers, along with those from CD biopsies and surgically resected tissue specimens. The diagnosis of non-specific colitis may sometimes be misleading. It is interpreted by some physicians as being borderline with Crohn's disease. Therefore, there is a chance that one of our positive control specimens may be from an actual CD patient.

The acid-fast stain on positive MAP cultures was performed at different stages of the incubation. It was noted early in the culture incubation that spheroplastic forms of MAP were reverting to the bacillus form. This was confirmed when the bacillus form became more dominant in the culture following

further incubation. As shown in Figure 1, a spheroplastic form of MAP (A) with partial cell wall materials was observed, compared to the remainder of the culture, which contained the bacillus form (B). This, in turn, confirms previous reports suggesting that the spheroplastic form is the virulent form of MAP in CD pathogenesis [11, 12, 19].

The presence of MAP in tissues of CD patients has been confirmed. A protocol describing the culture media and the types of specimen for investigating MAP's role in CD has been described.

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