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Detection and Verification of *Mycobacterium avium* subsp. *paratuberculosis* in Fresh Ileocolonic Mucosal Biopsy Specimens from Individuals with and without Crohn's Disease

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***Mycobacterium avium* subsp. *paratuberculosis* is a robust and phenotypically versatile pathogen which causes chronic inflammation of the intestine in many species, including primates. *M. avium* subsp. *paratuberculosis* infection is widespread in domestic livestock and is present in retail pasteurized cows' milk in the United Kingdom and, potentially, elsewhere. Water supplies are also at risk. The involvement of *M. avium* subsp. *paratuberculosis* in Crohn's disease (CD) in humans has been uncertain because of the substantial difficulties in detecting this pathogen. In its Ziehl-Neelsen staining-negative form, *M. avium* subsp. *paratuberculosis* is highly resistant to chemical and enzymatic lysis. The present study describes the development of optimized sample processing and DNA extraction procedures with fresh human intestinal mucosal biopsy specimens which ensure access to *M. avium* subsp. *paratuberculosis* DNA and maximize detection of these low-abundance pathogens. Also described are two nested PCR methodologies targeted at IS*900*, designated IS*900*[L/AV] and IS*900*[TJ1-4], which are uniquely specific for IS*900*. Detection of *M. avium* subsp. *paratuberculosis* in mucosal biopsy specimens was also evaluated by using mycobacterial growth indicator tube (MGIT) cultures (Becton Dickinson). IS*900*[L/AV] PCR detected *M. avium* subsp. *paratuberculosis* in 34 of 37 (92%) patients with CD and in 9 of 34 (26%) controls without CD (noninflammatory bowel disease [nIBD] controls) (*P* = 0.0002; odds ratio = 3.47). *M. avium* subsp. *paratuberculosis* was detected by IS*900*[L/AV] PCR in MGIT cultures after 14 to 88 weeks of incubation in 14 of 33 (42%) CD patients and 3 of 33 (9%) nIBD controls (*P* = 0.0019; odds ratio = 4.66). Nine of 15 (60%) MGIT cultures of specimens from CD patients incubated for more than 38 weeks were positive for *M. avium* subsp. *paratuberculosis*. In each case the identity of IS*900* from *M. avium* subsp. *paratuberculosis* was verified by amplicon sequencing. The rate of detection of *M. avium* subsp. *paratuberculosis* in individuals with CD is highly significant and implicates this chronic enteric pathogen in disease causation.**

The association between the chronic enteric pathogen *Mycobacterium avium* subsp. *paratuberculosis* and Crohn's disease (CD) in humans is poorly understood. The original studies of Chiodini and other researchers (5, 6) showed that *M. avium* subsp. *paratuberculosis* could be grown in conventional culture of specimens from the chronically inflamed intestines of individuals with CD, but from only about 5% of cases. The introduction of an IS*900*-specific PCR and its application, together with optimized processing procedures for surgically resected samples, led to the initial demonstration that *M. avium* subsp. *paratuberculosis* could be detected in the guts of 65% of individuals with CD (26). The results of subsequent PCR studies from other laboratories from 1994 to 1999 were not consistent, resulting in substantial uncertainty as to the role of *M. avium* subsp. *paratuberculosis* as a human pathogen (for a review, see reference 19). The recent demonstration that live *M. avium* subsp. *paratuberculosis* is present intermittently in retailed pasteurized cows' milk in the United Kingdom suggests that there is a risk that people in the United Kingdom maybe exposed to live *M. avium* subsp. *paratuberculosis* organisms by this route (14). Data from PCRs with bulk-tank milk in Switzerland also suggest that this risk may apply elsewhere (9). There is therefore a need to resolve the uncertainty associated with the presence of *M. avium* subsp. *paratuberculosis* in the tissues of patients with CD.

Studies with the intestines of sheep with the paucimicrobial form of Johne's disease and with gut tissues from humans with CD demonstrated that the Ziehl-Neelsen staining-negative form of *M. avium* subsp. *paratuberculosis* under these conditions is highly resistant to chemical and enzymatic lysis (33). The inclusion of an optimized mechanical disruption step during tissue processing was found to be essential to ensure reliable access to *M. avium* subsp. *paratuberculosis* DNA (19). The need for this mechanical disruption step has been supported by other work (23).

IS*900* belongs to a family of related insertion elements found in the order *Actinomycetales* that includes IS*1613* (GenBank accession no. AJ011837), IS*1626* (24), IS*901*/*902* (19, 22), IS*1110* (20), IS*116* (21), and IS*110* (4). Adjustment of PCR primer design and conditions has ensured that IS*900*-specific PCR does not produce an amplification product from these

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related elements. Recently, other IS*900*-like elements have been described at low copy numbers in rarely encountered environmental mycobacteria (10, 11). These elements can readily be distinguished from IS*900* by amplification product sequencing and the use of appropriately designed primer pairs. The present paper describes the application of optimized sample processing and a nested IS*900*-specific PCR, improved liquid cultures, and amplification product sequencing to the sensitive and specific detection and verification of *M. avium* subsp. *paratuberculosis* in fresh endoscopic ileocolonic mucosal biopsy specimens from individuals with and without CD.

MATERIALS AND METHODS

Mycobacterial strains and plasmids. The mycobacterial strains listed below were cultured in mycobacterial growth indicator tube (MGIT) liquid medium (Becton Dickinson, Oxford, United Kingdom), and genomic DNA was extracted as described below in the section on sample processing. The DNA was used to test for primer specificity as well as in positive and negative control reactions. A *Mycobacterium* species reported as strain 2333 was a gift from G. Bolske (11); *Mycobacterium* sp. strain WA-1, *Mycobacterium* species strain WA-2, and *Mycobacterium* species strain 22850 were gifts from D. Cousins (10); *M. avium* subsp. *paratuberculosis* strain K-10 was used for genome sequencing and was a gift from R. Barletta; *M. avium* subsp. *silvaticum* isolate 0012 was from a wood pigeon (2); *M. avium* subsp. *avium* strain MAA16 contained IS*1613* and was isolated from an AIDS patient; and *M. avium* subsp. *avium* strain NCTC 1551 containing IS*901*/*902*. A control plasmid designated pIDL60 containing a single copy of IS*900* cloned from a genome library of *M. avium* subsp. *paratuberculosis* strain Linda (ATCC43015) and a plasmid designated p1626 containing a single copy of the element IS*1626* (a gift from D. Kunimoto [24]) were also used.

Patients and samples. Patients undergoing a routine ileocolonoscopy as a normal part of their clinical care were prospectively enrolled in the study. Informed consent was obtained from each individual. The case definition of CD was established on the basis of standard clinical, radiological, endoscopic, and histopathological criteria. The control group, designated the noninflammatory bowel disease (nIBD) control group, comprised patients who were undergoing an ileocolonoscopy but who did not have a clinicopathological diagnosis of CD. Inclusion criteria were compliance with case definitions and the provision of informed consent. Exclusion criteria were treatment with rifabutin and clarithromycin combinations within the previous 1 year and anticoagulation therapy. Sampling from the CD and control groups was intercalated throughout the study.

Pairs of mucosal biopsy specimens (n = 2 to 8) were taken during ileocolonoscopy from the terminal ileum and/or colon of all patients with CD and the nIBD control patients. For patients with CD, biopsy specimens were obtained from inflamed edematous mucosa and not from the floor of the ulcers, as these may be densely fibrotic. Each mucosal sample was taken from the biopsy forceps with a sterile needle and transferred to a 1.5-ml sterile screw-cap reaction tube containing 0.5 ml of sterile 0.85% saline (molecular biology grade). Samples were given a double-letter code and were immediately taken to the laboratory. Tissue samples were not frozen at any time. The study was single blinded, with cases and controls intercalated.

DNA extraction. One letter-coded mucosal sample from each pair was processed for direct IS*900*-specific PCR testing. Tubes containing the biopsy specimens were first centrifuged at 10,000 × *g* for 3 min. The supernatant was removed; and the pellet was resuspended in 600 μl of mycobacterial lysis buffer (2 mM sodium EDTA [catlog no. E-7889; Sigma, Poole, United Kingdom], 400 mM NaCl [catalog no. S-5150; Sigma], 10 mM Tris HCl [pH 8.0; catalog no. T-3038Sigma], filtered [pore size, 0.2 μm] 0.6% sodium dodecyl sulfate [catalog no. L-4522; Sigma], and 33 μg of proteinase K [catalog no. P-2308; Sigma] per ml made up in sterile distilled nuclease-free water [catalog no. W-4502; Sigma] and transferred to a Lysing Matrix B Ribolyser tube (catalog no. 6911-100; Qbiogene, Noltingham, United Kingdom). The Ribolyser tube was incubated at an angle of 45° at 37°C for 2 h with shaking (200 rpm). The tubes were then either processed further through the extraction procedure or stored at −20°C for later testing in batches. The tubes were chilled on ice for 5 min and mechanically disrupted in the FastPrep Ribolyser (Qbiogene) at a setting of 6.5 ms^{-2} for 45 s and then immediately chilled again on ice for 15 min. A volume of 600 μl of phenol (pH 6.7; catalog no. P-4557; Sigma) saturated in 1× TE (10 mM Tris HCl, 1 mM sodium EDTA [pH 8.0]) was added, and the mixture was vortexed on a multitube mixing head (Vortex Genie; Scientific Industries, Alva, United Kingdom) for 20 s and then centrifuged at 10,000 × *g* for 1 min. The aqueous layer (550 μl) was transferred to a new screw-cap reaction tube containing an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1; catalog no. P3803 Sigma), and the mixture was saturated in 1× TE, vortexed for 20 s, and centrifuged (10,000 × *g* for 1 min). The aqueous layer (500 μl) was again transferred to a new tube containing 500 μl of chloroform-isoamyl alcohol (24:1), vortexed for 30 s, and centrifuged (10,000 × *g* for 1 min). The final aqueous layer (450 μl) was transferred to a new tube containing 90 μl of 10 M ammonium acetate and mixed thoroughly. One milliliter of ice-cold 100% ethanol was added to enable DNA precipitation at room temperature (RT) for 1 h. The samples were then centrifuged (10,000 × *g* for 20 min) at RT, and the pellets were washed in 750 μl of ice-cold 70% ethanol. The pellets were dried at RT for 30 min, resuspended in 50 μl of 1× TE, and allowed to redissolve at 4°C overnight.

Culture of biopsy specimens. The second of each pair of letter-coded biopsy specimens from each patient was decontaminated when fresh and subsequently cultured in separate MGIT culture tubes at 37°C for up to 18 months. Briefly, 0.5 ml of NaOH–*N*-acetyl-L-cysteine (BBL Mycoprep; Becton Dickinson) was added to each sample in 0.5 ml of saline, and the mixture was incubated at RT for 20 min with occasional mixing by inversion. The samples were then centrifuged (10,000 × *g* for 10 min), and the supernatant was removed by pipetting. The pellet was resuspended in 0.5 ml of TEN buffer (2 mM sodium EDTA, 400 mM NaCl, 10 mM Tris HCl [pH 8.0]; Sigma) and transferred to the tube containing 4.5 ml of MGIT medium (Becton Dickinson) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC), PANTA (40 U of polymyxin B per ml, 4 μg of amphotericin B per ml, 16 μg of nalidixic acid per ml, 4 μg of trimethoprim per ml, 4 μg of azlocillin per ml [final concentrations]), and 2 μg of mycobactin J (Allied Monitor, Fayette, Mo.) per ml. After incubation at 37°C for between 14 and 88 weeks, the cultures were mixed and 0.5 ml was removed aseptically for testing by the IS*900*-specific nested PCR as described below. MGIT cultures with visible growth were subcultured after 20 weeks onto 7H11 Middlebrook medium (with 10% OADC and 2 μg of mycobactin J per ml) and incubated at 37°C for up to 4 months. Subsequent acid-fast colonies were tested for IS*900* by inoculating one colony into 600 μl of mycobacterial lysis buffer in a Ribolyser tube and processed as described above for tissue DNA extractions and the IS*900*-specific PCR.

IS*900*-specific nested PCR and sequencing. Two nested PCR tests targeting IS*900*, designated IS*900*[L/AV] and IS*900*[TJ1-4], were used. For the IS*900*[L/AV] PCR, the first-round PCR mixture comprised 5 μl of DNA sample extract in a final volume of 50 μl with 2 μM (each) primers L1 (5′-CTT TCT TGA AGG GTG TTC GG-3′) and L2 (5′-ACG TGA CCT CGC CTC CAT-3′); 1× Expand High Fidelity reaction buffer containing 1.5 mM $MgCl_2$; 10% dimethyl sulfoxide; 200 μM (each) dATP, dGTP, dCTP, and dTTP; and 3.5 U of Expand High Fidelity *Taq* polymerase (no. 1759078; Expand High Fidelity PCR system; Roche, Lewes, United Kingdom). Cycling conditions were 1 cycle of 94°C for 5 min and then 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 3 min, followed by 1 cycle of 72°C for 7 min. To reduce the risk of amplicon contamination, the first-round PCR products were then treated with 1 U of uracil-DNA glycosylase (catalog no. 1775375; Roche) at RT for 10 min. For the nested PCR, 5 μl of the first-round reaction mixture was added to a 50-μl reaction mixture that had the same ingredients as the primary reaction mixture but with 2 μM (each) primers AV1 (5′-ATG TGG TTG CTG TGT TGG ATG G-3′) and AV2 (5′-CCG CCG CAA TCA ACT CCA G-3′) and 400 μM dUTP to replace dTTP. The cycling conditions were 1 cycle of 94°C for 5 min and then 40 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 3 min, followed by 1 cycle of 72°C for 7 min. Amplicons of the expected size (298 bp) were visualized with ethidium bromide on 1.5% agarose gels and were then excised and purified with a QIAquick gel extraction kit (Qiagen, Crawley, United Kingdom). Each amplicon was sequenced in both directions by using primers AV1 and AV2 as the sequencing primers.

The DNA sequences of the target regions in IS*900* and in IS*900*-like elements in mycobacterial species other than *M. avium* subsp. *paratuberculosis*, corresponding to the 298-bp amplification product from the IS*900*[L/AV] PCR, were aligned (Fig. 1a). A further alignment of the entire DNA sequences of all these elements was used to optimize the design of IS*900*-specific primer sets unique for the IS*900* element by using the Primer3 design facility available at the Whitehead Institute-Massachusetts Institute of Technology Center for Genome Research website (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/primer3_www.cgi). The resulting primer sets used in the PCR, designated the IS*900*[TJ1-4] PCR (Fig. 1b), were primer pair TJ1 (5′-GCT GAT CGC CTT GCT CAT-3′) and TJ2 (5′-CGG GAG TTT GGT AGC CAG TA-3′) in the first-round PCR and primer pair TJ3 (5′-CAG CGG CTG CTT TAT ATT CC-3′) and TJ4 (5′-GGC ACG GCT CTT GTT GTA GT-3′) in the nested PCR. The reaction conditions were the same as those described above for the IS*900*[L/AV] PCR, with the exception that annealing for both the primary and the secondary rounds

a

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GCCGAAGGAGATTGG-CCGCCCGCGGTCCCG-CGACGACTC-GACCGCTAATTGAGA---GATGCG-ATTGGAT--CGCTG-TGTAAGGACACGTCGGCG IS900
.................................................................................................... St.2333
.T............................................................................................A..... St.22850
...C.G.AG..............TC.............................................C............................. St.V-1
--...-.................AC...-.........................................-.......................C..... IS1626
.......................GC..T..........................................GT.......................C..... St.WA-1
                                                                                ..............A..... St.WA-2
......................AGC...T.........................................T............................. IS1613
CTT...A.GA..G.-.......TGCCGTTTT.G....GTC.T.G..A..G.......TCT..C...T.C.C...GA.....C.C.......CT..T.... IS901
..TTG.AAG..C..-........GCTG..T-.G...TT...T.G.....G.......TCT---......CT.C.......TG.T......A.T.CT.... IS1110
.T.CTCC.G...GCT....A.T..T.ATTGA.GA..TG.GGTC.T....GG..AG.-.....CCT..--.C.GCGG-.G..T.C.CC..G.-.A-...AA IS116
.A..CT.AT..GA..T...G..A.CGC..G.T.CGGCG.AAT.C.G.AC.GC.CGCGGGC.GCAG.T-C.GC..AA..TG.A..CGC.CGT...A.AC.A IS110

TGGTCGTCTGCTGGG-TTGATCTGGACAATGA-CGGTTACGGAG-GTGGTTGTGGCACAACCTGTCTGGGCG--GGCGTGGACGCCGGTAAGGCCGACCA IS900
...........C........................................................................................ St.2333
........C........C...TG....G..A.....C...........T.......................A....T.......T.....A..G..... St.22850
...........G....C..G.AG...TG.CAT.T.A.C.................GG....AAC.G.....C....T..C..T.....-...T....... St.V-1
.....................AG...TGCCA..T.A.C..........-C....AGC....AG.............T..C..T.....-...T....... IS1626
.....................AGAA..CGAG.....GA..........................G.....T....T...........G..A..G..... St.WA-1
...................TGGGAA..G.CA....AGC.T...............GC.....GT.G.....C....TA.T..T..T..G..A.T...... St.WA-2
.....................GAT...G..A..T.ACC.T.............A.G....AAC.G.....T.......T..T.....A...T....... IS1613
G...T...C..G...AC..CG----..G.CA--G.AG..GC-G.TA...CC.AAC.CG.C.GA..G....TG....TA.C....T.......T..ACT.. IS901
G...T...C.TG...AACATGGT....G.CA.G..AA..G.AG.TTC.A.G.A...GG.C.AGT.G.....T....TGC...T.T...C....AGC.... IS1110
..C.G...-A.G.T.G.G..A.G.AG.G--AGG.....G-A.CAC.C..C-A..----.T.GGA.A....TC....CA.C.....A..C....GGC.T.. IS116
CT.C..AGGC.G.CACG..CAACTC..C-...AA..GCG..AT.TTC.ACACCGAAGACGTGGGCG..TTCCTC..CCT.....T...C..A..G.CT.. IS110

TTACTGCATG-GTTATTAACGACGACGCGCAGCGATTGCTCTCGCAGCGGGTGGCCAACGACGAGGCCGCGCTG IS900
C......................................................................... St.2333
..........A......CG...G.....G.......C....T....G...T....................... St.28850
.C.T..TG.....C..CG...C.....G.A....GC.............A..TC.............T.TTGGA St.V-1
CC.T...G....C...CG.T.CG..G.GT......C....G..A-GA.....A...........AA....A... IS1626
....G..G.....C..CG...CT..G.GCA..TCG...a.G....G...C..C..T..T............... St.WA-1
CC.T...G.....C..CG...GT..T.G.A....GCA.T.G....TT..C...CT......T..A......... St.WA-2
CC.T...G.....C..CG.T.CA..G.GCA.C...C....T..T......A.CC.......T...AGTT..... IS1613
.C.-...G..C.CG..CG.T...AC..GAA..GTGG..TGG...A..AAAA.CC.G.....AC........A.C IS901
..G-G.TC..C...G.CG.....A.G.GCACCGCGG..T.G....GCAA.C.CA.......T..ACAAC..A.C IS1110
C.G-G..GGTG.CGG.CG...C.....G.G..ACG...T.....AC.AA....AT.............CA.G.C IS116
CC..G..-CAC.GAC.C.C.CCG.C..G.A..AAGG.C...GACA...A.C..C.....AG....C.GAGA... IS110
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b

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L1                   L2                  AV1                        AV2
CTTTCTTGCCGGGTGTTCGG ATGGAGGCGAGGTCACGT  ATGTGGTTGCTG---TGTTGG-ATGG  CTGGAGTTGATTGCGGCGG IS900
                     .............G....  ..........................  ................... St.2333
                     ....C..T...         ..T..TC............T......  G.TG.-.CC---....... St.V-1
........C......G.T.T CGACGC.T..A..T..C.  ...C.-....G.........C-...-  ......C....C.A...A. IS1626
                     ..C..C...T..A.T..C                              ...........CGACAT.. St.WA-1
                     ...AG..T...C....C.                              G.....C......GCA.C. St.WA-2
........C......G...T ....C...........C.  ...C.T....C.....C.GT......  ...C.AG....CCA..... IS1613
--....C.T.G..T..AAG.. T..CTAA.C.C..GGT..  .....T.-.T..CAAC.G.T..T..C  GAA..CC....C..CCA.. IS901
.CG..C.T...TTC..AG.T TG..CT....C..GT.C.  TC.CTC....C.GCC..A...TGC..  .GA..AC.C..C.AT.AAA IS1110
---..CCT...AA...G.G. .GC.T.AA......CG..  ...GCTCCAGGAGAA..GCC.CCC..  ..CACCC.C..C.AGA... IS116
.GG..C..C.A.AG.GC..C GCACG.T.CT...G.TCG  .CCG..CCCGGGAGG..CC.TCGG..  .G..CCG.CT.C.ACAA.C IS110

TJ1                 TJ2                 TJ3                   TJ4
GCTGATCGCCTTGCTCAT  TACTGGCTACCAAACTCCCG CAGCG-GCTGCTTTATATTCC ACTACAACAAGAGCCGTGCC IS900
................G.  C..C................  ......C...G....CC....  ......G............G St.2333
.......A..........  G..C............C....  ......--....C..C..C..  .T....G.GCC...AAG... IS1626
.T.....A.GC....G..  C..C.....T.....C....  ............C..C.....  .T....G...A.A.AAG... St.WA-1
.............T.G..  C..C.....T.....C...R  ...............C..C..  .T....G.GCC.A.AAG... St.WA-2
.......A..C.......  C..C..G.............  ............C.....C..  ......G.TCC...AAG... IS1613
.........G.A..GC.   CG..ATG.G.ACTC.GGG..  .C.A.-.TG.TG-...G....  .....TC..CCC..GCGC.G IS901
......GA.GG.....GC  C..GC.G..T..-..GA...  ..AGTC.G...GG...C.GA.  ...GACC..G..C.GCG..T IS1110
....C.G...C....GG.  G..C.AG..T..G..C....  ...AAC.TC.TC-..CG.G..  .....TC.G.GGC.AAG.G. IS116
C.C.C..A.G--.TCGCC  GGAGC.....GGCT....G.  .T..AA.G.CGCC..CC.G..  C.CC.GC.T.G.C.ACCAGG IS110
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FIG. 1. (a) Alignment of DNA sequences from IS*900* and related elements amplified with the second-round primers AV1 and AV2 in the nested IS*900*[L/AV] PCR, demonstrating sequence differences between these elements; (b) alignments of first- and second-round primers used in the IS*900*[L/AV] PCR and IS*900*[TJ1-4] PCR, demonstrating sequence differences in hybridization to target regions of related elements. Dots indicate the same base, and hyphens indicate deleted bases. For L1, L2, AV1, and AV2, no sequence data were available for strain 22850 and parts of strains WA-1, WA-2, V-1, and 2333. For TJ1, TJ2, TJ3, and TJ4, no sequence data were available for strains 22850 and V-1.

TABLE 1. Insertion elements of the IS*900* family ranked by their DNA sequence homology to IS*900* in *M. avium* subsp. *pseudotuberculosis*

Element	Organism	Copy no.	% Homology to IS*900*	Detection by[a]:	
				IS*900*[L/AV] PCR	IS*900*[TJ1-4] PCR
IS*900*	*M. avium* subsp. *paratuberculosis*	14–18	99.6–100	Y	Y
Strain 2333	*Mycobacterium* sp.	1	94.7 (1,043)[b]	Y	N
WA-1	*Mycobacterium* sp.	NK[c]	84.3 (1,204)	Y	N
WA-2	*Mycobacterium* sp.	NK	79.3 (1,159)	Y	N
Strain 22850	*Mycobacterium* sp.	NK	87 (186)	Y	N
IS*1613*	*M. avium* subsp. avium	4	83 (1,451)	N	N
IS*1626*	*M. avium* subsp. avium	1	80 (1,451)	N	N
IS*901/902*	*M. avium* subsp. avium	1–17	58.8 (1,451)	N	N
IS*1100*	*M. avium* subsp. avium	1	61 (1,451)	N	N
IS*116*	*Streptomyces clavuligerus*	1	65 (1,451)	N	N
IS*110*	*Streptomyces coelicolor*	1	54 (1,451)	N	N

[a] Y, yes; N, no.
[b] The values in parentheses are the length (in base pairs) of the DNA sequence for which homology was evaluated.
[c] NK, not known.

was carried out at 60°C. The sequences of the primers used for the IS*900*[TJ1-4] PCR differed from the sequence of the most closely related IS*900*-like element by 9 bp and yielded a specific final amplification product of 294 bp.

The following precautions were implemented to exclude amplicon contamination and to monitor the PCR products for amplicon contamination: the PCR master mixtures were made each day in a separate DNA-free room before sample extraction or product detection. Screw-cap reaction tubes were used at all times because flip-top caps tended to leak, especially during the phenol processing steps. Transfer of products between the first- and second-round PCRs was carried out in a laminar-flow cabinet in which a designated area had been exposed to UV irradiation overnight. Sample preparation and amplicon visualization and purification were carried out in separate dedicated rooms. Positive controls were processed separately after all samples, negative controls, or reagent controls had been processed. A negative dilution control tube (containing 5 μl of the 1× TE batch used to redissolve the DNA pellets), a positive process control tube, a negative process control tube, and a reagent control tube were included in each sample processing or PCR run. Coded samples shown to be IS*900*-specific PCR negative in duplicate were subsequently tested for PCR inhibition by repeating the PCR with the original DNA extract spiked with an estimated 1 to 10 copies of IS*900* plasmid pIDL60. DNA from coded samples shown to be IS*900*-specific PCR positive with the primary DNA extract were reamplified for amplicon sequencing as described above.

RESULTS

Table 1 ranks 10 members of the IS*900* family of insertion elements in the order *Actinomycetales* by sequence homology to IS*900*. The IS*900*[L/AV] nested PCR did not report the presence of IS*110*, IS*116*, IS*1110*, IS*901/902*, IS*1626*, or IS*1613.* This reaction did amplify the most closely related IS*900*-like elements from mycobacterial isolates designated *Mycobacterium* sp. strain 22850, *Mycobacterium* sp. strain WA-1, *Mycobacterium* sp. strain WA-2, and *Mycobacterium* sp. strain 2333 (Fig. 2). These non-IS*900* products could be differentiated from IS*900* itself by sequencing of the amplicon DNA, as demonstrated in Fig. 1a. The primers used for the IS*900*[TJ1-4] nested PCR (Fig. 1b) subsequently developed, together with the associated reaction conditions, were uniquely specific for IS*900* (Fig. 2).

Patient characteristics are shown in Table 2. The CD group consisted of 37 patients (21 males, 16 females) with endoscopically active inflammatory disease. Their ages ranged from 14 to 60 years (median age, 31 years; interquartile age range, 21 to 44 years). Their disease duration had ranged from 6 months to 20 years. Thirteen patients had ileal disease, 10 patients had colonic disease, and 14 patients had both. Fourteen patients had previously had a surgical resection, with granulomas identified histologically in the specimens resected from three patients. Granulomas were identified in the ileocolonoscopic mucosal biopsy specimens of a further four patients. None of the patients were receiving anti-*M. avium* subsp. *paratuberculosis* treatment with rifabutin and clarithromycin (17). At the time of sampling, 16 patients were being treated with prednisolone and 7 were being treated with azathioprine. Thirty of the CD patients were from the United Kingdom; four were from Ireland; and one each was from the United States, Germany, and the United Arab Emirates. The nIBD control group comprised 34 patients (15 males, 19 females) undergoing ileocolonoscopy who did not have a clinicopathological diagnosis of CD. Their ages ranged from 31 to 82 years (median age, 56 years; interquartile age range, 44 to 69 years). Of the 34 individuals, 10 were asymptomatic and were undergoing screening procedures, 5 had an episode of rectal bleeding, 3 were being investigated for anemia, 2 had colon cancer, and 1 had a tubular adenoma. Three individuals had a clinical diagnosis of irritable bowel syndrome (IBS). In addition, 10 individuals had previously undiagnosed abdominal pain and/or diarrhea; 1 of these was subsequently found to have what was labeled ischemic colitis in an inflammatory segment of the transverse colon, 1 had active diverticulitis of the sigmoid colon, one had lymphocytic colitis, and one was human immunodeficiency virus (HIV) positive. None of the individuals in the nIBD control group were receiving antibiotics, and all were from the United Kingdom.

The coded mucosal DNA extracts from 9 of 34 (26%) nIBD patients and 34 of 37 (92%) CD patients were positive when they were tested directly by the IS*900*[L/AV] nested PCR (P = 0.0002 by the χ^2 test; odds ratio = 3.47). The amplicon sequences were a 100% match with the IS*900* sequence in all cases. The IS*900*[TJ1-4] nested PCR was also positive when it was performed directly with each of seven selected IS*900*-positive DNA extracts. Final verification of IS*900* was obtained when sequencing of amplicons from these reactions again showed 100% identity with the IS*900* sequence of *M. avium* subsp. *paratuberculosis*. All negative reagent and process controls as well as dilution controls were PCR negative, demonstrating the absence of amplicon contamination. All samples

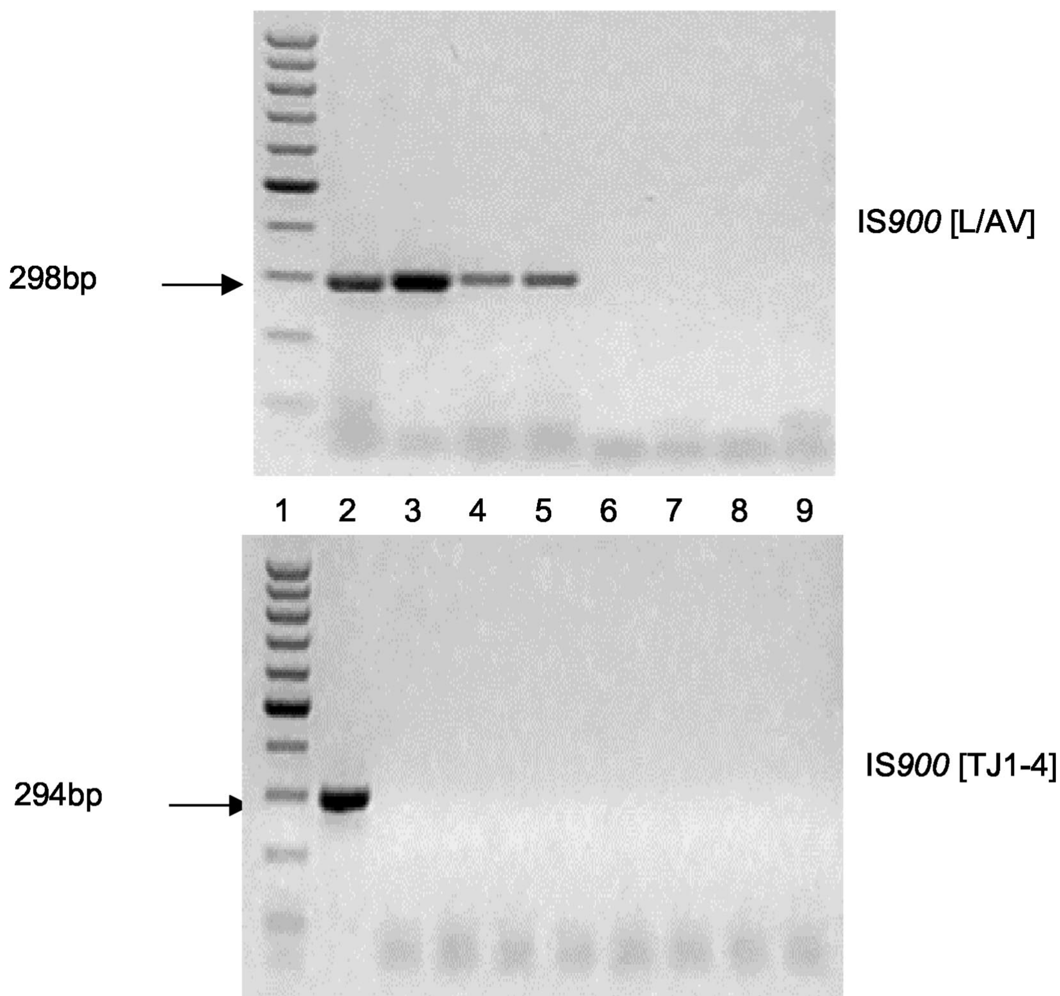

FIG. 2. PCR products from the IS*900*[L/AV] nested PCR (upper panel) and the IS*900*[TJ1-4] nested PCR (lower panel) obtained with DNA from the following sources: mucosal biopsy specimen from a patient with CD (lane 2), culture of *Mycobacterium* sp. strain 2333 (lane 3), culture of *Mycobacterium* sp. strain 28850 (lane 4), culture of *Mycobacterium* sp. strain WA-1 (lane 5), culture of *M. avium* subsp. *avium* containing IS*901* (lane 6), culture of *M. avium* subsp. *avium* containing IS*1613* (lane 7), a plasmid containing a single full copy of IS*1626* (lane 8), and a mucosal biopsy specimen from a patient without CD (lane 9). Lane 1, 100-bp ladder.

testing negative by the IS*900*[L/AV] nested PCR were retested after they were spiked with a low IS*900* copy number and were shown not to be inhibitory.

Of the tissue biopsy specimens from nine nIBD patients that were positive when they were directly tested by PCR, three were from patients with IBS, one was from a patient with a tubular adenoma of the colon, one was from a patient with histologically diagnosed lymphocytic colitis, one was from a patient with a segment of active diverticulitis in the sigmoid colon, one was from a patient with an inflammatory segment in the transverse colon designated ischemic colitis, one was from a patient with diarrhea and hyperthyroidism, and one was from a patient who was HIV positive. Both the HIV-positive and lymphocytic colitis patients were also PCR positive by MGIT culture after their biopsy specimens were incubated for 56 and 38 weeks, respectively. A 10th patient in the nIBD control group who had no endoscopic abnormality and whose mucosal biopsy specimen initially tested negative by direct PCR subsequently tested positive for *M. avium* subsp. *paratuberculosis* by MGIT culture after 38 weeks of incubation.

All three patients in the CD group who initially tested negative by the IS*900*[L/AV] nested PCR directly with the tissue DNA extract subsequently tested negative for *M. avium* subsp. *paratuberculosis* by MGIT culture after between 31 and 51 weeks of incubation. All three patients were from the United Kingdom. Two of them had terminal ileal disease and patchy involvement of the colon, and one of them had presented with orofacialgranulomatosis. The third PCR-negative patient in the CD group had a segment of left-sided Crohn's colitis, access to which was limited by the presence of a stricture at the rectosigmoid junction.

Among the whole group of 43 PCR-positive patients, both duplicates from all biopsy sites were PCR positive for 21 (49%) patients. Both duplicates from at least one biopsy site were PCR positive for 13 of the 43 (30%) patients, and one of the

TABLE 2. Patient details[a]

CD group							nIBD control group			
Patient no.	Sex	Age (yr)	Site	Duration (yr)	Gran.	Drugs	Patient no.	Sex	Age (yr)	Diagnosis
1	F	35	TI + C	9	+	P	38	M	45	Screening
2	F	31	TI	3			39	M	65	Ischemic colitis
3	M	49	TI + C	5	+	P	40	M	69	Anemia
4	M	42	C	1		P + A	41	M	65	Rectal bleeding
5	M	32	C	12		P	42	F	48	Rectal bleeding
6	M	50	TI	8		P + A	43	M	82	Colon carcinoma
7	M	25	TI	1		P	44	F	37	IBS
8	F	22	C	6		P + A	45	F	56	Screening
9	M	23	TI	5			46	F	81	Screening
10	M	25	TI + C	8			47	M	63	Tubular adenoma
11	F	31	TI	11			48	F	44	Hyperthyroid
12	M	42	C	8	+		49	F	39	Screening
13	M	19	TI + C	4		P	50	M	37	HIV
14	F	26	TI	6			51	M	65	Screening
15	F	26	C	1	+		52	F	65	Screening
16	F	42	TI + C	6			53	M	53	Rectal bleeding
17	F	49	TI	0.5			54	M	72	IBS
18	M	54	TI	20			55	F	31	Screening
19	M	20	TI + C	6	+	P	56	F	60	Screening
20	F	21	TI + C	4			57	F	68	Diarrhea
21	F	38	C	5		P	58	M	35	Diarrhea
22	F	14	TI + C	2			59	M	35	Rectal bleeding
23	M	29	TI + C	9		P + A	60	F	52	Diverticulitis
24	M	39	TI	4		P	61	F	77	Anemia
25	M	53	C	3		P	62	F	56	Screening
26	F	30	TI + C	10			63	F	59	Colon carcinoma
27	F	29	C	2		P	64	F	69	Abdominal pain
28	M	33	TI + C	5		P	65	M	54	Lymphocytic colitis
29	F	17	TI	1			66	F	36	Rectal bleeding
30	M	34	C	5			67	F	68	Diarrhea
31	F	21	TI + C	5	+		68	M	60	Rectal bleeding
32	M	50	TI	10			69	M	55	Diarrhea
33	M	32	TI + I	10		A	70	F	50	Screening
34	F	14	C	4			71	F	36	IBS
35	M	60	TI + I	20		A				
36	M	26	TI + C	13	+	P + A				
37	M	22	TI + R	5						

[a] Abbreviations: M, male; F, female; TI, terminal ileum; I, ileum; C, colon; R, rectum; Gran, granuloma detected histologically; P, prednisolone; A, azathioprine.

duplicates from only a single biopsy site was PCR positive for 9 (21%) patients.

MGIT cultures were not available for four patients in the CD group and one patient in the nIBD control group. Overall, 14 of the remaining 33 (42%) CD patients and 3 of 33 (9%) nIBD control patients were subsequently *M. avium* subsp. *paratuberculosis* positive by PCR with samples from the MGIT culture ($P = 0.0019$ by Fisher's exact test; odds ratio = 4.66). All PCR amplicons from the cultures showed a 100% identity with IS*900*. For the nIBD control group, none of the MGIT tissue cultures, including four which were positive by direct PCR, were IS*900*[L/AV] PCR positive for *M. avium* subsp. *paratuberculosis* after incubation for $<$38 weeks. For the CD group, none of the MGIT cultures were IS*900*[L/AV] PCR positive after incubation for $<$20 weeks. However, 5 of 14 (36%) samples from CD patients positive by MGIT culture were positive for *M. avium* subsp. *paratuberculosis* by the IS*900*[L/AV] nested PCR before 38 weeks of incubation. Cultures of samples from 9 of 15 (60%) CD patients incubated for more than 38 weeks were positive for *M. avium* subsp. *paratuberculosis*.

To demonstrate the growth of *M. avium* subsp. *paratuberculosis*, eight MGIT cultures of tissue shown to be IS*900* positive by direct testing were subcultured after 8 to 10 weeks of incubation. Six of the eight original MGIT cultures and all of the MGIT subcultures were subsequently shown to be IS*900* PCR positive after 38 weeks of incubation. However, the isolation of *M. avium* subsp. *paratuberculosis* from these positive MGIT cultures on solid medium or in axenic culture has not yet been achieved. These studies are ongoing. Five of 23 MGIT cultures which became cloudy due to bacterial growth after $>$12 weeks were subcultured onto solid mycobacterial culture medium. Colonies of acid-fast bacilli grew in 2 to 6 weeks, but they were all subsequently shown to be negative by the IS*900*[L/AV] nested PCR.

DISCUSSION

IS*900* is defined as a 1,451-bp multicopy element inserted into 14 to 18 conserved loci in the *M. avium* subsp. *paratuberculosis* genome, whose complete DNA sequence shares 99.6 to 100% homology with the IS*900* sequences deposited in GenBank. To date, IS*900*, as defined, is unique to *M. avium* subsp. *paratuberculosis*. The IS*900*[L/AV] nested PCR described here

did not detect any of the other elements in the IS*900* family with the exception of those reported in the mycobacterial isolates from Sweden (*Mycobacterium* sp. strain 2333) (11) and Australia (*Mycobacterium* sp. strain 28850, *Mycobacterium* sp. strain WA-1, and *Mycobacterium* sp. strain WA-2) (10). These rare elements can be excluded by amplicon sequencing. The IS*900*[TJ1-4] nested PCR was uniquely specific for IS*900* and *M. avium* subsp. *paratuberculosis*.

Previous work has shown that *M. avium* subsp. *paratuberculosis* is extraordinarily difficult to detect reliably and reproducibly by PCR tests applied directly to DNA extracted from human tissue and other samples (33). The use of suboptimal sample processing procedures results in false-negative results. The following steps in the DNA extraction procedure were found to be of importance: processing of the tissues when they fresh, never when they were frozen; mechanical disruption as described above to ensure access to *M. avium* subsp. *paratuberculosis* DNA; resuspension of the DNA pellets overnight at 4°C; the use of Expand HF *Taq* polymerase (Roche); and the adoption of nested PCR. The sensitivity of the reaction is greatly increased by the presence of multiple copies of IS*900.* Screw-cap tubes and not flip-top tubes should be used throughout. An important factor in the avoidance of PCR inhibition proved to be the individual testing of single fresh biopsy specimens.

The present study was designed to test human mucosal tissue samples under optimal conditions after the samples were brought directly to the laboratory from the endoscopy suite. Samples were tested on two occasions with the investigator blinded to the clinical diagnosis. Confidence in the results was ensured by rigorous retesting. The results for positive samples were confirmed by amplicon sequencing. Inhibition was excluded by spiking negative samples with the template at a low copy number. The presence of contamination artifacts was excluded by the extensive use of simultaneous controls, as described above.

The clinical control group consisted of patients with nIBD who were referred for endoscopy and who did not have a diagnosis of CD by established clinicopathological criteria; this group did not represent a healthy population. The difference in the age ranges of the CD and control groups reflects the fact that the peak incidence of CD is in the age range of 20 to 30 years, whereas the indication for ileocolonoscopy in cases of suspected cancer or other colonic conditions usually occurs in older people. An effect of these age differences on the percentage of individuals in whom *M. avium* subsp. *paratuberculosis* was detected in the present study cannot be excluded. Nine of the 34 nIBD control group patients were found to harbor *M. avium* subsp. *paratuberculosis*. In two of these patients, one with a tubular adenoma of the colon and the other with hyperthyroidism and diarrhea, the histology of the gut mucosa was normal. A third patient who was negative by direct PCR and in whom *M. avium* subsp. *paratuberculosis* was subsequently identified by a positive MGIT culture also had a histologically normal mucosa. One PCR-positive nIBD control group patient had an abnormal segment of transverse colon showing focal gland branching, mild chronic inflammatory infiltrate, vascular inflammation, and mucosal ulceration was diagnosed as probably having ischemic colitis. The mucosa sampled from a segment of sigmoid colon with diverticulitis in a fourth nIBD control group patient tested PCR positive. This is consistent with an *M. avium* subsp. *paratuberculosis* superinfection in a preexisting abnormality and is in line with the previous description of typical changes associated with CD affecting a segment of colon with diverticulitis (13). A fifth patient diagnosed with lymphocytic colitis had extensive intraepithelial lymphocytosis throughout the colon. A sixth patient who was positive for *M. avium* subsp. *paratuberculosis* by direct PCR as well as by MGIT culture was HIV positive. Biopsy specimens from the right and left colon and rectum of this patient showed occasional gland branching and increases in the numbers of inflammatory cells in the lamina propria, consistent with mild chronic inflammatory disease. This is the second description of the occurrence of *M. avium* subsp. *paratuberculosis* infection in an HIV-positive patient, although on this occasion the patient did not have clinical AIDS (25).

Three patients in the nIBD control group had IBS. All had suffered from abdominal discomfort and bloating, gas, and loose motions for some years; and all were clinically diagnosed as having IBS. In one of these patients, a pronounced disordered hypermotility state was seen in the right colon during endoscopy. The gut mucosa of none of these three nIBD control group patients showed any histological abnormality. All three were infected with *M. avium* subsp. *paratuberculosis*. Research from the Veterinary Institute at Massey University, Palmerston North, New Zealand (16), has shown that 8 of 12 *M. avium* subsp. *paratuberculosis*-infected sheep with naturally occurring Johne's disease and 5 of 14 sheep experimentally infected with *M. avium* subsp. *paratuberculosis* developed an enteric neuropathy that particularly affected the myenteric plexus. Distinct aggregations of mononuclear cells around nerves and ganglion cells were seen and were noted to resemble the lesions observed in human tuberculoid leprosy (16). Enteric neuropathy (12, 30) and abnormalities of enteric glial cells (3) are well described in humans and affect a substantial proportion of individuals with CD. Recent studies (32) from Sweden with full-thickness jejunum biopsy specimens demonstrated that 9 of 10 individuals with IBS had clear evidence of enteric neuropathy with lymphocytic infiltration around the ganglia and the nerve fibers of the myenteric plexus. Six of these patients with IBS also had neuronal degeneration. *M. avium* subsp. *paratuberculosis* expresses the HupB protein, which is also expressed by *Mycobacterium leprae* and which is involved in its initial interaction with Schwann cells around myelinated nerves (7, 31). Taken together, these studies suggest that enteric neuritis and neuropathy resembling those caused by *M. leprae* may develop in animals and humans as a result of *M. avium* subsp. *paratuberculosis* infection (18). Studies with larger numbers of individuals are necessary to investigate whether a relationship exists between *M. avium* subsp. *paratuberculosis* infection and the development of IBS.

The detection and verification of *M. avium* subsp. *paratuberculosis* in inflamed ileal and colonic mucosae from 92% of individuals with CD in this study, regardless of whether they were found microscopically to have granulomas, are highly significant. All four CD patients from Ireland, as well as those from the United States, Germany, and the United Arab Emirates, were *M. avium* subsp. *paratuberculosis* positive, suggesting that exposure to these pathogens occurs on an international basis. The high rate of detection of this organism in the present

study is in agreement with the isolation of *M. avium* subsp. *paratuberculosis* by culture from the guts of six of seven (86%) people with CD in the United States (27) and its localization by IS*900* in situ hybridization with fixed gut tissue from 27 of 33 (82%) CD patients in Italy (28). The *M. avium* subsp. *paratuberculosis* detection rate of 92% by incorporation of the mechanical disruption step and an optimized nested PCR is higher than the *M. avium* subsp. *paratuberculosis* detection rate of 19% by PCR in patients with CD reported by researchers in the United States and Denmark (8), who did not include the mechanical disruption step or a nested reaction. These comparisons emphasize the importance of optimized critical methods for the reliable detection of *M. avium* subsp. *paratuberculosis*. The detection of *M. avium* subsp. *paratuberculosis* by the IS*900*-specific PCR in all the biopsy specimens from throughout the gut from about half of the PCR-positive patients suggests that *M. avium* subsp. *paratuberculosis* infection can be extensive. We did not carry out repeated PCR tests of the liquid MGIT cultures over time in order to avoid risking contamination of the long-term cultures. Incubation times before PCR testing ranged from 14 to 64 weeks for the CD group and 14 to 88 weeks for the nIBD control group, with 38 weeks of incubation being the optimal time for PCR detection of *M. avium* subsp. *paratuberculosis* in CD patients. Sixty percent of MGIT cultures of samples from CD patients incubated for more than 38 weeks were positive for *M. avium* subsp. *paratuberculosis*. This suggests that PCR of MGIT cultures will be a useful additional system for the detection of *M. avium* subsp. *paratuberculosis*. Thirty-two MGIT cultures (18 PCR positive and 14 PCR negative) became cloudy after, but not before, 12 weeks of incubation. Microscopy demonstrated the presence of abundant acid-fast bacilli, indicative of the growth of other mycobacteria. When these fast growers were isolated on solid medium, all tested IS*900*-specific PCR negative. This is consistent with the known presence of mycobacteria in the guts of a high proportion of people.

The principal property which distinguishes *M. avium* subsp. *paratuberculosis* from all other candidate organisms in the causation of CD is the specific ability of *M. avium* subsp. *paratuberculosis* to cause chronic inflammation of the intestine of a broad range of histopathological types in many species of animals, including primates. Its presence in the overwhelming majority of individuals with chronic inflammation of the intestine of the CD type inevitably suggests causality. However, the presence of *M. avium* subsp. *paratuberculosis* in CD patients as a consequence of preexisting inflammation cannot be excluded at this stage. An association between *M. avium* subsp. *paratuberculosis* and disease causation is favored by the preliminary findings of each of four open-label clinical studies [1, 15, 29; A. Douglass, P. A. Cann, and M. G. Bramble, Gut **46**(Suppl. II)**:** A11, 2000], which suggest that drugs such as rifabutin and clarithromycin, which are more active against *M. avium* subsp. *paratuberculosis* than standard antituberculous therapy, may be followed by remission and healing of the inflamed intestine in CD patients. A randomized placebo-controlled trial of rifabutin, clarithromycin, and clofazimine treatment of CD is in progress (W. Selby, B. Crotty, T. Florin, and P. Pavli, J. Gastroenterol. Hepatol. **16:**A8, 2001).

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