JOURNAL OF CLINICAL MICROBIOLOGY, May 1993, p. 1241–1245
0095-1137/93/051241-05$02.00/0

Vol. 31, No. 5

Identification of Spheroplast-Like Agents Isolated from Tissues of Patients with Crohn's Disease and Control Tissues by Polymerase Chain Reaction

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Received 3 August 1992/Accepted 17 February 1993

***Mycobacterium paratuberculosis* has been isolated from tissue taken from patients with Crohn's disease and has been implicated in the etiology of this disease. On culture, the organisms appear initially as cell wall-deficient, spheroplast-like forms that are difficult to identify by conventional techniques. Here we examine 30 unidentified cultures by the polymerase chain reaction using primers specific for *M. paratuberculosis*, *Mycobacterium tuberculosis*, and *Mycobacterium avium* restriction fragment length polymorphism type A/I and also by a non-species-specific mycobacterial polymerase chain reaction. Six of these cultures, all from Crohn's disease, were shown to contain DNA from *M. paratuberculosis*. Cultures from both Crohn's disease and controls were found to contain mycobacterial DNA of unknown specific origin.**

The similarity of Crohn's disease (CD) (regional ileitis) to mycobacterial diseases in animals and humans was recognized in the earliest descriptions of the disease by both Dalziel (10) and Crohn (9), and this has long suggested a possible mycobacteria etiology. However, the absence of acid-fast bacilli in lesions and the inability to cultivate mycobacteria from tissues led to a retreat from this concept. Burnham et al. (2) isolated a strain of *Mycobacterium kansasii* from a mesenteric lymph node of a patient with CD. More recently, Chiodini et al. (3–5, 7) isolated a strain of *Mycobacterium paratuberculosis* from intestinal tissue from four patients with CD. Since *M. paratuberculosis* causes ileitis in ruminants in the form of Johne's disease, its isolation from tissue obtained from patients with CD was particularly interesting. DNA probes were previously used to demonstrate that the CD-isolated strains were indistinguishable from bovine *M. paratuberculosis* (23). Other laboratories have also isolated *M. paratuberculosis* from CD (11, 12, 15). *M. paratuberculosis* has also been detected in CD tissue by the polymerase chain reaction (PCR) in some studies (28, 31), although results from other studies have been negative (29, 36). In these and other studies, extremely slowly growing, pleomorphic, variably acid-fast, spheroplast-like organisms have been isolated from CD tissues and a smaller number of control tissue samples (2, 6, 11–13, 21). Because of the very poor growth of these cultures, it is impossible to identify these organisms by conventional techniques, even at the genus level. Without identification of these spheroplast-like agents, it is not possible to determine a specific association with CD or to separate pathogenic from environmental organisms. Using rRNA probes, Chiodini et al. demonstrated that the strains of *M. paratuberculosis* isolated from CD tissue first appeared as spheroplast-like agents (6), and they suggested that *M. paratuberculosis* is present in CD tissue in a spheroplast-like form. These observations were, however, specific to the spheroplasts that later transformed into *M. paratuberculosis*. McFadden et al. (26) used DNA probes to demonstrate that a strain isolated from another laboratory was not closely related to *M. paratuberculosis*. Moss et al. used PCR to demonstrate the presence of both *M. paratuberculosis* and *Mycobacterium avium* DNA in long-term cultures derived from CD and controls (27). The work described the use of PCR to detect mycobacterial DNA in similar microbial cultures derived from CD and control specimens. Four strains of *M. paratuberculosis*, all from CD specimens, have previously been isolated and identified from these cultures by conventional methods and with DNA probes (6, 11, 12, 15, 26). However, many cultures yielded only very slowly growing material that could not be identified by conventional means. Here we examine 30 of these unidentified cultures from 21 patients by PCR (32) to identify mycobacterial DNA. Primers designed to amplify *M. paratuberculosis* DNA were derived from the DNA sequence of the insertion sequence IS*900*, which is specific to *M. paratuberculosis* (14, 35). Recently, a strain of *M. avium* designated *M. avium* restriction fragment length polymorphism type A/I, which includes the wood-pigeon bacillus also known as *M. avium* subsp. *silvaticum* (34), which is able to cause Johne's disease in experimental infections (8) has also been found with DNA probes to be associated with some cases of Johne's disease in animals (19). This pathogen was recently identified in cultures from inflammatory bowel disease tissue with DNA probes (24), and we also aimed to detect this pathogen, if it was present in these samples. We therefore utilized PCR primers specific to sequences from the insertion sequence IS*901*, which is specific to the RFLP type A/I strain of *M. avium* (19, 20). DNA from the *Mycobacterium tuberculosis* complex was detected with primers designed to amplify a sequence from IS*6110* (IS*986/6110*), which is specific to the *M. tuberculosis* complex (16, 22). In addition, we wished to detect DNA from any other mycobacterial species that was not detected by the above primer combinations. We therefore utilized primers designed to amplify a fragment of the gene encoding the conserved 65-kDa antigen (33), now known to be the

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TABLE 1. Summary of culture results

Source of culture	Total no. of patients	No. of *M. paratuberculosis* isolates	No. of samples with unidentified growth in culture tubes
CD	28	4[a]	16
Ulcerative colitis	10	0	2
Control	13	0	3

[a] Previously described (3–7).

mycobacterial *groEL* gene, of *M. tuberculosis* and other mycobacteria (30). The *groEL* PCR has previously been shown to amplify DNA from a range of mycobacterial species but not from a collection of nonmycobacterial DNA samples (30). In order to detect a false-negative PCR due to inhibition by sample components and as a check on the sensitivity of amplification, we utilized modified templates engineered by site-directed mutagenesis as controls in our PCR (30).

MATERIALS AND METHODS

Cultures. Thirty unidentified cultures isolated from 21 different patients were obtained from R.C.'s laboratory (Table 1). These cultures had been inoculated with resected gastrointestinal tissues onto Herrold's egg yolk medium supplemented with mycobactin, as previously described (7). All cultures were coded and sent to the laboratory of J.J.M. for (blinded) PCR analysis.

Sample preparation. An inoculating loop was used to remove a small quantity of visible growth from the culture tubes. This growth was suspended in 100 μl of water in a 1.5-ml-volume screw-cap centrifuge tube and was boiled for 10 min. Glass beads (0.1 mm in diameter) were added to fill three-quarters of a 0.5-ml-volume microcentrifuge tube, the sample suspension was added, and the tube was shaken vigorously for 3 min with a Mini-Bead Beater (Stratech Scientific, Ltd., Luton, United Kingdom). The base of the microcentrifuge tube was then pierced with a fine needle, and the lysate was recovered by centrifuging into a 1.5-ml-volume centrifuge tube. This lysate was then added directly to PCRs. When PCR inhibition was detected, the sample was further purified by treatment with guanidinium isothiocyanate and DNA purified on silica particles, as described previously (1), before the PCR was repeated.

PCR. PCRs were performed by standard protocols as described previously (30, 32).

The primers for the detection of *M. paratuberculosis*, based on the sequence of IS*900*, were IS*900*-1, (209)TGGA CAATGACGGTTACGGAGGTGG(234), and IS*900*-2, (563) TGATCGCAGCGTCTTTGGCGTCGGT(538). The numbers in parentheses refer to the published sequence of IS*900* (14). The primers amplify a 354-bp fragment from *M. paratuberculosis* DNA. PCR parameters were 35 cycles of annealing (65°C, 45 s), extension (72°C, 150 s), and denaturation (94°C, 30 s).

The primers used for site-directed mutagenesis to engineer the modified template for IS*900* PCR were MTp1, TGAC GACGTTGGTTGATGGAGGCG, from the IS*900* sequence (375)TGACGACGTTGGCCGATGGAGGCG(398) with a 2-base (CC→TT) substitution, and MTp2, the complementary sequence of MTp1. IS*901* primers, specific for *M. avium* RFLP type A/I, were as previously described (19). The primers amplify a 1,104-bp product from *M. avium* RFLP type A/I. IS*986/6110* primers specific for *M. tuberculosis* were INS1 and INS2, as described previously (16). The primers amplify a 244-bp product from *M. tuberculosis* complex mycobacteria.

groEL primers were TB1 and TB2, as described previously (30), which amplified a 626-bp sequence from all mycobacteria tested. PCR products were electrophoresed through 1.5% agarose. When modified template was used, the samples were first digested with either *Hae*III (IS*900*) or *Xho*I (*groEL*) and electrophoresed through either 2% agarose or 6% polyacrylamide in Tris-borate buffer. Products were detected by staining with ethidium bromide and were photographed under UV illumination.

Synthesis and use of PCR-modified template. The standard IS*900* PCR product was modified by site-directed mutagenesis (17) to alter its restriction endonuclease digestion pattern. A standard PCR was performed with IS*900* primers to synthesize the wild-type IS*900* PCR product with 0.1 ng of *M. paratuberculosis* DNA as a template. A 0.1-μl volume of this product was used as the template in PCRs with (i) IS*900*-1 primer plus IS*900* MTp2 primer and (ii) IS*900*-2 primer plus IS*900* MTp1 primer. The products of these reactions were mixed and diluted 1:1,000, and 5 μl was used for a PCR with IS*900*-1 and -2. This product was electrophoresed through 1.5% agarose, excised, and purified with Gene-Clean (Bio 101, Inc., La Jolla, Calif.). The ends of the purified product were then filled in with Klenow fragment of *Escherichia coli* DNA polymerase I, phosphorylated with T4 polynucleotide kinase, and cloned into the *Sma*I site of the plasmid pUC18. DNA extracted from this clone was used as a modified template to spike PCR mixtures.

The *groEL* modified template was similarly constructed, as described previously (30).

For IS*900* PCRs, 0.09 fg (corresponding to approximately 20 target molecules) of modified template was added to each reaction mixture. For the *groEL* reaction, 0.2 fg (corresponding to approximately 50 target molecules) of modified template was added to the mixture.

RESULTS

In preliminary experiments with IS*900* primers in which samples were spiked with known amounts of *M. paratuberculosis* DNA, we found that some of the samples contained components that inhibited the PCR, leading to false-negative results. Since this decreased our confidence in negative results, we decided to construct a modified template that could be added to all IS*900* PCR mixtures that would indicate whether successful amplification had occurred in the tube but for which the PCR product was easily distinguishable from wild-type product. The 354-bp amplified fragment from IS*900* was therefore modified by site-directed mutagenesis by PCR (17). A 2-base substitution (CC→TT) was made in the *Hae*III restriction site at IS*900* position 385 (14) in the amplified product. This base substitution eliminated one of the four sites for *Hae*III in this amplified fragment so that upon digestion of the modified product, bands present at 178 and 39 bp were lost and a new band at 217 bp was produced. The modified amplified product was then cloned into pUC18. DNA was extracted from this clone and used to spike IS*900* PCR mixtures, and the reactions were then performed normally. In order to analyze the result, the amplified product of PCRs was digested directly (in the PCR buffer) with *Hae*III. Preliminary experiments were performed that demonstrated that so long as the amount of modified template added to each reaction mixture was no greater than 0.1 fg, corresponding to approximately

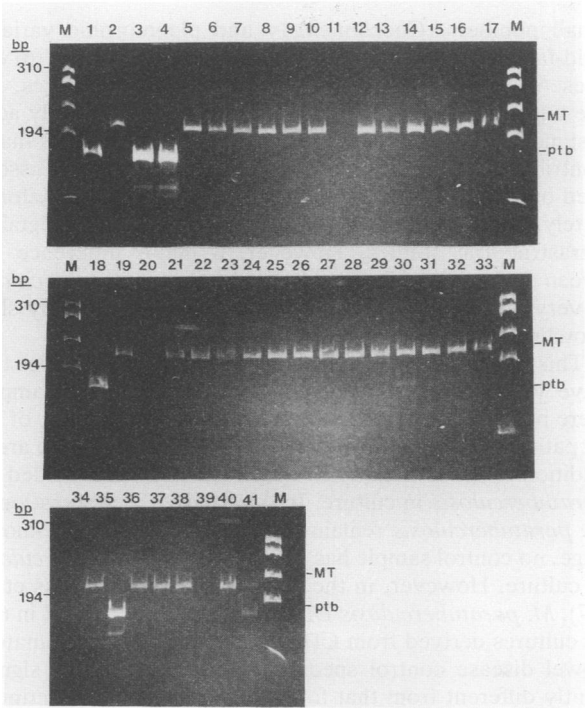

FIG. 1. IS*900* PCR products with modified template, digested with *Hae*III, electrophoresed through 6% polyacrylamide, stained with ethidium bromide, and photographed. Lanes: M, 300 ng of φX174 DNA fragment size markers digested with *Hae*III; 1, 18, and 41, 60 fg of *M. paratuberculosis* DNA; 2 through 17, 19 through 33, and 34 through 40, 30 randomized test samples plus 8 negative control samples. MT, modified template; ptb, PCR product band. Size markers (in base pairs) are on the left.

25 target molecules, the presence of modified template did not significantly reduce the sensitivity for detection of *M. paratuberculosis* DNA.

The positive, negative, and false-negative results for IS*900* PCR with the modified template can be seen in Fig. 1. The modified-template product band at 217 bp is visible in most samples (MT in Fig. 1). Positive amplification of *M. paratuberculosis* DNA is indicated by the presence of a PCR product band at 178 bp (ptb in Fig. 1, lanes 1, 3, and 4, etc). Unless only very small quantities (less than about 60 fg) of *M. paratuberculosis* DNA were present in the sample (Fig. 1, lane 41), the positive template products were not usually seen in *M. paratuberculosis*-positive samples, presumably because of competition for PCR reagents. A false-negative result is shown by the absence of any band (e.g., Fig. 1, lane 11). By being used to spike the reaction mixtures at a specific concentration (20 target gene copies per reaction mixture), the modified template also acts as an internal sensitivity control. A similarly constructed modified template was also utilized in the *groEL* PCRs as described previously (30).

The sensitivities (in femtograms) of the PCRs were determined with dilutions of homologous genomic mycobacterial DNA as the target and by detecting PCR product by ethidium bromide staining and were as follows: IS*900* PCR, 6; IS*901* PCR, 6; IS*986/6110* PCR, 6; and *groEL* PCR, 100. Therefore, all of the PCRs using insertion sequences as the target gave sensitivities equivalent to detecting one to two mycobacterial cells, whereas the sensitivity of the *groEL* PCR was about 1/20 of this.

All of the samples were subjected to all four PCRs. To minimize the possibility of sample contamination, samples were processed and analyzed in small batches (usually six samples). A positive result was recorded when a PCR product of the expected size was detected by ethidium bromide staining of agarose gels. When inhibition of PCR was detected (by the absence of modified template bands) with any of the samples, it was further diluted and/or purified until positive amplification was obtained. All reactions were performed at least in duplicate, and positive and negative controls were included in all runs. Two types of negative controls were always examined: (i) PCR reagents with no template DNA and (ii) samples containing only water that were subjected to the complete cycle of sample preparation in parallel with test samples. Any experiment in which any controls were positive was discarded. The results are presented in Table 2, and gels containing a representative group of samples are shown in Fig. 1. All samples were negative for both the IS*901* and IS*986/6110* PCRs, and therefore, these results are not presented. Table 2 also shows our estimate of how much visible growth was present in each tube.

Of the 30 samples examined, 6 were positive for IS*900*. Four positive samples may be seen in Fig. 1, lanes 3, 4, 30, and 35 (corresponding to samples 6b, 2, 11, and 12, respectively, in Table 2). Two further positives (samples 7c and 9b)

TABLE 2. PCR results for spheroplast-like agents

Culture no.[a]	Data[b]	Source[c]	Growth[d]	Result for:	
				IS*900*	*groEL*
1_a	1982	CD	+	−	−
1_b	1982	CD	+	−	−
1_c	1982	CD	++	−	+
2[e]	1985	CD	+++	+	+
3_a	1981	CD	++	−	+
3_b	1981	CD	+++	−	−
4	1984	CD	+++	−	+
5	1983	CD	+	−	−
6_a	1984	CD	+	−	−
6_b[e,f]	1984	CD	++	+	+
7_a	1983	CD	+	−	+
7_b	1983	CD	+	−	−
7_c[e]	1983	CD	+	+	+
8	1984	CD	+	−	−
9_a	1981	CD	++	−	−
9_b	1981	CD	+++	+	+
10_a	1984	CD	++	−	+
10_b	1984	CD	++	−	+
11[e,f]	1981	CD	+++	+	+
12[e]	1981	CD	++	+	−
13[e]	1982	CD	+++	−	+
14	1983	CD	+++	−	+
15	1984	CD	+++	−	+
16	1984	CD	+	−	−
17	1984	CD	+	−	−
18	1984	ML	+	−	−
19_a	1983	UC	++	−	+
19_b	1983	UC	+++	−	+
20	1983	Cancer	+++	−	−
21	1983	DV	+	−	+

[a] Multiple cultures from the same patient are designated by alphabetical subscripts.
[b] The year in which the culture tube was inoculated.
[c] Abbreviations: UC, ulcerative colitis; ML, melanosis coli; DV, diverticulitis.
[d] The quantity of visible growth in each tube was estimated by eye. + to +++, low to high levels of growth.
[e] Sample was subcultured.
[f] Subcultured growth was examined by electron microscopy.

were obtained upon purification of inhibiting samples. All IS*900*-positive samples were from the 25 CD samples. Altogether, 14 of the 30 samples were positive with the *groEL* PCR, 14 of 25 from CD and 3 of 5 from controls (data not shown).

Dilutions of most positive samples of up to 1:100 gave visible amplification products, indicating that a minimum of 100 target molecules were present in the original 4-μl sample aliquot added to the PCR mixtures and therefore that greater than 2,500 molecules were present in the original sample. As can be seen from Table 2, multiple cultures from the same patient did not always give the same result.

Samples that gave positive PCR results were examined by Ziehl-Neelsen staining, and these showed the presence of very scanty acid-fast rods plus variable acid-fast coccobacillary and pleomorphic forms very similar to the forms described previously for *M. paratuberculosis* spheroplasts (6). Since these cultures were very old and might therefore be expected to have anomalous staining characteristics, a number of samples (indicated in Table 1) giving positive PCR results were inoculated onto fresh media (Middlebrook 7H11 agar with 4 μg of mycobactin per ml). After 4 months of incubation, very tiny colonies (less than 1 mm) were barely visible in all tubes, except for the tube containing sample 13, in which no growth was visible. A culture of *M. paratuberculosis* inoculated at the same time showed good growth after a similar period of incubation. The subcultures in which tiny colonies were visible were examined by Ziehl-Neelsen staining, by carbon shadowing and electron microscopy, and by PCR. After Ziehl-Neelsen staining, very scanty acid-fast rods were seen again along with smaller amounts of pleomorphic forms. Electron microscopy of carbon-shadowed samples revealed scanty rods of sizes and shapes similar to those of *M. paratuberculosis* bacilli. All subcultures in which growth was visible gave the same PCR result as the original culture. After a further 3 months of incubation, the colonies were still very small in these subcultures, indicating that growth was still very slow and that the initial slow growth was not due to small amounts of inoculum.

As can be seen from Table 2, all samples that were positive for IS*900* were also positive for the *groEL* PCR, as expected, except for one sample, sample 12. This sample was subcultured, but the subculture continued to give the same anomalous result. It is possible that the *groEL* target sequence in this organism may have a minor sequence variation that prevents primer binding. A less likely possibility is that this sample contains a nonmycobacterial organism with IS*900*.

DISCUSSION

M. paratuberculosis has previously been isolated from CD tissue, and upon primary isolation, organisms appear as non-acid-fast coccobacillary forms that have the ultrastructural appearance of cell wall-deficient spheroplasts. However, most spheroplast-like agents isolated from CD and control tissues have not transformed in culture to typical mycobacterial forms and have therefore remained unidentified. Using PCR, we demonstrated here that DNA from a number of these cultures contained the insertion sequence IS*900*. IS*900* has so far not been found in any bacterium other than *M. paratuberculosis* (35), and we therefore conclude that these cultures contain *M. paratuberculosis*. Upon subculture, the agents retain the very slow growth rate of the parent cultures. The positive samples from both the original cultures and the subcultures contain acid-fast rods in very small numbers. Coccobacillary and pleomorphic variably acid-fast material similar to that previously described (6) was present in the original cultures and in the subcultures. We are uncertain as to whether the pleomorphic, variably acid-fast material represents a form of *M. paratuberculosis* that is contributing to the PCR result or the PCR signal is associated only with the small number of acid-fast rods. Unfortunately, insufficient material was available for any detailed ultrastructural studies. However, a major difference between the agents we have examined and *M. paratuberculosis* is very apparent, and that difference is the extremely slow growth rate of these agents.

This study increases the number of CD tissue samples that have yielded *M. paratuberculosis* in culture. Six samples were positive for *M. paratuberculosis*, representing 6 of the 28 patients from which samples were available. These are in addition to the four samples which have already yielded *M. paratuberculosis* in culture. In this study, the association of *M. paratuberculosis* remains specific to CD. To our knowledge, no control sample has yet yielded *M. paratuberculosis* in culture. However, in the study described by Moss et al. (27), *M. paratuberculosis* DNA was detected by PCR in 6 of 18 cultures derived from CD but in 1 of 6 non-inflammatory bowel disease control specimens, a frequency not significantly different from that for the CD group. The continued specific association between *M. paratuberculosis*, the cause of regional ileitis in ruminants, and CD, the cause of regional ileitis in humans, in our study remains provocative. However, remember that only a small percentage of CD tissue samples examined in a number of studies have yielded *M. paratuberculosis* in culture (25). *M. paratuberculosis* may be associated with CD in only a minority of patients. A significant proportion of samples were positive with the mycobacteria-specific *groEL* PCR, indicating that these spheroplast-like cultures did not contain DNA from *M. paratuberculosis*, *M. avium* RFLP type A/I, or *M. tuberculosis* but did contain DNA that was probably mycobacterial. However, these were identified from a similar proportion of controls as CD samples, and their significance is therefore uncertain. A significant proportion of the spheroplast-like samples remain unidentified, since they did not give a positive PCR. Many of these were, however, from tubes in which very little growth was visible.

Spheroplast-like agents have long been suspected to be present in mycobacterial diseases, including tuberculosis, in which they have been previously proposed to be responsible for the persistence of infection following successful treatment (18). The phenomenon of persistence has increased relevance presently because of the AIDS pandemic, since human immunodeficiency virus infection is strongly associated with reactivation of tuberculosis. The study of spheroplast-like agents that may be involved in persistence has been severely hampered by the inability to specifically identify unusual agents isolated from mycobacterial diseases. We here demonstrate that organisms isolated from CD tissue having some of the characteristics of spheroplast-like agents may be specifically identified and studied by PCR.

Finally, the use of a modified template in PCRs may be of value in any PCRs in which unsuccessful amplification due to inhibition or other causes (e.g., operator error) is suspected. The technique may also be of value in quantitative PCR.

ACKNOWLEDGMENT

This work was supported by the Crohn's and Colitis Foundation of America, Inc.

REFERENCES

1. **Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.
2. **Burnham, W. R., J. E. Lennard-Jones, J. L. Stanford, and R. G. Bird.** 1978. Mycobacteria as a possible cause of inflammatory bowel disease. Lancet **ii:**693–696.
3. **Chiodini, R. J.** 1988. Identification of mycobacteria from Crohn's disease by restriction polymorphism of the 5S ribosomal DNA genes, p. 509–514. *In* R. P. MacDermott (ed.), Inflamatory bowel disease: current status and future approaches. Elsevier Science Publications, Amsterdam.
4. **Chiodini, R. J.** 1989. Crohn's disease and the mycobacteriosis: a review and comparison of the two disease entities. Clin. Microbiol. Rev. **2:**90–117.
5. **Chiodini, R. J., H. J. Van Kruiningen, R. S. Merkal, W. R. Thayer, and J. A. Coutu.** 1984. Characteristics of an unclassified *Mycobacterium* species isolated from patients with Crohn's disease. J. Clin. Microbiol. **20:**966–971.
6. **Chiodini, R. J., H. J. Van Kruiningen, W. R. Thayer, and J. A. Coutu.** 1986. Spheroplastic phase of mycobacteria isolated from patients with Crohn's disease. J. Clin. Microbiol. **24:**357–363.
7. **Chiodini, R. J., H. J. Van Kruiningen, W. R. Thayer, R. S. Merkal, and J. A. Coutu.** 1984. Possible role of mycobacteria in inflammatory bowel disease. I. An unclassified *Mycobacterium* species isolated from patients with Crohn's disease. Dig. Dis. Sci. **29:**1073–1079.
8. **Collins, P., P. R. J. Mathews, A. McDiarmid, and A. Brown.** 1983. The pathogenicity of *Mycobacterium avium* and related mycobacteria for experimental animals. J. Med. Microbiol. **16:**27–35.
9. **Crohn, B., L. Ginzberg, and G. Oppenheimer.** 1932. Regional ileitis, a pathological and clinical entity. JAMA **99:**1323–1329.
10. **Dalziel, T. K.** 1913. Chronic interstitial enteritis. Br. Med. J. **ii:**1068–1070.
11. **Gitnick, G., J. Collins, B. Beaman, et al.** 1989. Preliminary report on isolation of mycobacteria from patients with Crohn's disease. Dig. Dis. Sci. **34:**925–932.
12. **Graham, D. Y., D. C. Markesich, and H. H. Yoshimura.** 1987. Mycobacteria and inflammatory bowel disease. Results of culture. Gastroenterology **92:**436–442.
13. **Graham, D. Y., D. C. Markesich, and H. H. Yoshimura.** 1989. Mycobacteria as the cause of Crohn's disease. Gastroenterology **97:**1354–1356. (Letter.)
14. **Green, E. P., M. L. V. Tizard, M. T. Moss, et al.** 1989. Sequence and characteristics of IS900, and insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. Nucleic Acids Res. **17:**9063–9073.
15. **Haagsma, J., C. Mulder, A. Eger, J. Bruins, J. Ketel, and G. Tytgat.** 1988. *Mycobacterium* species isolated from patients with Crohn's disease, p. 535–537. *In* R. P. MacDermott (ed.), Inflammatory bowel disease: current status and future approaches. Elsevier Science Publishers, Amsterdam.
16. **Hermans, P. W. M., D. van Soolingen, J. W. Dale, A. R. J. Schuitema, R. A. McAdam, D. Catty, and J. D. A. van Embden.** 1990. Insertion element IS*986* from *Mycobacterium tuberculosis*: a useful tool for diagnosis and epidemiology of tuberculosis. J. Clin. Microbiol. **28:**2051–2058.
17. **Higuchi, R.** 1989. Using PCR to engineer DNA, p. 61–70. *In* H. A. Erlich (ed.), PCR technology. Principles and applications for DNA amplification. Stockton Press, New York.
18. **Imaeda, T.** 1984. Cell wall deficient forms, p. 671–680. *In* G. P. Kubica and L. G. Wayne (ed.), The mycobacteria. Marcel Dekker, Inc., New York.
19. **Kunze, Z. M., F. Portaels, and J. J. McFadden.** 1992. Biologically distinct subtypes of *Mycobacterium avium* differ in possession of insertion sequence IS*901*. J. Clin. Microbiol. **30:**2366–2372.
20. **Kunze, Z. M., S. Wall, R. Appelberg, M. T. Silva, F. Portaels, and J. J. McFadden.** 1991. IS901, a new member of a widespread class of atypical insertion sequences, is associated with pathogenicity in *Mycobacterium avium*. Mol. Microbiol. **5:**2265–2272.
21. **Markesich, D. C., D. Y. Graham, and H. H. Yoshimura.** 1988. Progress in culture and subculture of spheroplasts and fastidious acid-fast bacilli isolated from intestinal tissues. J. Clin. Microbiol. **26:**1600–1603.
22. **McAdam, R. A., P. W. M. Hermans, D. van Soolingen, et al.** 1990. Characterization of a *Mycobacterium tuberculosis* insertion sequence belonging to the IS*3* family. Mol. Microbiol. **4:**1607–1613.
23. **McFadden, J. J., P. D. Butcher, J. Thompson, R. J. Chiodini, and J. Hermon-Taylor.** 1987. The use of DNA probes identifying restriction-fragment-length polymorphisms to examine the *Mycobacterium avium* complex. Mol. Microbiol. **1:**283–291.
24. **McFadden, J. J., J. Collins, B. Beaman, M. Arthur, and G. Gitnick.** 1992. Mycobacteria and Crohn's disease: DNA probes identify the wood-pigeon strain of *Mycobacterium avium* and *Mycobacterium paratuberculosis* from human tissue. J. Clin. Microbiol. **30:**3070–3073.
25. **McFadden, J. J., and P. Seechurn.** 1992. Mycobacteria and Crohn's disease. Molecular approaches, p. 259–271. *In* R. MacDermott and W. Stenson (ed.), Inflammatory bowel disease. Elsevier Press, New York.
26. **McFadden, J. J., J. Thompson, E. Hull, S. Hampson, J. Stanford, and J. Hermon-Taylor.** 1988. The use of cloned DNA probes to examine organisms isolated from Crohn's disease tissue, p. 515–520. *In* R. P. MacDermott (ed.), Inflammatory bowel disease: current status and future approaches. Elsevier Science Publishers, Amsterdam.
27. **Moss, M. T., J. D. Sanderson, M. L. V. Tizard, et al.** 1992. Polymerase chain reaction detection of *Mycobacterium paratuberculosis* and *Mycobacterium avium* subsp. *silvaticum* in long term cultures from Crohn's disease and control tissues. Gut **33:**1209–1213.
28. **Quirke, P., D. Dockey, G. R. Taylor, F. A. Lewis, P. Hawkey, and D. Graham.** 1991. Detection of *Mycobacterium paratuberculosis* in Crohn's disease. Gut **32:**A572.
29. **Rosenberg, W. M. C., J. L. Bell, and D. P. Jewel.** 1991. *Mycobacterium paratuberculosis* DNA cannot be detected in Crohn's disease. Gastroenterology **100:**A611.
30. **Saboor, S., N. J. Johnson, and J. J. McFadden.** 1992. Detection of mycobacteria in sarcoidosis and tuberculosis with polymerase chain reaction. Lancet **339:**1012–1015.
31. **Sanderson, J. D., M. T. Moss, M. L. V. Tizard, and J. Hermon-Taylor.** 1992. *Mycobacterium paratuberculosis* in Crohn's disease tissue. Gut **33:**890–896.
32. **Sauki, R. K., S. Scharf, F. Faloona, et al.** 1985. Enzymic amplification of the β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230:** 1350–1354.
33. **Shinnick, T. M.** 1987. The 65-kilodalton antigen of *Mycobacterium tuberculosis*. J. Bacteriol. **169:**1080–1088.
34. **Thorel, M.-F., M. Krichevsky, and V. V. Lévy-Frébault.** 1990. Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., and *Mycobacterium avium* subsp. *silvaticum* subsp. nov. Int. J. Syst. Bacteriol. **40:**254–260.
35. **Vary, P. H., P. R. Andersen, E. Green, J. Hermon-Taylor, and J. J. McFadden.** 1990. Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. J. Clin. Microbiol. **28:**933–937.
36. **Wu, S. W. P., C. C. Pao, and J. Chan.** 1991. Lack of mycobacterial DNA in Crohn's disease tissue. Lancet **337:**174–175.