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BRIEF COMMUNICATIONS

Detection and differentiation of *Mycobacterium avium* and *Mycobacterium genavense* by polymerase chain reaction and restriction enzyme digestion analysis

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Mycobacteria are acid-fast, facultative intracellular bacilli that are highly resistant to environmental conditions and can survive in soil for months.[4,14] *Mycobacterium avium* causes a slow spreading, granulomatous infection seen predominantly in birds and in humans with AIDS.[14] Once organisms are ingested, they disseminate to the intestine and internal organs, resulting in chronic weight loss, diarrhea, emaciation, and eventually death. Tubercles developing at the site of infection in the intestinal wall allow for constant shedding into the environment.[4]

Recently a new species of mycobacteria, *Mycobacterium genavense*, has been identified and shown to cause infection in birds, in immunocompromised humans, and in other animals including dogs.[2,6,8,9,11] In Switzerland, an epidemiologic study undertaken between 1986 and 1995 identified *M. genavense* as the most common etiologic agents of mycobacteriosis in birds.[7] In both birds and humans, *M. avium* and *M. genavense* result in nearly identical clinical signs following infection and have primarily been differentiated through sequencing of the 16S ribosomal RNA (rRNA) gene.[2,8,10] Moreover, dual infections have been reported.[10]

Currently, there are many limits to the detection of *M. avium* and *M. genavense* infection. Culture may take up to 4 weeks and requires the use of special media because mycobacteria are slow growing and fastidious, making this method inefficient and expensive.[4,5] Primary growth of *M. genavense* on conventional solid mycobacterial medium has not been demonstrated,[2,8] which creates a serious problem for detection and diagnosis of *M. genavense* infection, particularly for veterinary diagnostic laboratories. Because of the potential biological hazards of liquid culture, standard mycobacterial protocols for most veterinary diagnostic laboratories are typically limited to solid media, such as Middlebrook or Lowenstein-Jensen. It is therefore possible that many *M. genavense* infections go undiagnosed in animals.

Techniques that utilize the polymerase chain reaction (PCR) have recently been developed for the detection and differentiation of mycobacterial species. Several published PCR protocols rely on sequencing of amplicons from various genes, including rRNA genes.[1,12,15,16] However, the need for sequencing following amplification limits the usefulness of these protocols as rapid diagnostic assays for species differentiation. In part because of this difficulty in differentiating mycobacterial species, *M. genavense* was only recently recognized to be different from *M. avium*. Little is known, therefore, about the epidemiology of *M. genavense* infection in either birds or humans. The possibility of zoonotic infection between immunocompromised individuals and their pets makes rapid detection and differentiation of these bacteria very important.

A PCR assay based on the amplification of a portion of the 65-kDa heat shock protein gene (*hsp65*) has previously been developed.[3] This gene contains sequences that are unique to various species of mycobacteria and contains portions that are universally conserved[13] and may therefore be useful both for detection and differentiation of mycobacterial species. Here, we describe the development of an assay that will rapidly differentiate *M. avium* from *M. genavense*. PCR was performed on analogous portions of the *hsp65* of both organisms, and PCR products were cloned and sequenced. A restriction enzyme map was developed that allowed identification of restriction enzyme sites unique to each of the mycobacterial species. This combination of PCR followed by restriction enzyme digestion allows efficient detection and differentiation of *M. avium* and *M. genavense*.

Previously identified mycobacterial samples were obtained from a number of different species and sources (Table 1). *Mycobacterium avium* isolates were grown on Middlebrook 7H10 plates[a] at 37 C; *M. genavense* isolates were grown in Middlebrook 7H9 broth[a] at 37 C. Mycobacterial DNA was extracted from lysates of cultured organisms by a previously described method using zirconium beads[b] and guanidine thiocyanate.[c,1]

For PCR amplification, each 50 μl of reaction mixture contained 10–100 ng of DNA lysate, 200 μM (each) deoxynucleotide triphosphate,[d] 1 μM (each) primers, 1.25 U of *Taq* polymerase,[d] 10 mM Tris HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$. Samples were amplified in a thermocycler[e] for 30 cycles (1 cycle consists of denaturation for 45 seconds at 95 C, annealing for 45 seconds at 60 C, and extension for 60 seconds at 72 C). The primer sequences are primer MKMTB13A: 5′-AGG-CGA-TGG-ACA-AGG-T-3′, and primer MPTB II: 5′-CCT-CGA-TGC-GGT-GCT-TGC-3′.These primers amplify a 693-bp fragment from both the *M. avium* and *M. genavense hsp65* genes. The fragment corresponds to the region between nucleotides 609 and 1,310 of the *Mycobacterium paratuberculosis hsp65* sequence in the GenBank database (R. Budjoso, accession number X74518).

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Table 1. Origins of the Mycobacterium isolates used.

Sample no.	Isolate	Origin	Source*
1	*M. avium* TMC 724	chicken	1
2	*M. avium* 101	human	1
3	*M. avium* 94-6621	wood duck	2
4	*M. avium* 97-102586	teal	2
5	*M. avium* 20919-3049	bovine	3
6	*M. avium* 20011-2911	bovine	3
7	*M avium* 19992-2905	bovine	3
8	*M. avium* 18	bovine	4
9	*M. avium* 25-291	chicken	4
10	*M. genavense* 51233	human	5
11	*M. genavense* 51234	human	5
12	*M. genavense* 2281	human	5
13	*M. genavense* 4782	human	5
14	*M. genavense* 5283	human	5

* 1 = I. Orme, Colorado State University, Ft. Collins, CO; 2 = Washington Animal Disease Diagnostic Laboratory, Pullman, WA; 3 = National Veterinary Services Laboratory, Ames, IA; 4 = American Type Culture Collection, Rockville, MD; 5 = M. Coyle, Harborview Medical Center, Seattle, WA.

To facilitate sequencing, fresh PCR products (<1 day old) were initially cloned into the pCR 2.1 vector by using a TA Cloning Kit[f] according to the manufacturer's recommended protocols. Both mycobacterial species were subsequently sequenced multiple times, and consensus sequences were compared (Fig. 1). Restriction enzyme maps were generated from consensus sequences to identify enzymes that would differentiate the bacteria upon digestion of amplified PCR product. Restriction enzyme digestion of PCR products was performed according to the manufacturers' recommendations. The resulting products were electrophoresed in a 1% agarose gel and were visualized with Gel Star[g] nucleic acid gel stain. Several enzymes, *Sal*I,[h] *Bste*II,[h] and *Bgl*I,[h] were effective in distinguishing *M. avium* from *M. genavense* (Table 2). Nine independent confirmed isolates of *M. avium* were examined and compared with 5 confirmed *M. genavense* isolates. *Sal*I and *Bste*II cut the 5 *M. genavense* PCR products but did not cut the PCR products of the 9 *M. avium* isolates. Conversely, *Bgl*I cut the *M. avium* PCR products but did not cut the *M. genavense* PCR products. For each of the 14 isolates, the restriction enzyme patterns generated were consistent with previous culture results and correctly identified *M. avium* and *M. genavense* in all cases (Fig. 2; some data not shown).

The ability of the assay to differentiate *M. avium* from *M. genavense* in clinical specimens was then evaluated. Granulomatous lesions were obtained from a chicken and a red-lored Amazon parrot, both of which had died from a mycobacterial infection. DNA was extracted from frozen tissues according to manufacturer's suggested protocols.[i] PCR was performed on the extracted DNA according to the protocol described above, and the resulting 693-bp fragments were digested with *Bste*II and *Bgl*I restriction enzymes. Restriction digests were consistent with *M. genavense* infection in the red-lored Amazon and *M. avium* infection in the chicken (data not shown). Unfortunately, because no samples were submitted for culture, the results from PCR cannot be compared with culture results.

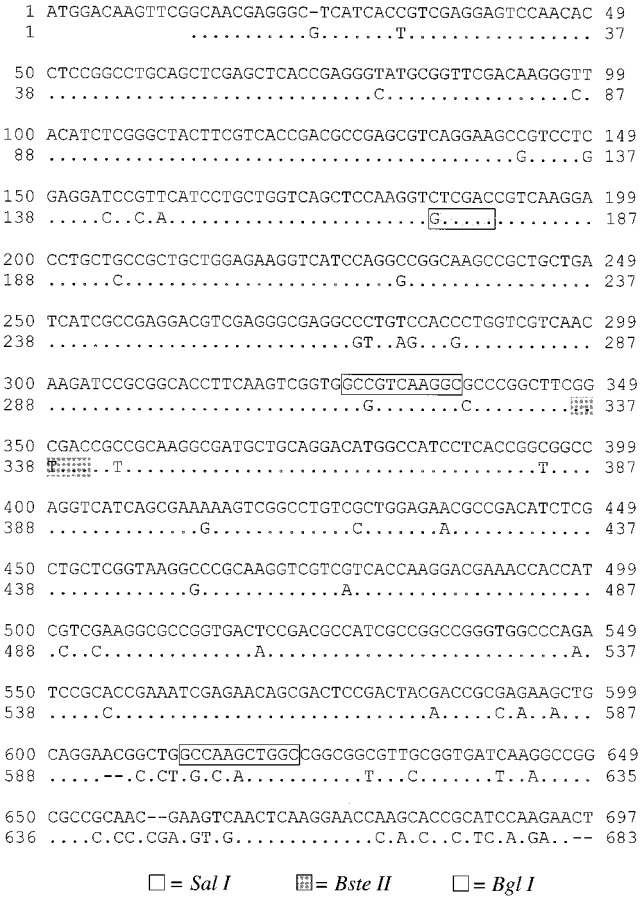

Figure 1. Alignment of *M. avium* and *M. genavense* consensus sequences. *Mycobacterium avium* was used as the reference sequence (top). Only nucleotide differences are shown in the *M. genavense* sequence (bottom). Dashes indicate nucleotide deletions. Restriction enzyme sites unique to *M. avium* or *M. genavense* are labeled as follows: □ = *Sal*I; ▨ = *Bste*II; □ = *Bgl*I.

Because of the recent distinction of *M. avium* and *M. genavense* as unique species, very little information is known about the transmission, virulence factors, or host range of *M. genavense*. By identifying molecular differences between these 2 organisms and creating a rapid test to differentiate

Table 2. Fragment sizes resulting from digestion with restriction enzymes that differentiate *Mycobacterium avium* and *M. genavense.*

Enzyme	Fragments (bp)	
	M. avium	*M. genavense*
*Sal*I	693*	173, 520
*Bste*II	693*	336, 357†
*Bgl*I	333, 285, 75‡	693*

* Does not contain this restriction enzyme site.

† These restriction fragments are very similar in size and typically appear as a single band under the stated electrophoresis conditions.

‡ Because of its small size, the 75-bp band is not consistently visible under the stated electrophoresis conditions.

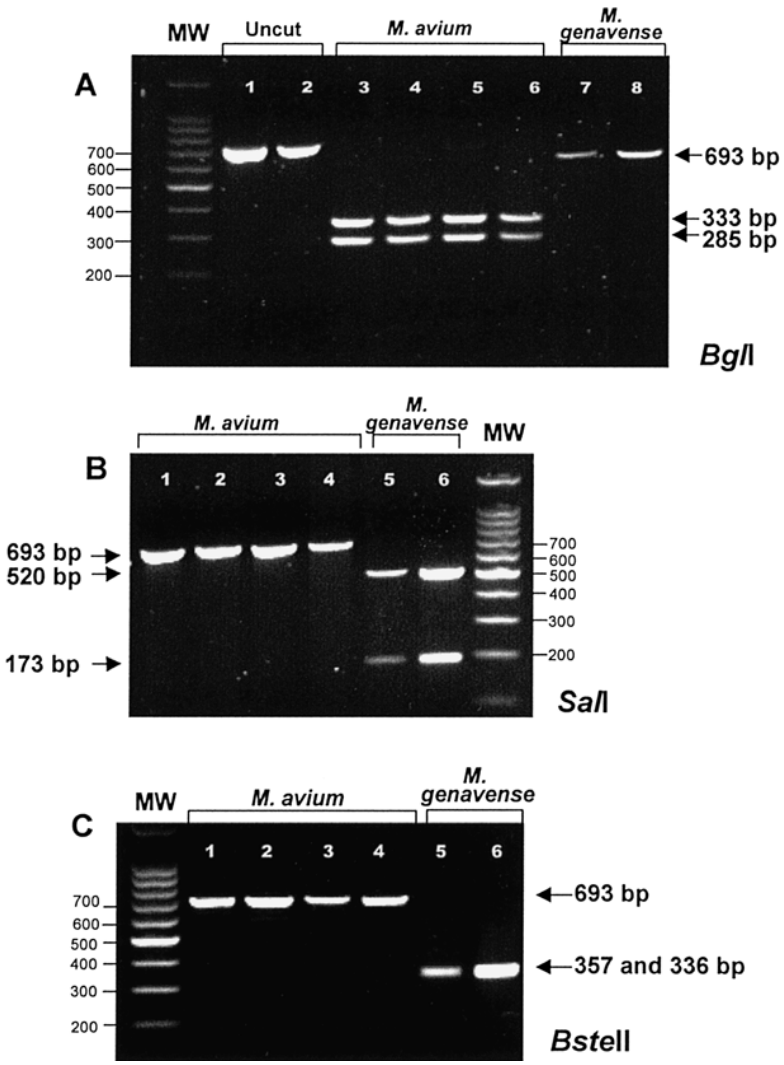

Figure 2. Restriction enzyme digestion profiles of PCR-amplified mycobacteria. MW = molecular mass markers. Restriction digests of amplicons from all *M. avium* or *M. genavense* isolates were identical to the patterns shown. **A**. Uncut PCR amplicons of *M. avium* (lane 1) and *M. genavense* (lane 2). Digestion with *Bgl*I of *M. avium* isolates TMC 724, 101, 94-6621, and 20919-3049 (lanes 3–6) and of *M. genavense* isolates 51233 and 51234 (lanes 7, 8). **B**. *Sal*I digestion profile for the same isolates of *M. avium* (lanes 1–4) and *M. genavense* (lanes 5, 6). **C**. *Bste*II digestion profile for the same isolates of *M. avium* (lanes 1–4) and *M. genavense* (lanes 5, 6).

them, important epidemiologic information can be gathered to learn more about *M. genavense.* The ability to detect *M. genavense* directly from infected tissues could be of great value to veterinary diagnostic laboratories, where mycobacterial culture in liquid medium is not always feasible. Moreover, by rapidly detecting these infections in birds, the possibility of zoonotic transmission from pet to owner may be reduced. The experiments described here using PCR amplification and restriction enzyme digestion of a portion of the *hsp65* gene offer a rapid and simple assay for the detection of and distinction between *M. avium* and *M. genavense.*

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Sources and manufacturers

a. Remel, Lenexa, KS
b. BioSpec Products, Bartlesville, OK.
c. Sigma Chemical Co., St. Louis, MO.
d. Boehringer Mannheim, Indianapolis, IN.
e. GeneAmp 2400, Perkin-Elmer, Norwalk, CT.
f. Invitrogen, San Diego, CA.
g. FMC Bioproducts, Rockland, ME.
h. Gibco BRL, Life Technologies, Frederick, MD.
i. QI Amp Tissue Kit, Qiagen, Valencia, CA.

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Immunohistochemical detection of *Mycobacterium paratuberculosis* in formalin-fixed, paraffin-embedded bovine tissue sections

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Paratuberculosis (Johne's disease) in cattle, a chronic granulomatous enteric disease caused by *Mycobacterium paratuberculosis* (*Mycobacterium avium* subspecies *paratuberculosis*), is diagnosed by clinical observation of chronic diarrheal disease followed by bacterial culture of feces and histopathologic identification of acid-fast bacilli in macrophages in the lamina propria and submucosa of the intestine. The control of paratuberculosis is limited by the lack of rapid, specific diagnostic tests. Microbial culture of fecal samples is routinely used to diagnose the disease, but growth of the organism requires a minimum of 4–8 weeks. Alternatively, histopathologic diagnosis by examination of infected tissues, typically by identification of multifocal accumulations of macrophages and detection of intracellular acid-fast organisms in the lamina propria of the intestine, takes less time but has low sensitivity and specificity.[10] Acid-fast techniques can also have false-positive reactions because they detect the environmental mycobacteria *Nocardia* and *Corynebacteria.*[8]

Immunohistochemical detection of antigens of *M. paratuberculosis* in tissue sections could lead to a more specific and sensitive diagnostic method. Previous studies have used serum from infected goats, cattle, and rabbits to detect *M. paratuberculosis* in formalin-fixed, paraffin-embedded tissue sections.[4–7,9,10] In 1 study, immunohistochemistry was a better indicator of infection than acid-fast stains because it detected more infected animals.[10] Unfortunately, the antibodies used in these studies were made by individual laboratories and are unavailable for widespread use. The goal of the current study was to use a commercial readily available antibody to detect *M. paratuberculosis* in formalin-fixed, paraffin-embedded bovine tissues and to compare, by morphometric analysis, the sensitivity of immunohistochemistry to acid-fast stain.

An immunofluorescence technique was used to show reactivity between the commercially available rabbit anti-*Mycobacterium bovis* serum[a] and *M. paratuberculosis.* The commercially available antibody was produced by immunization of a rabbit with cell lysates from *M. bovis.* After fixation for 10 minutes in 4 C acetone on a slide, *M. paratuberculosis* were rehydrated for 5 minutes in 0.01 M phosphate-buffered saline (pH 7.2) (PBS) and incubated with the primary antibody diluted 1:100 in PBS at room temperature for 1 hour. After incubation, the unbound primary antibody was removed by washing in PBS for 5 minutes, and goat serum was applied for 10 minutes to block nonspecific bind-

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