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Rapid Differentiation of *Mycobacterium avium* and *M. paratuberculosis* by PCR and Restriction Enzyme Analysis

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***Mycobacterium avium* subsp. *avium* (*M. avium*) and *M. avium* subsp. *paratuberculosis* (*M. paratuberculosis*), intracellular bacteria that can cause chronic granulomatous enteritis in cattle, are difficult to distinguish on the basis of growth and biochemical characteristics. We report the development of a PCR-based strategy for the rapid differentiation of isolates of *M. avium* from isolates of *M. paratuberculosis*. Restriction fragment length polymorphism was identified by PCR amplification and subsequent restriction enzyme digestion with *Pst*I of a 960-bp fragment of the 65-kDa heat shock protein (*hsp65*) from 21 clinical isolates of *M. paratuberculosis* and 14 isolates of *M. avium*. These results indicate that a restriction fragment length polymorphism in the *hsp65* gene can be used for the rapid differentiation of clinical isolates of *M. paratuberculosis* and *M. avium*.**

Members of the *Mycobacterium avium* complex are a family of intracellular bacteria that includes *M. avium* subsp. *avium* (*M. avium*), *M. intracellulare*, and *M. avium* subsp. *paratuberculosis* (*M. paratuberculosis*) (17, 24). These organisms cause significant disease in food-producing animals, including poultry, swine, and cattle (2, 13, 22, 23). *M. paratuberculosis*, the causative agent of Johne's disease in ruminants (5), continues to pose a significant economic and health problem, with a high prevalence in cattle throughout the United States. Poor detection methods have hindered efforts to eradicate the disease (2, 13, 27).

Both *M. avium* infection and *M. paratuberculosis* infection can cause chronic granulomatous enteritis in cattle and other ruminants (1, 10, 16). It is important to determine the causative mycobacterial species in these cases because of the consequences for the individual infected cattle as well as the herd. *M. paratuberculosis*-positive cattle are sent to slaughter, and the disease is reportable in some states (20). The significance of an *M. avium* isolate and its potential for contagion within a cattle herd are unknown; however, the disease is sporadic.

Currently, differentiation of *M. avium* and *M. paratuberculosis* involves evaluation of phenotypic and biochemical growth characteristics. These methods are not always specific in distinguishing between the two subspecies (3, 8). Bacteria from feces must be cultured for a minimum of 2 weeks for *M. avium* isolates and from 4 weeks to 4 months for *M. paratuberculosis* isolates (12). Cultures are assessed for mycobactin dependence, which is considered a feature characteristic of *M. paratuberculosis* (15, 19). However, some strains of *M. avium* are also mycobactin dependent on primary culture, making this an unreliable method for differentiating these subspecies (10, 11). In addition, culture methods are labor-intensive and expensive, making them potentially unsuitable for epidemiologic surveys involving large numbers of animals.

Techniques that use PCR have recently been developed for the detection and differentiation of mycobacterial species. However, many of the methods for analysis of an amplified product do not lend themselves to the rapid differentiation of *M. avium* and *M. paratuberculosis* (25, 26). In addition, these techniques may not detect the presence of more than one mycobacterial species (6, 25). Recently, several investigators have described techniques for the rapid detection and identification of mycobacteria to the species level on the basis of PCR and then restriction enzyme analysis (15, 21). However, the ability of these assays to differentiate between the closely related species *M. avium* and *M. paratuberculosis* was not examined.

Because of the importance of distinguishing *M. paratuberculosis* from *M. avium* in samples isolated from cattle feces, we evaluated PCR-restriction enzyme analysis strategies for differentiation of clinical isolates of *M. avium* ($n = 14$) and *M. paratuberculosis* ($n = 21$) (Table 1). *M. paratuberculosis* isolates were grown and maintained on Herrold's egg yolk medium with mycobactin J at 37°C; *M. avium* isolates were grown on Middlebrook 7H10 plates at 37°C. The mycobacterial isolates used in the study were identified by growth characteristics, mycobactin dependence, and identification by PCR amplification of the species-specific insertion sequence IS*900* or IS*901*. Possession of the insertion sequence IS*900* has been shown to be specific for *M. paratuberculosis* (7, 8). In addition, some strains of *M. avium*, designated restriction fragment length polymorphism (RFLP) type A/I, contain a repetitive element related to IS*900*, termed IS*901* (8, 9). PCR was performed on all mycobacterial samples by previously described protocols (8). The results are presented in Table 1.

Following species identification, we initially evaluated the abilities of two previously described PCR-restriction enzyme assays (15, 21) to differentiate *M. avium* and *M. paratuberculosis* through amplification of a fragment of the gene that encodes the 65-kDa heat shock protein (*hsp65*). These assays, which use primers from conserved regions that span variable regions and then restriction enzyme digestion of the amplified product, yield characteristic restriction digestion patterns for a large number of different mycobacterial species and can differentiate closely related mycobacterial species.

To perform PCR, lysates of mycobacterial DNA from cultured organisms were prepared with the GeneReleaser kit (National Scientific Supply Co.) by the manufacturer's recom-

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TABLE 1. Origins of the isolates used and presence of insertion sequences

Sample	Isolate	Origin	Insertion sequence	Source[a]
1.	*M. avium* 94-6621	Wood duck	IS*901*	1
2.	*M. avium* 19499-2822	Bovine	None	2
3.	*M. avium* 19992-2905	Bovine	None	2
4.	*M. avium* 19993-2906	Bovine	None	2
5.	*M. avium* 19996-2909	Bovine	None	2
6.	*M. avium* 20010-2910	Bovine	None	2
7.	*M. avium* 20011-2911	Bovine	None	2
8.	*M. avium* 20599-3008	Bovine	None	2
9.	*M. avium* 20919-3049	Bovine	None	2
10.	*M. avium* 21626-3131	Bovine	None	2
11.	*M. avium* 16409-2414	Bovine	IS*901*	2
12.	*M. paratuberculosis* 369276-6	Bovine	IS*900*	3
13.	*M. paratuberculosis* 369276-8	Bovine	IS*900*	3
14.	*M. paratuberculosis* 3203-2	Bovine	IS*900*	3
15.	*M. paratuberculosis* 3203-3	Bovine	IS*900*	3
16.	*M. paratuberculosis* 2245	Bovine	IS*900*	3
17.	*M. paratuberculosis* 3251	Bovine	IS*900*	3
18.	*M. paratuberculosis* 3594	Bovine	IS*900*	3
19.	*M. paratuberculosis* 3203-1	Bovine	IS*900*	3
20.	*M. paratuberculosis* ATCC 19698	Bovine	IS*900*	4
21.	*M. paratuberculosis* 92-2290	Bovine	IS*900*	1
22.	*M. paratuberculosis* 94-5791A	Bovine	IS*900*	1
23.	*M. paratuberculosis* 94-5791B	Bovine	IS*900*	1
24.	*M. paratuberculosis* 94-112	Bovine	IS*900*	1
25.	*M. paratuberculosis* 94-158	Bovine	IS*900*	1
26.	*M. paratuberculosis* 94-715A	Bovine	IS*900*	1
27.	*M. paratuberculosis* 94-715B	Bovine	IS*900*	1
28.	*M. paratuberculosis* 94-9585	Bovine	IS*900*	1
29.	*M. paratuberculosis* 94-9705A	Bovine	IS*900*	1
30.	*M. paratuberculosis* 94-9705B	Bovine	IS*900*	1
31.	*M. paratuberculosis* 2377	Bovine	IS*900*	3
32.	*M. paratuberculosis* 4599	Bovine	IS*900*	3
33.	*M. avium* 101	Human	None	5, 6
34.	*M. avium* TMC 724	Chicken	IS*901*	5
35.	*M. avium* 18[b]	Bovine	IS*901*	4

[a] Sources: 1, Washington Animal Disease Diagnostic Laboratory, Pullman; 2, National Veterinary Services Laboratory, Ames, Iowa; 3, M. T. Collins, University of Wisconsin, Madison; 4, American Type Culture Collection, Rockville, Md.; 5, I. Orme, Colorado State University, Ft. Collins; 6, L. Bermudez, Kuzell Institute for Arthritis and Infectious Diseases, San Francisco, Calif.

[b] Formerly *M. paratuberculosis* 18.

mended protocols. For the first assay tested (21), published primer sequences corresponding to a portion of *hsp65* were synthesized on a DNA synthesizer (ABI 381A). These primers amplify a 441-bp fragment between positions 396 and 836 of the published gene sequence for *M. tuberculosis* (18, 21).

To determine whether an RFLP exists between the 441-bp fragments generated by PCR of *M. avium* and *M. paratuberculosis* DNAs, representative samples of PCR products from each subspecies were individually digested with the following restriction enzymes: *Alu*I, *Bam*HI, *Bst*EII, *Bst*NI, *Eco*RI, *Eco*RV, *Hae*II, *Hin*dIII, *Hin*fI, *Kpn*I, *Not*I, *Pst*I, *Pvu*II, *Sac*I, *Sal*I, *Sfu*I, *Sma*I, *Sst*I, *Taq*I, and *Xho*I. No RFLPs were detected between the two subspecies on the basis of digestion with these enzymes. Subsequent sequencing of the 441-bp fragments from representative samples of *M. avium* and *M. paratuberculosis* revealed identical sequences (7a).

The ability of a second PCR-restriction enzyme assay (15) to differentiate *M. paratuberculosis* and *M. avium* was also examined. For this assay, primers that amplify an approximately 1,380-bp portion of the *hsp65* gene between positions 234 and 1612 of the published sequence for *M. tuberculosis* were synthesized (15, 18). However, we were unable to consistently amplify the *hsp65* gene from either *M. paratuberculosis* or *M. avium*, despite repeated attempts to optimize the amplification protocol. It is not known why consistent amplification did not occur.

Although the *hsp65* gene is highly conserved among mycobacterial species, comparison of DNA and protein sequences from *M. paratuberculosis*, *M. tuberculosis*, and *M. leprae* reveals variability at the 3′ end of this gene. To capitalize on this variability, new primers from conserved regions that span a variable region at the 3′ end of the mycobacterial *hsp65* gene were synthesized. These primers amplify a 960-bp fragment between positions 837 and 1796 of the published gene sequence for *M. tuberculosis* (18). The primer sequences are as follows: primer TB13, 5′-GGC-TAC-ATC-TCG-GGG-TAC-TTC-3′; primer 65-1400, 5′-CTC-GGT-GGT-CAG-GAA-CAG-C-3′.

For PCR amplification, each 50 μl of reaction mixture contained DNA lysate, 200 μM (each) deoxynucleotide triphosphates, 1 μM (each) primers, 1.25 U of *Taq* polymerase, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, and 5% glycerol (added as a cosolvent to enhance the amplification [28, 29]). Samples were amplified in a thermocycler (9600; Perkin-Elmer) for 35 cycles (1 cycle was denaturation for 45 s at 95°C, annealing for 45 s at 60°C, and extension for 60 s at 72°C). To detect RFLPs between the 960-bp products resulting from PCR amplification of *M. avium* and *M.*

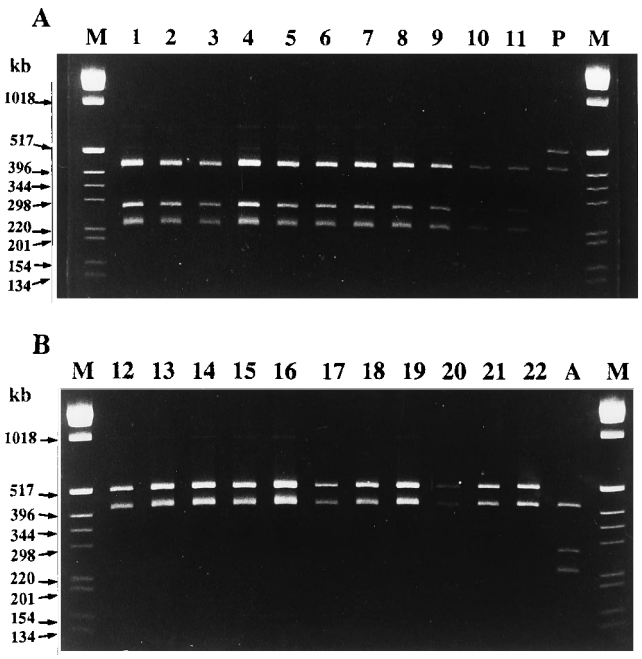

FIG. 1. *Pst*I digestion profiles of PCR-amplified mycobacterial DNA. Lane numbers correspond to the mycobacterial isolates listed in Table 1. Lane M, molecular mass markers. (A) *Pst*I digestion profile of *M. avium* isolates (lanes 1 to 11). The digestion profile of an *M. paratuberculosis* isolate (lane P) is presented for comparison. (B) *Pst*I digestion profile of *M. paratuberculosis* isolates (lanes 12 to 22). The digestion profile of an *M. avium* isolate (lane A) is presented for comparison.

paratuberculosis DNAs, representative samples of each subspecies were amplified and then digested with the following restriction enzymes: *Bst*NI, *Eco*RV, *Hin*dIII, *Kpn*I, *Pst*I, *Sst*I, and *Xho*I. The digestion products were electrophoresed on a MetaPhor agarose gel (FMC Bioproducts) and were visualized by ethidium bromide staining.

A RFLP that differentiated *M. avium* from *M. paratuberculosis* was identified only in the digestion products of *Pst*I. This restriction enzyme produced three bands of approximately 415, 280, and 240 bp from the *M. avium* isolates tested, but it produced only two bands of approximately 515 and 415 bp from the *M. paratuberculosis* isolates tested. To determine whether the RFLP patterns generated by *Pst*I could consistently distinguish *M. avium* from *M. paratuberculosis*, all 35 isolates listed in Table 1 were subjected to the PCR-*Pst*I digestion. The restriction enzyme products were electrophoresed and were visualized by ethidium bromide staining. Results for 22 of the mycobacterial samples are depicted in Fig. 1A and B. The RFLP patterns generated by PCR amplification and subsequent *Pst*I restriction enzyme digestion were identical among all isolates of each subspecies and consistently distinguished *M. avium* from *M. paratuberculosis*.

In order to directly determine the locations of the nucleotide sites conferring the RFLP, the DNA sequences of the PCR amplicons from one isolate of *M. avium* and one isolate of *M. paratuberculosis* were analyzed. Unincorporated nucleotides and primers were separated from amplified DNA by filtration through Microcon 100 microconcentrators (Amicon, Inc., Beverly, Mass.). Sequencing reactions with the *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems Inc., Foster City, Calif.) were performed with 5 μl (approximately 250 ng) of PCR-amplified DNA as template and 3.2 pmol of either the forward or the reverse primer. The unincorporated dye terminators and primers were separated from the extension products by spin column purification (Centri-Sep; Princeton Separations Inc., Adelphia, N.J.). The products were dried in a vacuum centrifuge, resuspended in 4 μl of loading buffer (deionized formamide and 50 mM EDTA [5:1; pH 8.0]), heat denatured for 2 min at 90°C, and immediately loaded onto an acrylamide gel in an automated DNA sequencer (model 377; Applied Biosystems Inc.). Both strands were sequenced two times, and the data were assembled and edited with EDIT SEQ, ALIGN, and SEQMAN programs (DNASTAR, Madison, Wis.). The results of the analysis indicate that the *Pst*I polymorphism results from a T ↔ G transversion at position 861 of the *hsp65* open reading frame. The single isolate of *M. avium* examined had the *Pst*I recognition sequence 5′-CTG CAG at positions 859 through 864 of the *hsp65* gene, whereas the *M. paratuberculosis* isolate had the nucleotide sequence 5′-CTTCAG at the same position, resulting in the loss of a *Pst*I recognition site. The differences detected by sequencing variable portions of the *hsp65* gene suggest that this could be a useful technique for the molecular typing of mycobacterial organisms for epidemiologic studies.

Interestingly, the RFLP pattern generated by PCR of *M. avium* 18 (formerly *M. paratuberculosis* 18) was identical to those of the other *M. avium* isolates. In addition, consistent with previously published data, this isolate did not possess the IS*900* insertion sequence characteristic of *M. paratuberculosis* isolates, but it did possess an IS*901* insertion sequence, which appears to be found in many animal-associated *M. avium* isolates (8). Thus, our data corroborate those of other investigators which indicate that this isolate is *M. avium* (4, 8).

In summary, using PCR amplification and restriction enzyme digestion of a portion of the *hsp65* gene, we have demonstrated distinct, characteristic RFLPs from diverse isolates of *M. avium* and *M. paratuberculosis*. Because of the importance of differentiating these closely related mycobacteria in cattle specimens, the resulting test could be useful as a diagnostic assay for the rapid identification of the mycobacterial species responsible for Johne's disease-like symptoms in cattle. We are developing an immunomagnetic bead technique to rapidly detect and determine the species of mycobacteria from fecal specimens (14, 30). Immunomagnetic bead extraction coupled with PCR amplification-restriction enzyme digestion of the purified mycobacterial organisms could provide a powerful tool for the rapid detection and differentiation of mycobacterial species isolated from clinical samples.

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