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Highly Specific and Quick Detection of *Mycobacterium avium* subsp. *paratuberculosis* in Feces and Gut Tissue of Cattle and Humans by Multiple Real-Time PCR Assays▽

Can Imirzalioglu,[1] Heinrich Dahmen,[2] Torsten Hain,[1] Andre Billion,[1] Carsten Kuenne,[1] Trinad Chakraborty,[1] and Eugen Domann[1]*

Institute of Medical Microbiology, Frankfurter Strasse 107, D-35392 Giessen, Germany,[1] and Veterinary Practice, Burgring 9, D-54595 Pruem, Germany[2]

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***Mycobacterium avium* subsp. *paratuberculosis* is the causative agent of Johne's disease (JD) in cattle and may be associated with Crohn's disease (CD) in humans. It is the slowest growing of the cultivable mycobacteria, and culture from clinical, veterinary, food, or environmental specimens can take 4 months or even longer. Currently, the insertion element IS*900* is used to detect *M. avium* subsp. *paratuberculosis* DNA. However, closely related IS*900* elements are also present in other mycobacteria, thus limiting its specificity as a target. Here we describe the use of novel primer sets derived from the sequences of two highly specific single copy genes, MAP2765c and MAP0865, for the quantitative detection of *M. avium* subsp. *paratuberculosis* within 6 h by using real-time PCR. Specificity of the target was established using 40 *M. avium* subsp. *paratuberculosis* isolates, 67 different bacterial species, and two intestinal parasites. Using the probes and methods described, we detected 27 (2.09%) *M. avium* subsp. *paratuberculosis*-positive stool specimens from 1,293 individual stool samples by the use of either IS*900* or probes deriving from the MAP2765c and MAP0865 genes described here. In general, bacterial load due to *M. avium* subsp. *paratuberculosis* was uniformly low in these samples and we estimated 500 to 5,000 *M. avium* subsp. *paratuberculosis* bacteria per gram of stool in assay-positive samples. Thus, the methods described here are useful for rapid and specific detection of *M. avium* subsp. *paratuberculosis* in clinical samples.**

Mycobacterium avium subsp. *paratuberculosis* is the causative agent of Johne's disease (JD) in cattle, and it has been suggested that this microorganism may be associated with Crohn's disease (CD) in humans (14, 17). *M. avium* subsp. *paratuberculosis* belongs to the mycobacterial species *M. avium*, which is currently subdivided into three subspecies (33): *M. avium* subsp. *avium* (synonym, *M. avium*), *M. avium* subsp. *paratuberculosis* (synonym, *M. paratuberculosis*), and *M. avium* subsp. *silvaticum* (synonym, *M. silvaticum*). At the subspecies level, *M. avium* subsp. *paratuberculosis* can be differentiated phenotypically from *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum* by its dependence on mycobactin (51) and genotypically by the presence of multiple copies of the insertion element IS*900* (2, 5, 13, 22, 45).

JD (or paratuberculosis) is a chronic, granulomatous severe form of gastroenteritis with progressive weight loss and emaciation affecting domestic and wild ruminants, e.g., cattle, sheep, goats, red deer, and rabbits worldwide (12, 30, 31). Infected livestock periodically shed *M. avium* subsp. *paratuberculosis* via feces and milk, which results in environmental distribution, where *M. avium* subsp. *paratuberculosis* can survive for extended periods (55). Milk pasteurization trials showed that high-temperature and short-duration standard pasteurization procedures do not effectively kill *M. avium* subsp. *paratuberculosis* in milk, as clinical strains of *M. avium* subsp. *paratuberculosis* have been shown to be more thermally tolerant than either *M. bovis* or *Coxiella burnetii*, the current milk pasteurization standard microorganisms (10, 44, 47, 48). Therefore, human contact can result from the consumption of inadequately pasteurized milk or raw milk or of certain other dairy products, fecally contaminated vegetables, contaminated beef, or even water (12, 14, 34, 41, 47, 48).

CD is a chronic inflammatory disease of the gastrointestinal tract in humans, affecting in particular the terminal ileum, with symptoms of general malaise, weight loss, abdominal pain, and diarrhea (4, 12, 16, 40). A hallmark of CD is the histological proof of a granulomatous inflammation, which is also characteristic of JD and other mycobacterial diseases, leading to suggestions that *M. avium* subsp. *paratuberculosis* may be associated with CD in humans (see references 12 [and references therein], 8, 15, 16, 27, and 28).

Currently, there is controversy as to whether *M. avium* subsp. *paratuberculosis* (i) is an innocent bystander that has merely colonized the intestine of Crohn's patients, (ii) could be a secondary infection but not a cause of the disease, (iii) could be the primary infectious agent and the cause of CD, (iv) acts as a superantigen, or (v) modifies the immune response in CD (6, 12, 15, 25, 32, 37, 42, 43, 50). One of the major obstacles to resolution of the debate on the role of *M. avium* subsp. *paratuberculosis* in CD and the controversial studies published is the requirement of reliable and contemporary detection and identification of *M. avium* subsp. *paratuberculosis* in complex

* Corresponding author. Mailing address: Institute of Medical Microbiology, Frankfurter Strasse 107, D-35392 Giessen, Germany. Phone: 49 641-9941287. Fax: 49 641-9941259. E-mail: eugen.domann@mikrobio.med.uni-giessen.de.

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TABLE 1. Specific primers and TaqMan probes used in this study to detect *M. avium* subsp. *paratuberculosis*

Target (GenBank accession no.)	Oligonucleotide sequence	Reference(s) or source
Amplicon I IS900 (AE016958.1)		
Forward	5′-AAT GAC GGT TAC GGA GGT GGT-3′	21, 22
Reverse	5′-GCA GTA ATG GTC GGC CTT ACC-3′	
Probe	FAM-TCC ACG CCC GCC CAG ACA GG-TAMRA	
Amplicon II 251 (AF445445)		
Forward	5′-GCA AGA CGT TCA TGG GAA CT-3′	2, 38
Reverse	5′-GCG TAA CTC AGC GAA CAA CA-3′	
Probe	FAM-CTG ACT TCA CGA TGC GGT TCT TC-TAMRA	
Amplicon III f57 (X70277)		
Forward	5′-TAC CGA ATG TTG TTG TCA CCG-3′	35, this study
Reverse	5′-TGG CAC AGA CGA CCA TTC AA-3′	
Probe	FAM-CCG GTC CCA GGT GTG TTC GAG TTG-TAMRA	
Amplicon IV MAP0865 (AE016958.1)		
Forward	5′-GCG CGG CCA GTA TGG ATA TA-3′	19, 22, this study
Reverse	5′-GAC TCA ACC CAA CGA GCT CC-3′	
Probe	FAM-AGA TGC CTC TCC GAT GCT CGA TGG-TAMRA	
Amplicon V_1 16S rRNA gene		
Universal forward	5′-TCC TAC GGG AGG CAG CAG T-3′	23
Universal reverse	5′-GGA CTA CCA GGG TAT CTA ATC CTG TT-3′	
Universal probe	FAM-CGT ATT ACC GCG GCT GCT GGC AC-TAMRA	
Amplicon V_2 18S rRNA gene		
Universal forward	5′-GCG GAT CCG CGG CCG CTG GTT GAT CCT GCC AGT-3′	26
Universal reverse	5′-GCG GAT CCG CGG CCG CGG CAG GTT CAC CTA C-3′	

specimens such as blood, biopsy samples, breast milk, and feces. *M. avium* subsp. *paratuberculosis* is the slowest growing of the cultivable mycobacteria, and primary culture from clinical, veterinary, food, or environmental specimens can take 4 months or even longer (12, 24, 44, 55). Moreover, the characteristics of *M. avium* subsp. *paratuberculosis* in JD and in CD seem to be totally different: in CD, *M. avium* subsp. *paratuberculosis* appears as non-acid-fast coccobacilli with the ultrastructure of spheroplasts (cell-wall-deficient forms) that do not transform into characteristic *M. avium* subsp. *paratuberculosis* organisms until after several months of incubation (4).

In this study, we aimed at establishing highly specific multiple quantitative real-time PCR (qrt-PCR) assays based on the published genome sequence of *M. avium* subsp. *paratuberculosis* strain K-10 to enable rapid and unequivocal detection and identification of *M. avium* subsp. *paratuberculosis* directly from clinical, veterinary, food, or environmental specimens as well as from pure cultures. The method developed allows the quick and reliable detection and quantification of *M. avium* subsp. *paratuberculosis* directly from stool samples within 6 h at reasonable costs.

MATERIALS AND METHODS

Human clinical samples and data. Patient fecal and tissue specimens represented clinical routine diagnostic samples for the detection of pathogenic bacteria and parasites. Testing for the presence of *M. avium* subsp. *paratuberculosis* DNA was performed in addition to DNA detection of conventional enteropathogenic bacteria and parasites. Positive results were reported. Clinical data from patients with positive results were obtained after informed consent by record review. The study was approved by the Ethics Board of the Justus-Liebig-University of Giessen, Faculty of Medicine.

Cattle samples. Feces and gut biopsy specimens were obtained from healthy cows and from cows with suspected paratuberculosis. The symptoms of clinical paratuberculosis are chronic diarrhea and progressive weight loss, whereas subclinically infected animals mainly exhibit decreased milk production. The guts were dissected into small pieces and subdivided into inflamed and noninflamed tissue samples based on microscopic examination.

Microorganisms and standard cultivation. The mycobacterial and nonmycobacterial species used in the study are listed here (see Table 2). The strains were obtained from the German Resource Center for Biological Material (DSMZ) and from the strain collection of the Institute of Medical Microbiology, Justus-Liebig University of Giessen.

M. avium subsp. *paratuberculosis* was cultivated on BD BBL Herrold's egg yolk agar with Mycobactin J and ANV (Becton Dickinson, Heidelberg, Germany) and in modified Middlebrook 7H9 medium for up to 4 months as previously described (12, 39). All remaining bacteria were grown under optimal conditions on appropriate media as previously recommended (3).

Acid-fast staining. The acid-fast staining of specimens was done by the Ziehl-Neelsen procedure, and stained specimens examined under conditions of oil immersion. Dissected biopsy samples from gut tissue of cows were stained as previously described for tissue sections of bisons (20).

DNA extraction. DNA was extracted from pure bacterial cultures with a RTP Spin Bacteria DNA kit (Invitek, Berlin, Germany). The total DNA from human and animal fecal specimens was extracted with a PSP Spin Stool DNA kit and from tissue with an Invisorb Spin Tissue minikit (Invitek) as recommended by the vendor.

Parasitic DNA from *Entamoeba histolytica* and from *Giardia lamblia* was obtained from the Bernhard-Nocht Institute for Tropical Medicine (Hamburg, Germany).

Quantitative real-time PCR (qrt-PCR) and conventional PCR. The new primers and probes used were designed with "Primer Express" version 1.0 software (Applied Biosystems, Foster City, CA). The internal probes were labeled with the fluorescent reporter dye 5-carboxyfluoroscein (FAM) on the 5′ end and the quencher dye N′,N′,N′,N′-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3′ end. The qrt-PCR was accomplished as described previously (21, 38). Protozoal universal 18S small-subunit rRNA (SSU-rRNA) detection was performed by conventional PCR using eukaryote-specific primers (Table 1) as described previously (26). The amounts of specific target sequences present in unknown samples were calculated by measuring the threshold cycle (C_T) values and using standard curves generated with a series of known quantities of target sequences. The C_T value represented the cycle at which the copy of the amplified target

sequence intersected the threshold or baseline. Briefly, an inoculation loop of the *M. avium* subsp. *paratuberculosis* DSM 44133 type strain grown on Herrold's egg yolk was carefully resuspended in phosphate-buffered saline (PBS) and serially diluted. The total amounts of cells were counted using a Neubauer counting chamber. We determined the total cell numbers, since complex biological material may contain both viable and dead bacteria.

Detection of *Salmonella enterica* serovar *enteritidis*, *Campylobacter jejuni*, *Yersinia enterocolitica*, *Clostridium difficile*, *Entamoeba histolytica*, and *Giardia lamblia*. The total DNA of human fecal specimens was extracted with a PSP Spin Stool kit (Invitek) as described above. *S. enteritidis*, *C. jejuni*, *Y. enterocolitica*, *C. difficile*, *E. histolytica*, and *G. lamblia* were detected by specific real-time PCRs as previously described (18, 29, 52, 53, 54).

Multiple displacement amplification (MDA) of IS*900*-positive fecal specimens. MDA was performed with the extracted DNA of amplicon I (IS*900*)-positive but amplicon II (MAP2765c [251])-, III (MAP0865 [f57])-, and IV (MAP0865)-negative fecal samples. A commercially available GenomiPhi DNA amplification kit (Amersham Biosciences, Uppsala, Sweden), which utilizes MDA to exponentially amplify genomic DNA, was used, following the manufacturer's instructions. Briefly, 1 μl of DNA extract was added to 9 μl of sample buffer containing random hexamer primers and heated to 95°C. The chilled sample was mixed with 9 μl of reaction buffer and 1 μl of enzyme mix. The mixture was incubated for 14 h at 30°C and afterwards subjected to heat inactivation for 10 min at 65°C.

Bioinformatics. DNA sequences were aligned with MegAlign version 5.0 software (DNASTAR Inc., Madison, WI) and compared with sequences deposited in the GenBank, EMBL, DDBJ, and PDB databases using the BLASTn basic local alignment search tool (1). The published genome of *M. avium* subsp. *paratuberculosis* strain K-10 (22; GenBank accession number AE016958.1) was analyzed and depicted with the GenomeViz bioinformatics tool (11). The standard curves to calculate the amounts of *M. avium* subsp. *paratuberculosis* in complex stool and biopsy specimens were generated with SigmaPlot 2000 version 6.00 software (SPSS GmbH Software, Munich, Germany).

RESULTS

Development of MAP0865-specific real-time PCRs. Fragment f57 (GenBank accession no. X70277) has been previously described and used as a highly specific 620-bp-long probe for detection of *M. avium* subsp. *paratuberculosis* and diagnosis of Johne's disease (35, 49). The nucleotide-nucleotide BLAST (BLASTn) analysis we performed revealed that it was located in gene MAP0865, one of the 4,350 predicted open reading frames (ORFs) of *M. avium* subsp. *paratuberculosis* strain K-10 (Fig. 1) (22). Fragment f57 covered 48.7% of this 1,272-bp-long ORF with an identity of 100% (Fig. 1). Another BLASTn analysis using the nucleotide sequence of ORF MAP0865 with at least 3.7 million sequences deposited in databases GenBank, EMBL, DDBJ, and PDB revealed it to be unique. Consensus was detected only with deposited MAP0865 and fragment f57 sequences.

By the use of Primer Express software, two new TaqMan amplicons with primers and probes were designed for this *M. avium* subsp. *paratuberculosis*-specific MAP0865 ORF. The first one, designated amplicon III, covered both fragment f57 and ORF MAP0865, while the second, designated amplicon IV, covered only ORF MAP0865 (Fig. 1, Table 1).

Specificities of the MAP0865-specific real-time PCRs. The specificities of the new real-time PCRs using amplicons III and IV were demonstrated by the analysis of DNA from panels of 40 *M. avium* subsp. *paratuberculosis* isolates, 17 other mycobacterial species, 13 Gram-positive bacterial species, 20 Gram-negative bacterial species (including 8 different species causing gastroenteritis), 7 obligate anaerobic bacterial species, and 2 intestinal parasite species (Table 2). The results showed that the real-time PCRs with amplicons III and IV, as well as those performed with previously developed amplicons I (IS*900*) and II (MAP2765c [251]), specifically amplified only the *M. avium* subsp. *paratuberculosis* DNA but not the DNA of the remaining bacteria (Table 2), indicating that the entire ORF MAP0865 is unique for *M. avium* subsp. *paratuberculosis*.

All DNA extracts gave positive results using 16S rRNA gene universal amplicon V_1 for bacteria or 18S rRNA gene universal amplicon V_2 for parasites (Table 2). The nucleic acid concentrations of the DNA extracts were calculated to be at ~50 ng/μl.

Positions of element IS*900*, ORF MAP2765c (251), and ORF MAP0865 (f57) on the chromosome of *M. avium* subsp. *paratuberculosis* strain K-10. A previously published genome-scale comparison of *M. avium* subsp. *paratuberculosis* with its closely related subspecies *M. avium* subsp. *avium* revealed potential new diagnostic sequences (2). Among these, target 251 with a length of 540 bp has been identified as a valuable new sequence for specific amplification of *M. avium* subsp. *paratuberculosis* DNA (38; GenBank accession no. AF445445). Nucleotide sequence alignments indicated that target 251 is located in gene MAP2765c of *M. avium* subsp. *paratuberculosis* strain K-10 (Fig. 1) (22).

Genome visualization using the GenomeViz bioinformatics tool (11) revealed a random distribution of 17 copies of IS*900* on the *M. avium* subsp. *paratuberculosis* chromosome and that both ORF MAP0865 (f57) and ORF MAP2765c (251) exist as singular sequences at diametrically opposite positions (Fig. 1). Eleven copies of IS*900* and MAP0865 (f57) were located on the positive strand, whereas the remaining six copies of IS*900* and MAP2765c (251) were located on the negative strand (Fig. 1). This underlines the utility of IS*900* as a highly sensitive PCR target for *M. avium* subsp. *paratuberculosis* due to its high copy number on the *M. avium* subsp. *paratuberculosis* chromosome. Nevertheless, ORFs MAP0865 and MAP2765c are highly specific PCR targets that can be used to identify *M. avium* subsp. *paratuberculosis* and to confirm IS*900*-specific PCRs.

Optimization of PCR conditions and annealing temperature of IS*900*-, MAP2765c (251)-, MAP0865 (f57)-, and MAP0865-specific real-time PCRs. In order to determine the optimal PCR buffer conditions and the optimal primer annealing temperature for all four amplicons, we analyzed the efficiencies of the real-time PCRs with $MgCl_2$ concentrations in the range of 1.0 to 5.0 mM and temperatures in the range of 55 to 65°C (data not shown). The DNA used was extracted from ~10^8 cells of *M. avium* subsp. *paratuberculosis*, and the experiments were done in triplicate. For all amplicons (I through IV), the optimal $MgCl_2$ concentration was 3.5 mM and the annealing temperature 57.8°C. The ideal results obtained consisted of a C_T value of 14.4 for amplicon I (IS*900*), a C_T value of 18.2 for amplicon II (MAP2765c [251]), and a C_T value of 16.2 for amplicons III (MAP0865 [f57]) and IV (MAP0865) (Fig. 2). The order with respect to sensitivity and efficiency of the TaqMan-PCR was amplicon I first, amplicons III and IV second, and amplicon II third. In cases of amplicon I-positive but amplicon II- to IV-negative specimens, multiple displacement amplification (MDA) was used to exponentially amplify genomic DNA in the DNA extracts of the tested samples. The results suggest the use of MDA as a routine tool for amplification of specimens when low concentrations of *M. avium* subsp. *paratuberculosis*-specific DNA are suspected.

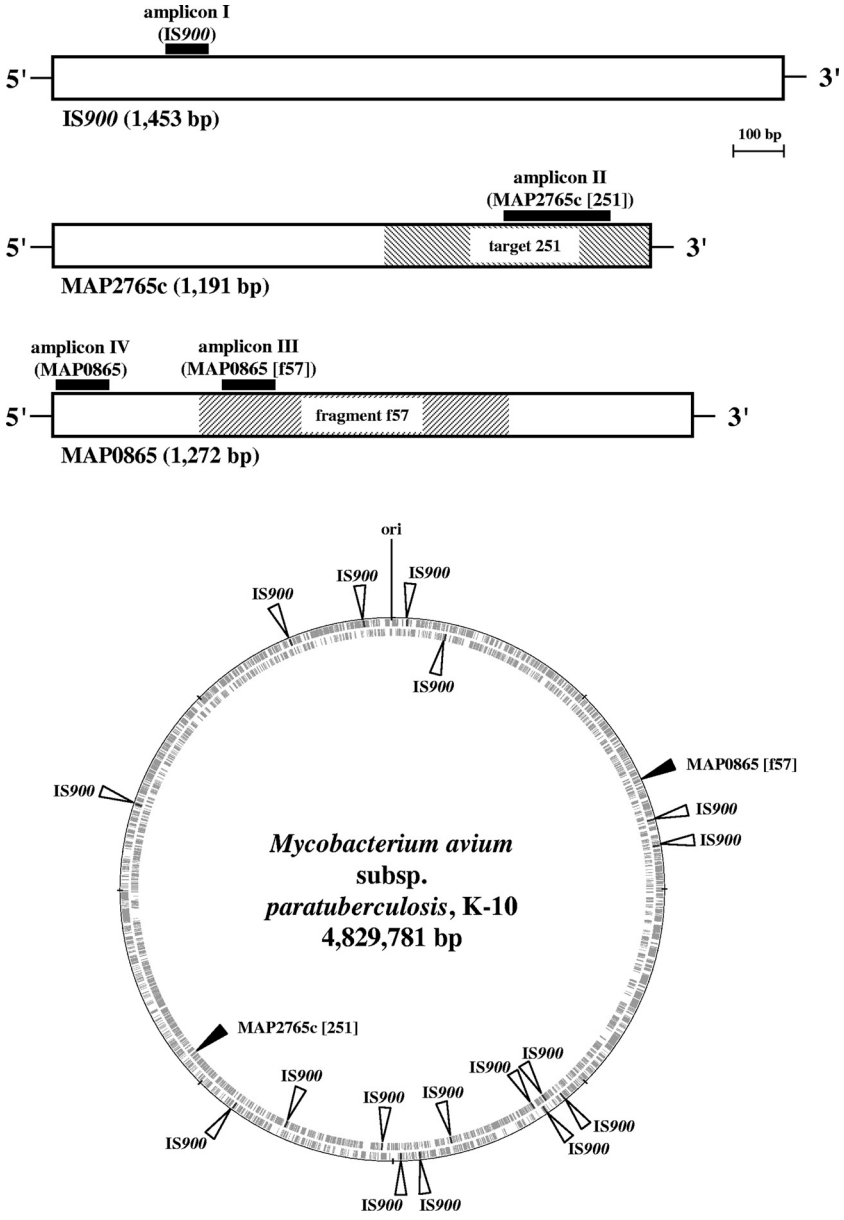

FIG. 1. Graphic presentation of the *M. avium* subsp. *paratuberculosis*-specific chromosomal regions IS*900*, MAP2765c (target 251), and MAP0865 (fragment f57) and their positions in the *M. avium* subsp. *paratuberculosis* K-10 genome (GenBank accession no. AE016958.1). Outer circle, plus strand; inner circle, minus strand. The homology analyses and the graphic presentations were done using the methods BLAST, Clustal W (1), and GenomeViz (11). The black boxes represent specific TaqMan PCR amplicons I through IV, including forward and reverse primers and the internal probe labeled with FAM and TAMRA (Table 1). The amplicons were designed using Primer Express software (Applied Biosystems, Foster City, CA).

The sensitivity of real-time PCR depends on the DNA extraction method used. Since the real-time PCR with amplicon I (IS*900*) showed the highest efficiency, serial dilutions of the genomic DNA of *M. avium* subsp. *paratuberculosis* type strain DSM 44133 were used as templates to assess its sensitivity under the conditions mentioned above. The detection limit of the real-time PCR with amplicon I (IS*900*) was (theoretically) determined to be 1 to 10 CFU/reaction and therefore was identical to the results obtained previously (21). To identify *M. avium* subsp. *paratuberculosis* and to calculate directly the amount of the bacteria in complex biological material such as feces and tissue without cultivation, we assessed the efficiency of the DNA extraction methods with two commercially available kits: a stock solution of 7.3×10^7 *M. avium* subsp. *paratuberculosis* bacteria was serially diluted to 7.3×10^6, 7.3×10^5, 7.3×10^4, 7.3×10^3, 7.3×10^2, and 7.3×10^1 bacteria, and the DNA of the bacteria from each dilution step was extracted using commercially available kits for stool and tissue specimens (Invitek, Berlin, Germany). The stool kit had a detection limit of ≥70 cells/reaction, whereas the detection limit of the tissue kit was ≥10 cells/reaction. Both analyses confirmed very high correlation, as demonstrated by determinations of linear regression (r^2) (Fig. 3).

TABLE 2. Bacterial isolates (and their sources) used in this study[a]

Bacterium	Source	PCR result with indicated amplicon					
		I	II	III	IV	V_1	V_2
M. avium subsp. *paratuberculosis* type strain	DSM 44133	+	+	+	+	+	−
M. avium subsp. *paratuberculosis*	DSM 44135	+	+	+	+	+	−
M. avium subsp. *paratuberculosis* (n = 40)	Field isolates	+	+	+	+	+	−
M. avium subsp. *paratuberculosis*	Patient isolate	+	+	+	+	+	−
M. avium subsp. *avium*	DSM 44158	−	−	−	−	+	−
M. avium subsp. *silvaticum*	DSM 44175	−	−	−	−	+	−
Mycobacterium tuberculosis (n = 10)	Patient isolates	−	−	−	−	+	−
Mycobacterium marinum	Patient isolate	−	−	−	−	+	−
Mycobacterium kansasii	Patient isolate	−	−	−	−	+	−
Mycobacterium chelonae	Patient isolate	−	−	−	−	+	−
Mycobacterium intracellulare	Patient isolate	−	−	−	−	+	−
Mycobacterium abscessus	Patient isolate	−	−	−	−	+	−
Staphylococcus aureus	Patient isolate	−	−	−	−	+	−
Streptococcus agalactiae	Patient isolate	−	−	−	−	+	−
Lactobacillus spp. (n = 5)	Patient isolates	−	−	−	−	+	−
Lactococcus lactis	Patient isolate	−	−	−	−	+	−
Enterococcus faecalis (n = 3)	Patient isolates	−	−	−	−	+	−
Enterococcus faecium	Patient isolate	−	−	−	−	+	−
Listeria monocytogenes	Patient isolate	−	−	−	−	+	−
Salmonella enteritidis (n = 2)	Patient isolates	−	−	−	−	+	−
Salmonella typhimurium (n = 2)	Patient isolates	−	−	−	−	+	−
Salmonella infantis	Patient isolate	−	−	−	−	+	−
Salmonella typhi	Patient isolate	−	−	−	−	+	−
Campylobacter jejuni	Patient isolate	−	−	−	−	+	−
Yersinia enterocolitica	Patient isolate	−	−	−	−	+	−
Shigella flexneri	Patient isolate	−	−	−	−	+	−
Enterohemorrhagic *Escherichia coli* (EHEC)	Patient isolate	−	−	−	−	+	−
Proteus mirabilis (n = 3)	Patient isolates	−	−	−	−	+	−
Citrobacter diversus (n = 2)	Patient isolates	−	−	−	−	+	−
Enterobacter cloacae	Patient isolate	−	−	−	−	+	−
Enterobacter sakazakii	Patient isolate	−	−	−	−	+	−
Escherichia coli	Patient isolate	−	−	−	−	+	−
Hafnia alvei	Patient isolate	−	−	−	−	+	−
Helicobacter pylori	Patient isolate	−	−	−	−	+	−
Clostridium difficile	Patient isolate	−	−	−	−	+	−
Fusobacterium nucleatum	Patient isolate	−	−	−	−	+	−
Propionibacterium acnes	Patient isolate	−	−	−	−	+	−
Bacteroides fragilis	Patient isolate	−	−	−	−	+	−
Prevotella intermedia	Patient isolate	−	−	−	−	+	−
Veillonella spp.	DSM 2008	−	−	−	−	+	−
Atopobium spp.	DSM 15829	−	−	−	−	+	−
Entamoeba histolytica	BNI	−	−	−	−	−	+
Giardia lamblia	BNI	−	−	−	−	−	+

[a] Positive results for amplicons I through V are indicated by a plus sign, and negative results are indicated by a minus sign. *n*, number of isolates; DSM, strain derived from the German Resource Center for Biological Material (DSMZ); BNI, parasite DNA obtained from the Bernhard-Nocht Institute for Tropical Medicine; amplicon I, IS*900*; amplicon II, MAP2765c (251); amplicon III, MAP0865 (f57); amplicon IV, MAP0865; amplicon V_1, universal 16S rRNA gene; amplicon V_2, universal 18S rRNA gene.

Microscopic detection and cultivation of *M. avium* subsp. *paratuberculosis* in feces and gut tissue from cattle with Johne's disease. We examined the feces and dissected gut tissue from 12 butchered cows by Ziehl-Neelsen staining and culture. Three cows were healthy, and the remaining nine were suspected to be afflicted with Johne's disease. Diseased cows 1 to 4, 6 to 9, and 12 showed acid-fast bacteria in both feces and inflamed gut tissue, and pure cultures of *M. avium* subsp. *paratuberculosis* could be obtained after 3 to 6 months of incubation (Fig. 2A, Table 3). The noninflamed gut tissue samples were uniformly negative for *M. avium* subsp. *paratuberculosis*. Healthy cows 5, 10, and 11 were negative for *M. avium* subsp. *paratuberculosis* in all samples examined (Table 3). All *M. avium* subsp. *paratuberculosis* isolates were identified by the specific real-time PCRs with amplicons I (IS*900*), II (MAP2765c [251]), III (MAP0865 [f57]), and IV (MAP0865). The growth of all *M. avium* subsp. *paratuberculosis* isolates was mycobactin dependent. Furthermore, the isolates were assigned to the *M. avium* complex by partial sequencing of the 16S rRNA genes and by performing a Genotype *Mycobacterium CM/AS* test (Hain Lifescience GmbH, Nehren, Germany).

Evaluation of the *M. avium* subsp. *paratuberculosis*-specific real-time PCRs using original bovine gut tissue and feces. The total DNA from inflamed and noninflamed dissected gut tissue and from feces of healthy and diseased cows was extracted with commercially available kits for tissue and stool specimens (Invitek, Berlin, Germany). All of the diseased cows with positive *M. avium* subsp. *paratuberculosis* culture results were also positive for specific real-time PCR amplicons I to IV (Table 3).

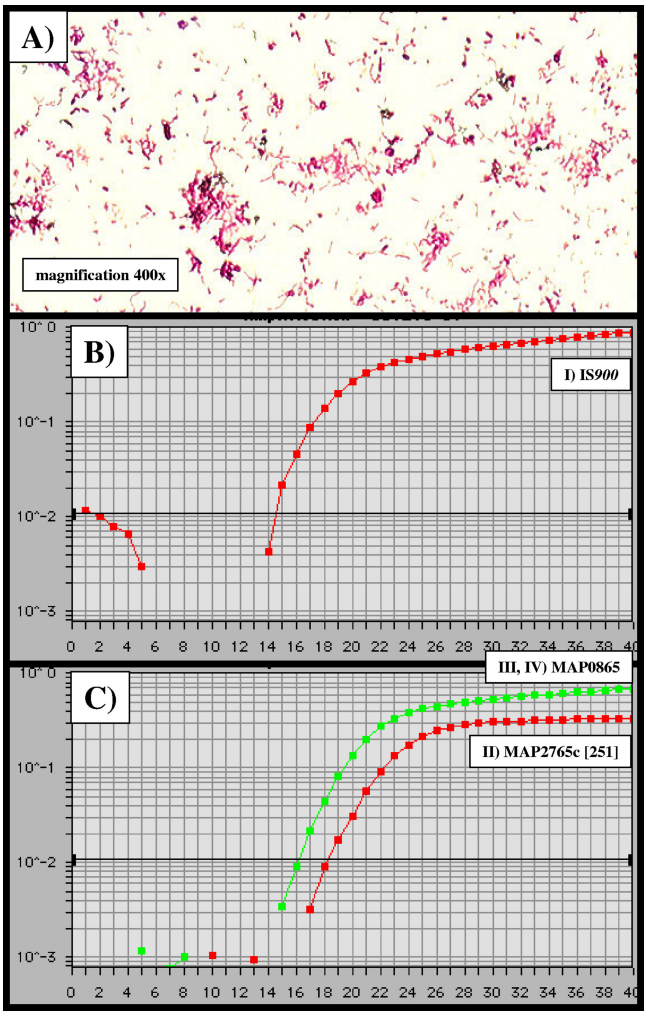

FIG. 2. Temperature optimization and sensitivity of IS*900*-, MAP2765c-, and MAP0865-specific TaqMan PCRs. Using a DNA extract of ~10^8 CFU of the *M. avium* subsp. *paratuberculosis* DSM 44133 type strain, the optimal annealing temperature for all amplicons I through IV was determined to be uniformly 57.8°C. (A) Ziehl-Neelsen staining of the *M. avium* subsp. *paratuberculosis* wild-type isolate obtained from feces of cow 7 (Table 3). (B and C) *x* axis, C_T values; *y* axis, ΔR_n (FAM reporter signal divided by the ROX [carboxy-X-rhodamine] passive reference signal). (B) amplicon I (IS*900*), C_T value = 14.4; (C) amplicon II (MAP2765c [251]), C_T value = 18.2; amplicons III (MAP0865 [f57]) and IV (MAP0865), C_T value = 16.2.

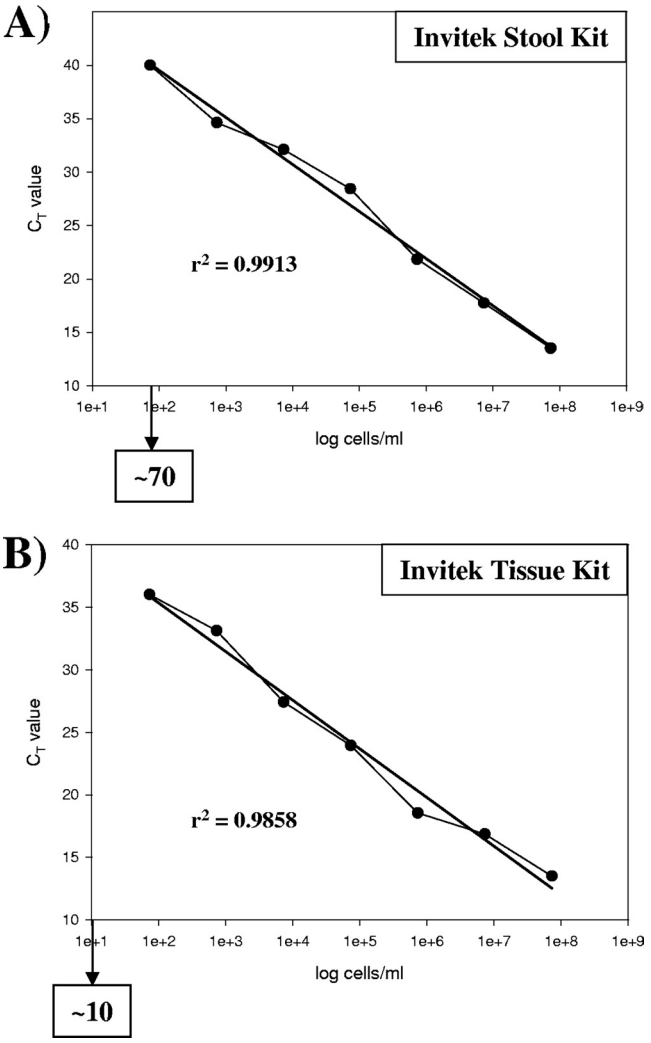

FIG. 3. Calculation of the detection limits for *M. avium* subsp. *paratuberculosis* by using commercially available DNA extraction kits and IS*900* TaqMan PCR (amplicon I). The DNA of 1 ml of serial dilutions of the *M. avium* subsp. *paratuberculosis* DSM 44133 type strain was extracted to generate standard curves and to determine the linear regression (r^2) and the detection limit (indicated by arrows) for each method. (A) Invitek stool kit (r^2 = 0.9913); detection limit, ≥70 cells. (B) Invitek tissue kit (r^2 = 0.9858); detection limit, ≥10 cells. The calculations were done with Sigma Plot software (SPSS Inc. Software, Munich, Germany).

Using the standard curves (Fig. 3), the amount of *M. avium* subsp. *paratuberculosis* was calculated to be in the range of ~2×10^4 to 6×10^7 bacteria per gram of inflamed gut tissue and ~1×10^7 to 2×10^9 bacteria per gram of feces. Thus, the concentration of *M. avium* subsp. *paratuberculosis* in feces was continuously ~30- to 500-fold higher than in inflamed parts of the intestinal tissue. All of the tissue samples from healthy *M. avium* subsp. *paratuberculosis* culture-negative cows as well as the noninflamed tissue samples of diseased cows were also negative in the *M. avium* subsp. *paratuberculosis*-specific real-time PCRs (Table 3).

Detection of *M. avium* subsp. *paratuberculosis* in stool specimens and gut tissue of patients with diarrhea. We examined consecutive stool specimens of 1,293 hospitalized patients with mild or severe symptoms of diarrhea for the presence of *S. enteritidis*, *C. jejuni*, *Y. enterocolitica*, *C. difficile*, *E. histolytica*, *G. lamblia*, and *M. avium* subsp. *paratuberculosis*. The total DNA was extracted with an Invitek stool kit, and the *M. avium* subsp. *paratuberculosis* analysis was done with highly sensitive amplicon I (IS*900*). Twenty-seven patients (2.09%) gave positive results, and the concentrations of *M. avium* subsp. *paratuberculosis* were calculated in the range of ~5×10^2 to 5×10^3 bacteria per gram of stool by the use of the corresponding standard curve (Fig. 3A). The results were confirmed by using amplicons II through IV. For 6 patients, the results for amplicons II to IV remained negative following initial amplification despite a positive amplicon I result, suggesting the presence of non-*M. avium* subsp. *paratuberculosis* mycobacterial DNA.

TABLE 3. Detection of *M. avium* subsp. *paratuberculosis* in bovine gut tissue and feces by amplicon I (IS*900*)-, amplicon II (MAP2765c [251])-, amplicon III (MAP0865 [f57])-, and amplicon IV (MAP0865)-specific TaqMan PCR, by acid-fast staining, and by culture ($n = 12$)[a]

Cattle and specimen category	Acid-fast staining result	Culture result	TaqMan PCR result for indicated amplicon			
			I	II	III	IV
Diseased ($n = 9$)						
Noninflamed gut tissue	−	−	−	−	−	−
Inflamed gut tissue	+	+	+	+	+	+
Feces	+	+	+	+	+	+
Healthy ($n = 3$)						
Noninflamed gut tissue	−	−	−	−	−	−
Feces	−	−	−	−	−	−

[a] Diseased cattle ($n = 9$) were suspected to be infected with *M. avium* subsp. *paratuberculosis* because of typical symptoms of Johne's disease (paratuberculosis).

However, following MDA, the presence of *M. avium* subsp. *paratuberculosis* was then confirmed by positive PCR results for amplicons II to IV for all 6 specimens. All of the 26 *M. avium* subsp. *paratuberculosis*-positive patients gave negative test results for *S. enteritidis*, *C. jejuni*, *Y. enterocolitica*, *E. histolytica*, and *G. lamblia*. Only one of these patients, who suffered from pseudomembranous colitis, subsequently tested positive for *C. difficile*.

Among the 1,293 patients, we identified 11 patients with chronic inflammatory bowel disease. Of these, 6 patients had clinically confirmed CD and an additional 5 were classified with ulcerative colitis (UC). Only two of the 27 *M. avium* subsp. *paratuberculosis*-positive individuals were CD patients. Additionally, a small piece of gut tissue from one CD patient also gave *M. avium* subsp. *paratuberculosis*-positive test results. The identification was confirmed by reamplification with amplicons II through IV, acid-fast staining, and culture. The remaining four CD and five UC patients gave negative results for M. *avium* subsp. *paratuberculosis*.

DISCUSSION

The most prominent target used in several studies to detect DNA of *M. avium* subsp. *paratuberculosis* by PCR is the insertion element IS*900* (19, 21). The multicopy (17 copies) nature of the sequence on the *M. avium* subsp. *paratuberculosis* chromosome makes it ideal as a target sequence for the detection of *M. avium* subsp. *paratuberculosis*, since it exhibits a higher level of sensitivity compared to the use of single-copy genes as targets (22, 45). We analyzed consecutive stool specimens of 1,293 hospitalized patients by the use of target IS*900*. Twenty-seven (2.09%) of the cohort gave positive test results for IS*900*. Only two of these patients suffered from CD. The bacterial load was persistently low and was calculated in the range of 500 to 5,000 *M. avium* subsp. *paratuberculosis* bacteria per gram of stool. In addition, an analyzed section of gut tissue from one CD patient was also positive for IS*900* and the bacterium could be isolated by culture. It was not possible to isolate *M. avium* subsp. *paratuberculosis* from the 26 other patients, since the analyses were performed retrospectively with stored DNA extracts.

Unfortunately, the specificity of target IS*900* is not 100%, since IS*900* insertion elements with close sequence homology are also present on the chromosomes of *M. cookii*, *M. marinum*, *M. paraffinicum*, and *M. scrofulaceum* isolates (7, 9, 21, 37, 40). Furthermore, polymorphisms detected in IS*900* as variants of *M. avium* subsp. *paratuberculosis* have been previously described; such variants should be interpreted as suggestive of the presence of a *Mycobacterium* organism other than *M. avium* subsp. *paratuberculosis* until the detection has been confirmed by independent methods (38). Therefore, to enhance the specificity of *M. avium* subsp. *paratuberculosis* detection, it is indispensable to use multiple *M. avium* subsp. *paratuberculosis*-specific targets. So far, several specific targets have been used by employing different techniques: IS*900* and target 251 by real-time PCR (21, 38, 44, 49), ISMap*02* by a nested PCR method (46), and f57 sequences by hybridization and by PCR (35, 44, 49). Also, the completed genome sequence of *M. avium* subsp. *paratuberculosis* strain K-10 and comparative genome analysis with the closely related species *M. avium* subsp. *avium*, including experimental studies to identify *M. avium* subsp. *paratuberculosis*-specific genomic regions, revealed miscellaneous potential new diagnostic targets (2, 19, 22, 35, 38). In order to improve detection, we used several *M. avium* subsp. *paratuberculosis*-specific targets in one assay for detection and also used quantitative TaqMan real-time PCR, since the technology is highly sensitive and specific.

For a multiple real-time PCR assay, we chose IS*900*, target 251, and the f57 sequence, which are randomly distributed on the *M. avium* subsp. *paratuberculosis* chromosome (Fig. 1, bottom). When BLASTn analysis and bioinformatic GenomeViz software were used, target 251 and sequence f57 were found in genes MAP2765c and MAP0865 of the published *M. avium* subsp. *paratuberculosis* K-10 strain, respectively (Fig. 1, top). Computer-aided analysis of the entire MAP0865 ORF revealed that it was unique for *M. avium* subsp. *paratuberculosis*, since no corresponding sequences were detected in publicly available databases. To establish real-time PCR assays for this sequence, two new TaqMan targets were generated: amplicon III, located in the f57 sequence, and amplicon IV, located at the 5′ end of ORF MAP0865 (Fig. 1, top). Each PCR was run separately and independently in corresponding unique microtiter wells but under the same conditions with respect to PCR buffer and temperature profiles. Even though the conditions were identical, the results showed that the sensitivities of the individual PCRs were different (Fig. 2B and C). The highest sensitivity was obtained with the IS*900* PCR and the lowest with the MAP2765c (251) PCR. Amplicons III (MAP0865 [f57]) and IV (MAP0865) showed identical levels of efficacy and intermediate levels of sensitivity between those of IS*900* PCR and MAP2765c (251) PCR. Because of the identical efficacy results determined under the applied conditions, only one curve, representing both amplicons, is depicted (Fig. 2C). The high sensitivity of the IS*900* PCR is attributed to the multicopy nature of this element, which is present as 17 copies on the *M. avium* subsp. *paratuberculosis* K-10 chromosome (Fig. 1, bottom). The sensitivities determined for amplicons II through IV were lower, since ORFs MAP2765c and MAP0865 are singular targets (Fig. 1, bottom). We also attribute the

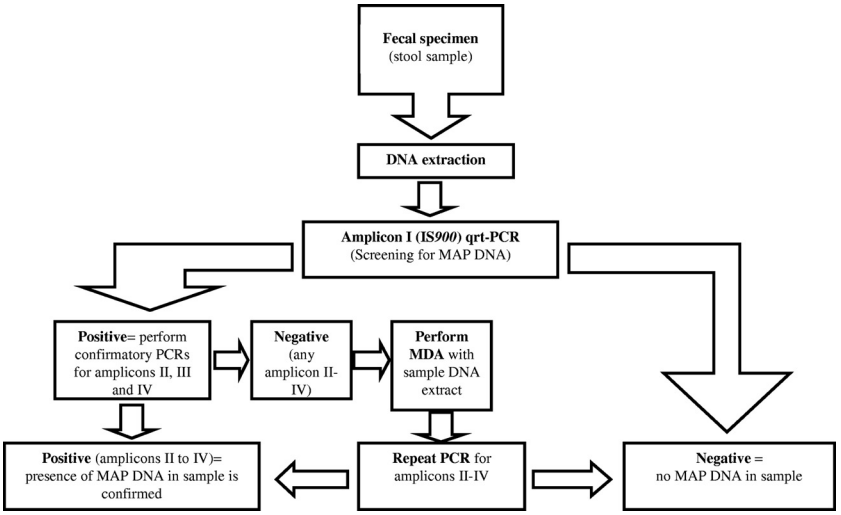

FIG. 4. Suggested workflow for the detection of *M. avium* subsp. *paratuberculosis* DNA in stool samples. MDA, multiple displacement amplification. Amplicon I = IS*900*; amplicon II = MAP2765c (251); amplicon III = MAP0865 (f57); amplicon IV = MAP0865.

lower sensitivity of the MAP2765c PCR to its size (203 bp), which affected the efficacy of the PCR methods used. The recommended size for TaqMan-based amplicons is 70 to 150 bp (36), and both amplicons III and IV of MAP0865 represent an optimal size of 101 bp, which resulted in identical efficacy results for these independent PCRs.

The specificities of newly designed quantitative real-time PCR amplicons III and IV, along with those of the previously described specific amplicons I and II, were demonstrated using 2 *M. avium* subsp. *paratuberculosis* type strains, 40 field isolates of *M. avium* subsp. *paratuberculosis* (human, animal, and environmental origin), and 13 species of Gram-positive bacteria, 20 species of Gram-negative bacteria, 7 species of anaerobic bacteria, and 2 species of intestinal parasites (Table 2). Positive signals were obtained only for the *M. avium* subsp. *paratuberculosis* strains; all of the other microorganisms tested gave uniformly negative results. The results for the pure microorganisms were achieved with DNA extracted by an RTP Spin Bacteria kit. Since stool and tissue specimens are complex and represent difficult samples for DNA extraction and PCR with respect to potential DNA-degrading enzymes and PCR inhibitors and to standardize methodology, we used commercially available stool and tissue kits from Invitek (see Materials and Methods). DNA extraction is generally achieved within 1 h. The detection limits for *M. avium* subsp. *paratuberculosis* determined using the tissue and the stool kits for DNA extraction of tissue and stool specimens were marginally above the theoretical detection limit of 1 to 10 CFU and determined to be ~10 CFU and ~70 CFU, respectively (Fig. 3).

The applicability of the DNA extraction methods and the specific quantitative real-time PCR assays used was assessed by acid-fast staining and by culture using tissue and stool specimens of three healthy cattle and of nine diseased cattle with Johne's disease. The real-time PCR results were 100% identical to the results of microscopic analysis and the culture (Table 3) but could be accomplished in 6 h. The quantification obtained by qrt-PCR showed that the examined cattle shed very large amounts of *M. avium* subsp. *paratuberculosis* calculated to be in the range of approximately 1×10^7 to 2×10^9 bacteria per gram of feces, which was ~20,000- through ~400,000-fold higher than in human feces. Fecal samples from cattle are easily obtained and are therefore superior to other specimens such as biopsy specimens or blood samples for analysis. In particular, the results we obtained from gut tissue of infected cattle strongly depended on the biopsy specimens used. Noninflamed gut tissue from diseased cattle invariably gave negative results, whereas inflamed gut tissue gave positive results (Table 3).

The presence of *M. avium* subsp. *paratuberculosis* in human stool specimens and one biopsy specimen as detected with target IS*900* was confirmed by reamplifying the samples with amplicons II through IV. This means that all samples that gave IS*900*-positive test results truly represented *M. avium* subsp. *paratuberculosis*. We are well aware that our human samples were single snapshots randomly collected and want to emphasize that our results for CD and UC patients are not meant to be representative. However, the results clearly demonstrate that the technology of DNA extraction and qrt-PCR utilized is appropriate for analysis of stool specimens and gut tissue for the presence of *M. avium* subsp. *paratuberculosis* and could therefore substantially contribute to the current debate about the role of *M. avium* subsp. *paratuberculosis* in CD. Further studies are necessary, and we suggest using several consecutive stool specimens, which are noninvasive compared to biopsy specimens and with which we were able to demonstrate high sensitivity and specificity for the detection of *M. avium* subsp. *paratuberculosis*.

In conclusion, we demonstrate the development of qrt-PCR amplicons III and IV, which, in combination with amplicons I and II, enable unequivocal detection, identification, and quantification of *M. avium* subsp. *paratuberculosis* directly from clinical, veterinary, food, and environmental specimens as well as from pure cultures. For IS*900*-positive but amplicon II- to IV-negative specimens, we suggest the use of MDA and the consecutive repetition of the confirmatory PCRs. A suggested workflow is shown in Fig. 4. Fecal specimens are true alterna-

tives for the analysis of patients and cattle because of their noninvasiveness and simplicity in collection. Our data presented here provide a basis for further structured studies of the potential role of *M. avium* subsp. *paratuberculosis* in CD and other human diseases.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Bannantine, J., P. E. Baechler, Q. Zhang, L. Li, and V. Kapur.** 2002. Genome scale comparison of *Mycobacterium avium* subsp. *paratuberculosis* with *Mycobacterium avium* subsp. *avium* reveals potential diagnostic sequences. J. Clin. Microbiol. **40:**1303–1310.
3. **Chapin, K. C., and T.-L. Lauderdale.** 2007. Reagents, stains, and media: bacteriology, p. 334–364. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), Manual of clinical microbiology, 9th ed. ASM Press, Washington, DC.
4. **Chiodini, R. J.** 1989. Crohn's disease and the mycobacterioses: a review and comparison of two disease entities. Clin. Microbiol. Rev. **2:**90–117.
5. **Collins, D. M., and G. W. de Lisle.** 1986. Restriction endonuclease analysis of various strains of *Mycobacterium paratuberculosis* isolated from cattle. Am. J. Vet. Res. **47:**2226–2229.
6. **Committee on Diagnosis and Control of Johne's Disease.** 2003. Johne's disease and Crohn's disease, p. 104–120. *In* Diagnosis and control of Johne's disease: report for the National Research Council of the National Academy of Science. The National Academic Press, Washington, DC.
7. **Cousins, D. V., et al.** 1999. Mycobacteria distinct from *Mycobacterium avium* subsp. *paratuberculosis* isolated from the faeces of ruminants possess IS*900*-like sequences detectable IS*900* polymerase chain reaction: implications for diagnosis. Mol. Cell Probes **13:**431–442.
8. **Dalziel, T. K.** 1913. Chronic interstitial enteritis. Br. Med. J. **2:**1068–1070.
9. **Englund, S., G. Bolske, and K. E. Johansson.** 2002. An IS*900*-like sequence found in a *Mycobacterium* sp. other than *Mycobacterium avium* subsp. *paratuberculosis.* FEMS Microbiol. Lett. **209:**267–271.
10. **Gao, A., L. Mutharia, S. Chen, K. Rahn, and J. Odumeru.** 2002. Effect of pasteurisation on survival of *Mycobacterium paratuberculosis* in milk. J. Dairy Sci. **85:**3198–3205.
11. **Ghai, R., T. Hain, and T. Chakraborty.** 2004. GenomeViz: visualizing microbial genomes. BMC Bioinformatics **5:**198.
12. **Grant, I. R.** 2005. Zoonotic potential of *Mycobacterium avium* subsp. *paratuberculosis*: the current position. J. Appl. Microbiol. **98:**1282–1293.
13. **Green, E. P., et al.** 1989. Sequence and characteristics of IS*900*, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. Nucleic Acids Res. **17:**9063–9073.
14. **Harris, N. B., and R. G. Barletta.** 2001. *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. Clin. Microbiol. Rev. **14:**489–512.
15. **Hermon-Taylor, J.** 2001. Protagonist: *Mycobacterium avium* subspecies *paratuberculosis* is a cause of Crohn's disease. Gut **49:**755–756.
16. **Hermon-Taylor, J., and T. J. Bull.** 2002. Crohn's disease caused by *Mycobacterium avium* subsp. *paratuberculosis*: a public health tragedy whose resolution is long overdue. J. Med. Microbiol. **51:**3–6.
17. **Hermon-Taylor, J., et al.** 2000. Causation of Crohn's disease by *Mycobacterium avium* subspecies *paratuberculosis*. Can. J. Gastroenterol. **14:**521–539.
18. **Hoorfar, J., P. Ahrens, and P. Radström.** 2000. Automated 5′ nuclease PCR assay for identification of *Salmonella enterica*. J. Clin. Microbiol. **38:**3429–3435.
19. **Hruska, K., M. Bartos, P. Kralik, and I. Pavlik.** 2005. *Mycobacterium avium* subsp. *paratuberculosis* in powdered infant milk: paratuberculosis in cattle—the public health problem to be solved. Vet. Med.-Czech **50:**327–335.
20. **Huntley, J. F. J., R. H. Whitlock, J. P. Bannantine, and J. R. Stabel.** 2005. Comparison of diagnostic detection methods for *M. avium* subsp. *paratuberculosis* in North American bison. Vet. Pathol. **42:**42–51.
21. **Kim, S. G., et al.** 2002. Development and application of quantitative polymerase chain reaction assay based on the ABI 7700 system (TaqMan) for detection and quantification of *Mycobacterium avium* subsp. *paratuberculosis.* J. Vet. Diagn. Invest. **14:**126–131.
22. **Li, L., et al.** 2005. The complete genome sequence of *Mycobacterium avium* subspecies *paratuberculosis*. Proc. Natl. Acad. Sci. U. S. A. **102:**12344–12349.
23. **Martin, F. E., M. A. Nadkami, N. A. Jacques, and N. Hunter.** 2002. Quantitative microbiological study of human carious dentine by culture and real-time PCR: association of anaerobes with histopathological changes in chronic pulpitis. J. Clin. Microbiol. **40:**1698–1704.
24. **McFadden, J. J., P. D. Butcher, R. J. Chiodini, and J. Hermon-Taylor.** 1987. Crohn's disease-isolated mycobacteria are identical to *Mycobacterium paratuberculosis*, as determined by DNA probes that distinguish between mycobacterial species. J. Clin. Microbiol. **25:**796–801.
25. **Mendoza, J. L., R. Lana, and M. Díaz-Rubio.** 2009. *Mycobacterium avium* subspecies *paratuberculosis* and its relationship with Crohn's disease. World J. Gastroenterol. **15:**417–422.
26. **Müller, A., et al.** 2000. Detection of *Isospora belli* by polymerase chain reaction using primers based on small-subunit ribosomal RNA sequences. Eur. J. Clin. Microbiol. Infect. Dis. **19:**631–634.
27. **Naser, S. A., D. Schwartz, and I. Shafran.** 2000. Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from breast milk of Crohn's disease patients. Am. J. Gastroenterol. **95:**1094–1095.
28. **Naser, S. A., G. Ghobrial, C. Romero, and J. F. Valentine.** 2004. Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's disease. Lancet **364:**1039–1044.
29. **Nogva, H. K., A. Bergh, A. Holck, and K. Rudi.** 2000. Application of the 5′-nuclease PCR assay in evaluation and development of methods for quantitative detection of *Campylobacter jejuni*. Appl. Environ. Microbiol. **66:** 4029–4036.
30. **Olsen, I., G. Sigurgardottir, and B. Djonne.** 2002. Paratuberculosis with special reference to cattle. Vet. Q. **24:**12–28.
31. **Ott, S. L., S. J. Wells, and B. A. Wagner.** 1999. Herd-level economic losses associated with Johne's disease on US dairy operations. Prev. Vet. Med. **40:**179–192.
32. **Parrish, N. M., et al.** 2009. Absence of *Mycobacterium avium* subsp. *paratuberculosis* in Crohn's patients. Inflamm. Bowel Dis. **15:**558–565.
33. **Pfyffer, G. E.** 2007. *Mycobacterium*: general characteristics, laboratory detection, and staining procedures, p. 543–572. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), Manual of clinical microbiology, 9th ed. American Society for Microbiology Press, Washington, DC.
34. **Pierce, E. S.** 2009. Possible transmission of *Mycobacterium avium* subspecies *paratuberculosis* through potable water: lessons from an urban cluster of Crohn's disease. Gut Pathog. **23:**17.
35. **Poupart, P., M. Coene, H. Vanheuverswyn, and C. Cocito.** 1993. Preparation of a specific RNA probe for detection of *Mycobacterium paratuberculosis* and diagnosis of Johne's disease. J. Clin. Microbiol. **31:**1601–1605.
36. **Proudnikov, D., et al.** 2003. Optimizing primer-probe design for fluorescent PCR. J. Neurosci. Methods **123:**31–45.
37. **Quirke, P.** 2001. Antagonist: *Mycobacterium avium* subspecies *paratuberculosis* is a cause of Crohn's disease. Gut **49:**757–760.
38. **Rajeev, S., Y. Zhang, S. Sreevatsan, A. S. Motiwala, and B. Byrum.** 2005. Evaluation of multiple genomic targets for identification and confirmation of *Mycobacterium avium* subsp. *paratuberculosis* isolates using real-time PCR. Vet. Microbiol. **105:**215–221.
39. **Schwartz, D., et al.** 2000. Use of short-term culture for identification of *M. avium* subsp. *paratuberculosis* in tissue from Crohn's disease patients. Clin. Microbiol. Infect. **6:**303–307.
40. **Semret, M., C. Y. Turenne, and M. A. Behr.** 2006. Insertion sequence IS*900* revisited. J. Clin. Microbiol. **44:**1081–1083.
41. **Shankar, H., et al.** 2010. Presence, characterization, and genotype profiles of *Mycobacterium avium* subspecies *paratuberculosis* from unpasteurized individual and pooled milk, commercial pasteurized milk, and milk products in India by culture, PCR, and PCR-REA methods. Int. J. Infect. Dis. **14:**121–126.
42. **Shivananda, S., et al.** 1996. Incidence of inflammatory bowel disease across Europe: is there a difference between north and south? Results of the European Collaborative Study in Inflammatory Bowel Disease (EC-IBD). Gut **39:**690–697.
43. **Sibartie, S., et al.** 2010. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) as a modifying factor in Crohn's disease. Inflamm. Bowel Dis. **16:**296–304.
44. **Slana, I., M. Liapi, M. Moravkova, A. Kralova, and I. Pavlik.** 2009. *Mycobacterium avium* subsp. *paratuberculosis* in cow bulk tank milk in Cyprus detected by culture and quantitative IS*900* and F57 real-time PCR. Prev. Vet. Med. **89:**223–226.
45. **Soumya, M. P., R. M. Pillai, P. X. Antony, H. K. Mukhopadhyay, and V. N. Rao.** 2009. Comparison of faecal culture and IS*900* PCR assay for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine faecal samples. Vet. Res. Commun. **33:**781–791.
46. **Stabel, J. R., and J. P. Bannantine.** 2005. Development of a nested PCR method targeting a unique multicopy element, ISMap*02*, for detection of *Mycobacterium avium* subsp. *paratuberculosis* in fecal samples. J. Clin. Microbiol. **43:**4744–4750.
47. **Sung, N., and M. T. Collins.** 1998. Thermal tolerance of *Mycobacterium paratuberculosis*. Appl. Environ. Microbiol. **64:**999–1005.
48. **Sung, N., and M. T. Collins.** 2000. Effect of three factors in cheese produc-

tion (pH, salt and heat) on *Mycobacterium avium* subsp. *paratuberculosis* viability. Appl. Environ. Microbiol. **66:**1334–1339.
49. **Tasara, T., and R. Stephan.** 2005. Development of an F57 sequence-based real-time PCR assay for detection of *M. avium* subsp. *paratuberculosis* in milk. Appl. Environ. Microbiol. **71:**5957–5968.
50. **Thomas Dow, C.** 2008. Cows, Crohn's and more: is *Mycobacterium paratuberculosis* a superantigen? Med. Hypotheses **71:**858–861.
51. **Thorel, M. F., M. Krichevsky, and V. V. Levy-Frebault.** 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.
52. **van den Berg, R. J., et al.** 2005. Prospective multicenter evaluation of a new immunoassay and real-time PCR for rapid diagnosis of *Clostridium difficile*-associated diarrhea in hospitalized patients. J. Clin. Microbiol. **43:**5338–5340.
53. **Verweij, J. J., et al.** 2004. Simultaneous detection of *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* in fecal samples by using multiplex real-time PCR. J. Clin. Microbiol. **42:**1220–1223.
54. **Vishnubhatla, A., et al.** 2000. Rapid 5′ nuclease (TaqMan) assay for detection of virulent strains of *Yersinia enterocolitica*. Appl. Environ. Microbiol. **66:**4131–4135.
55. **Whittington, R. J., D. J. Marshall, P. J. Nicholls, I. B. Marsh, and L. A. Reddacliff.** 2004. Survival and dormancy of *Mycobacterium avium* subsp. *paratuberculosis* in the environment. Appl. Environ. Microbiol. **70:**2989–3004.