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# Detection of Fastidious Mycobacteria in Human Intestines by the Polymerase Chain Reaction

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The aim of this study was to determine whether difficult-to-grow mycobacteria are present in human intestines. Intestinal tissue samples were subjected to both mycobacterial culture and a polymerase chain reaction (PCR) assay. After detection by PCR, species identity was determined by hybridizing the amplified 16S rRNA gene fragments with species-specific oligonucleotides. Intestinal biopsies from 63 patients with noninflammatory bowel diseases (n = 22), Crohn's disease (n = 31), or ulcerative colitis (n = 10) were analyzed. Culture and PCR revealed mycobacteria in four (6%) and 25 (40%) samples, respectively. Samples positive by PCR were negative with all probes specific to nine common cultivable species but were positive with the *Mycobacterium genavense*-specific probe in 68% of cases. Mycobacterial isolates were identified as *Mycobacterium gordonae* and *Mycobacterium chelonae*. Findings were similar in Crohn's disease samples compared to non-Crohn's disease samples. This study shows that difficult-to-grow mycobacteria can be detected by PCR in large and similar proportions of inflamed intestinal tissue from patients with inflammatory bowel disease and intestinal tissue that appears normal from patients with noninflammatory bowel disease.

The combination of enzymatic nucleic acid amplification techniques with 16S rRNA-based molecular phylogeny has revealed the existence of a greater diversity of mycobacteria than has previously been shown by culture (1). These molecular biology techniques have allowed characterization of difficult-to-grow or formerly noncultivable mycobacterial species that can be pathogenic in patients with AIDS (2) or in apparently immunocompetent individuals (3).

In intestinal tissue samples from individuals free of mycobacterial disease, most laboratories using culture techniques have reported the detection of mycobacteria in 5 to 10% of cases, compared to 30 to 100% of cases reported by centers using the polymerase chain reaction (PCR) (4–6). The scanty detection of mycobacteria by culture compared to PCR could be due to the presence of difficult-to-grow or noncultivable mycobacterial species. This situation would be consistent with the suspected environmental origin of mycobacteria in the intestines (7) and the recent demonstration that a majority of environmental species cannot be cultured (8). Other possible explanations for such discrepancies are a lower detection limit of mycobacteria (i.e., the minimum number of mycobacteria required for detection) using PCR compared to culture (9) and differences between groups of subjects included in PCR- and culture-based studies.

A PCR-based strategy allowing the detection of almost any described mycobacterial species and the identification of the most frequently encountered ones has been developed (10). The conditions of this PCR assay have been adapted so that its detection limit is higher than that of mycobacterial culture (i.e., 5000 compared to 50 to 100 mycobacteria/sample, respectively) (11, 12).

The presence of noncultivable mycobacteria in human intestinal tissue was analyzed by subjecting tissue samples to mycobacterial culture and PCR. Mycobacterial species detectable by each technique in tissue that appeared normal from patients with noninflammatory bowel diseases and in tissue affected

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**Table 1:** Characteristics of the patients and tissue samples.

| Characteristics | Noninflammatory disease group (n = 22) | Ulcerative colitis group (n = 10) | Crohn's disease group (n = 31) |
|---|---|---|---|
| Mean age in years (range) | 62 (16–85) | 46 (24–66) | 40 (21–65) |
| Mean duration of disease in years (range) | – | 7 (0–21) | 8 (0–45) |
| No. of men (%) | 16 (73) | 7 (70) | 13 (42) |
| No. receiving corticotherapy (%) | 0 | 2 (20) | 9 (29) |
| Sampling procedure | | | |
| Endoscopy (%) | 17 (77) | 8 (80) | 22 (71) |
| Surgery (%) | 5 (23) | 2 (20) | 9 (29) |
| No. of positive samples | | | |
| Culture (%) | 0 | 0 | 4 (13) |
| 16S PCR (%) | 11 (50) | 4 (40) | 10 (32) |

–, not applicable.

by inflammatory changes (Crohn's disease or ulcerative colitis) were compared.

## Materials and Methods

*Patients.* Ileal or colonic samples were obtained during endoscopy (n = 47) or laparotomy (n = 16) from patients without human immunodeficiency virus type 1 or 2 or mycobacterial disease. Patient characteristics are shown in Table 1. In patients without inflammatory change (n = 22), the diagnoses included colonic polyps (n = 15), diverticulitis (n = 3), irritable bowel syndrome (n = 3), and iron deficiency without gastrointestinal cause (n = 1). Biopsies were taken from areas of tissues that appeared normal in the noninflammatory disease group and from inflamed areas in 41 patients with inflammatory bowel diseases (31 with Crohn's disease and 10 with ulcerative colitis). Diagnoses were made on established clinical, endoscopic, histopathological, and radiological criteria. No patient was taking antimycobacterial chemotherapy at the time of tissue sampling.

*Tissue Processing.* Samples intended for analysis using molecular biology techniques were placed immediately in sterile plastic containers and stored at −20°C until processed. Of the samples intended for culture, those obtained during endoscopy were kept at 4°C and processed within 24 h, whereas those obtained during laparotomy were frozen immediately and stored at −20°C until processed.

*Culture Techniques.* For each patient, a whole endoscopic biopsy or a full-thickness surgical specimen (0.5 g) was processed for culture. Specimens were homogenized in a mortar containing 1 ml of saline decontaminated with 1 ml of HCl 1 M over 30 min and neutralized with NaOH 1 M. The decontaminated suspensions were inoculated (0.2 ml/tube) onto Löwenstein-Jensen, Ogawa, and Ogawa supplemented with mycobactin J media, as described previously (13).

Tubes were incubated at 33°C for four to six days in a slanted position, with caps slightly loose to allow for evaporation of the inoculum. Tubes were then tightly closed and incubated at 33°C for at least 12 months or until growth was evident. When growth developed, colonies were transferred onto a fresh tube of the same medium on which a positive primary culture had been obtained.

The mycobacteria were identified according to standard methods (14). Standard recommendations (15) for the prevention of culture contamination, including the simultaneous processing within a laminar flow hood of no more than three specimens as well as UV illumination of work areas at regular intervals, were followed meticulously.

*Molecular Biology Techniques.* For each case, DNA was extracted from a whole endoscopic biopsy or a 2 to 5 $mm^3$ transmural section of a surgical specimen and subjected to a nested PCR assay targeting the 16S rRNA mycobacterial gene, as described previously (11, 16).

Species identity of mycobacteria detected by PCR was determined by oligonucleotide-specific capture plate hybridization (OSCPH) with probes Av, Chel, Gena, Intra, Tb3, Scrof, and Xen, as described previously (10). An additional probe ($rmV_3$, 5' AACCCGGACCTTCGTCGATG), which gives positive results with amplified products from most slow-growing mycobacteria (except *Mycobacterium simiae*, *Mycobacterium genavense*, *Mycobacterium intermedium*, *Mycobacterium terrae*, *Mycobacterium nonchromogenicum*, *Mycobacterium scrofulaceum*, *Mycobacterium cookii*, *Mycobacterium triviale*, *Mycobacterium xenopi*, *Mycobacterium farcinogenes*, *Mycobacterium celatum*, and *Mycobacterium shimoidei*) was used under the same conditions. Direct DNA sequencing was performed in five cases, as previously described (16).

*Controls.* Endoscope and endoscope-automated washer samples were obtained from all material in use in our endoscopy unit (1 series of samples in each case, but 2 series for the colonoscopes), as described previously (17). After centrifugation (3000 g for 30 min), sediments were resuspended in 1 ml of sterile water. Aliquots of 100 and 600 μl were subjected to detection of mycobacteria by 16S PCR and culture, respectively, as described above (except the decontamination step before culture, which consisted of adding HCl to a final concentration of 0.03M) (3).

Standard recommendations for the prevention of PCR contamination were followed meticulously, including the simultaneous processing of no more than six specimens as well as the exclusive use of disposable equipment and filter tips. Polymerase chain reaction-negative controls consisted of reagent-only tubes. Polymerase chain reaction-positive controls consisted of fragments of a tissue sample negative for the detection of mycobacteria by culture as well as by 16S and IS*900* PCR, which had been spiked with 5,000 *Mycobacterium paratuberculosis* (reference strain ATCC 19698) (18). Poly-

**Table 2:** Identification of mycobacteria detected by 16S polymerase chain reaction (PCR) and culture, using oligonucleotide-specific capture plate hybridization (OSCPH) and standard culture techniques, respectively.

| Case no. | 16S PCR | OSCPH with probes | | Culture |
|---|---|---|---|---|
| | | Gena | $rmV_3$* | |
| Noninflammatory group | | | | |
| 1 | + | + | – | – |
| 2 | + | + | – | – |
| 3 | + | + | – | – |
| 4 | + | – | + | – |
| 5 | + | – | + | – |
| 6 | + | – | + | – |
| 7 | + | – | + | – |
| 8 | + | + | + | – |
| 9 | + | + | + | – |
| 10 | + | + | + | – |
| 11 | + | + | + | – |
| Ulcerative colitis group | | | | |
| 12 | + | – | + | – |
| 13 | + | – | + | – |
| 14 | + | + | + | – |
| 15 | + | + | + | – |
| Crohn's disease group | | | | |
| 16 | + | + | – | – |
| 17 | + | + | – | – |
| 18 | + | + | – | – |
| 19 | + | + | – | – |
| 20 | + | + | – | – |
| 21 | + | – | + | – |
| 22 | + | – | + | – |
| 23 | + | + | + | – |
| 24 | + | + | + | – |
| 25 | + | + | – | *M. chelonae* |
| 26 | – | | | *M. gordonae* |
| 27 | – | | | *M. gordonae* |
| 28 | – | | | *Mycobacterium* spp. |

*The $rmV_3$ probe gives positive results with amplified products from slow-growing mycobacteria, with a few exceptions, including *Mycobacterium genavense* (see text for details). All samples were negative with probes specific to *Mycobacterium avium/Mycobacterium paratuberculosis, Mycobacterium chelonae, Mycobacterium xenopi, Mycobacterium intracellulare, Mycobacterium tuberculosis* complex, and *Mycobacterium scrofulaceum*.
+, positive; –, negative.

merase chain reaction controls were subjected to every step of the procedure together with the samples in a 1:6 ratio. The presence of PCR inhibitors was tested for in each sample by spiking a reaction mixture with 100 fg of mycobacterial DNA.

*Statistical Analyses.* Differences in proportion were tested by the chi-square test with Yates' correction or Fisher's exact test as appropriate; p values $< 0.05$ were considered statistically significant.

## Results

Of the 63 samples analyzed by each technique, four (6%) yielded mycobacterial isolates (1 or 2 cfu on 1 tube in each case) in culture, whereas the 16S PCR detected mycobacterial DNA in 25 (40%) cases (Table 1). No correlation was observed between results obtained with the two techniques; only a single sample was positive by both culture and PCR (Table 2). Tissue that appeared normal from patients with noninflammatory disease was as likely to contain mycobacteria as inflamed tissue from patients with Crohn's disease or ulcerative colitis, as assessed by culture or PCR.

Results of species identity determination using standard culture methods and OSCPH for all samples found to be positive by culture and PCR are shown in Table 2. Mycobacterial isolates were identified as *Mycobacterium gordonae* (2 cases) and *Mycobacterium chelonae* (1 case). In one case, species identity could not be determined (*Mycobacterium* spp.) due to poor in vitro growth in primary culture. Amplified 16S rRNA gene fragments were shown by OSCPH to derive

from *Mycobacterium genavense* in 68% of cases. Other slow-growing mycobacterial species were evidenced in 64% of cases by hybridization with the $rmV_3$ probe. In 32% of cases, amplified DNA fragments hybridized with both the $rmV_3$ and the *Mycobacterium genavense*-specific probe. Probes specific to *Mycobacterium avium/Mycobacterium paratuberculosis*, *Mycobacterium chelonae*, *Mycobacterium intracellulare*, *Mycobacterium tuberculosis* complex, *Mycobacterium scrofulaceum*, and *Mycobacterium xenopi* gave negative results with all 25 PCR-positive samples. The sample that yielded *Mycobacterium chelonae* in culture was, in OSCPH, positive with the *Mycobacterium genavense*-specific probe but negative with the *Mycobacterium chelonae*-specific probe.

DNA sequences of the hypervariable regions A and B (1), corresponding to *Escherichia coli* 16S rRNA positions 127 to 242 and 430 to 500 (19), were obtained in five cases, starting from the original DNA extract. In three culture-negative cases that were positive with the *Mycobacterium genavense*-specific probe alone, DNA sequencing confirmed the species identity by revealing the characteristic sequence signature of *Mycobacterium genavense* and the short helix 18 (1,20). In two culture-negative cases that were positive with both the Gena and the $rmV_3$ probe, the DNA sequence could not be determined due to the mixture in similar proportions of two different PCR products.

Culture yielded mycobacteria from Crohn's disease tissue exclusively, but the difference between isolation rates from Crohn's disease tissue compared to other tissue was not statistically significant (4/31 vs. 0/32, $p > 0.05$). DNA from either *Mycobacterium genavense* or an "$rmV_3$ probe +" species or both was identified in similar proportions of specimens in the Crohn's disease, ulcerative colitis, and noninflammatory disease groups.

All controls for possible endoscope or endoscope-automated washer contamination were always negative in culture and PCR; reagent-only control samples with each PCR also were negative, suggesting that we had no contamination. Polymerase chain reaction was inhibited in three cases of Crohn's disease and two cases of noninflammatory disease.

## Discussion

Mycobacteria were detected more frequently by PCR (40% of cases) than by culture (6% of cases) in specimens obtained from the same site during a single endoscopic or surgical procedure ($p < 0.00005$). Polymerase chain reaction or endoscope-related contamination (21) is unlikely to have occurred; the previously described extensive precautions for the prevention of contamination were followed rigorously, and controls were repeatedly negative. Our 16S PCR requires 5000 mycobacteria/sample for a positive result (11) compared to 50 to 100 mycobacteria/sample for isolation by culture (12), suggesting that our results are attributable to the ability of PCR to detect difficult-to-grow or noncultivable mycobacteria.

The possibility that PCR-positive samples contained DNA from dead organisms cannot be excluded. Mycobacteria are susceptible to gastric, biliary, and pancreatic secretions (22) but can be found alive in stools (23, 24), probably if a sufficient number of bacilli are ingested. Alterations of culture results due to freezing and thawing of specimens (which concerned surgical samples alone) were probably not significant, as surgical samples inoculated for culture were large (25) and yielded three of the four mycobacterial isolates obtained.

*Mycobacterium genavense* was identified in 68% of our 16S PCR-positive specimens, but it did not grow in any culture. Isolating this species from nonsterile specimens (even those containing huge amounts of acid-fast bacilli) is difficult because *Mycobacterium genavense* has specific growth requirements and is highly susceptible to decontamination procedures (26–28; unpublished data). This probably accounts for a large underestimation of the distribution of this species. Careful laboratory examinations recently suggested that *Mycobacterium genavense* was responsible for more than 10% of disseminated nontuberculous mycobacterial infections in patients with AIDS (29); previous reports of infection with this organism have been anecdotal. Our results further suggest that *Mycobacterium genavense* could be a ubiquitous opportunistic organism that frequently colonizes the gut by ingestion.

Half of the samples positive in OSCPH with the Gena probe were also positive with the $rmV_3$ probe. This latter oligonucleotide recognizes the long helix 18, which is characteristic of slow-growing mycobacteria but absent in *Mycobacterium genavense*. In two of these samples, DNA sequencing showed that two different PCR products were present in similar proportions. Consequently, OSCPH was able to recognize at least two different DNA sequences present in a single speci-

men. Compared to direct DNA sequencing, OSCPH is less expensive and easier to perform; furthermore, it can identify many specimens within a few hours (10). However, at the time of this study, only probes corresponding to nine species commonly identified in laboratories were available. Better knowledge of the pathogenicity of mycobacterial species described since the introduction of molecular biology techniques will allow selection of the most clinically relevant ones to design new probes.

Our 6% isolation rate of mycobacteria from intestinal tissue samples is similar to that reported by others (4). *Mycobacterium chelonae* and *Mycobacterium gordonae*, both environmental species, have been identified. To our knowledge, the isolation of *Mycobacterium gordonae* from human intestinal tissue has not been reported previously. This saprophytic species can be found alive in water, even after high-dose chlorination (30). We speculate that tap water is a source for acquisition of this species in humans, as has been reported with other species (e.g., *Mycobacterium avium*) (31). Mycobacteria detected by culture were probably present in very low numbers, as suggested by the recovery of only 1 or 2 cfu in each case and the absence of detection with our somewhat insensitive 16S PCR. A single sample was positive with both culture and PCR. For this sample the mycobacterial isolate was identified as *Mycobacterium chelonae*, whereas the PCR product was shown to derive from *Mycobacterium genavense* in OSCPH. Numbers of *Mycobacterium chelonae* present in this sample were probably low compared to numbers of *Mycobacterium genavense*, since *Mycobacterium chelonae* was not detected by PCR.

Inflammation of the mucosa was not found to favour colonization by *Mycobacterium genavense* or by cultivable mycobacteria. The suggested role of some mycobacterial species (i.e., *Mycobacterium paratuberculosis* and *Mycobacterium avium* subsp. *silvaticum*) in Crohn's disease (4, 32) is not supported by our findings or by other recent reports (6, 11, 33, 34). However, with the techniques used, the possible implication of some mycobacteria cannot be evaluated. These include fastidious mycobacteria different from *Mycobacterium genavense* and cultivable species present in spheroplast form at a low density.

In conclusion, this study shows that noncultivable mycobacteria can be detected by molecular biology techniques in large and similar proportions of inflamed intestinal tissue from patients with inflammatory bowel diseases and intestinal tissue that appears normal from patients with noninflammatory bowel diseases. *Mycobacterium genavense*, which can cause systemic infections in patients with AIDS, predominates. Other species may still be uncharacterized. The analysis of both environmental specimens (e.g., tap water) and intestinal tissue samples by molecular biology techniques should improve our knowledge of the distribution of the mycobacterial flora, which are probably major sources of infection in immunocompromised patients and whose incidence has been underestimated by culture-based studies.

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