Definitive Differentiation between Single and Mixed Mycobacterial Infections in Red Deer (*Cervus elaphus*) by a Combination of Duplex Amplification of p34 and f57 Sequences and Hpy188I Enzymatic Restriction of Duplex Amplicons

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Severe emaciation and mortalities suggestive of mycobacterial infections were recently reported for both adult and young wild red deer (*Cervus elaphus*) in the southeastern part of Belgium. In deer, tuberculous lesions are not pathognomonic of *Mycobacterium bovis* infection due to gross and microscopic similarities with lesions caused by *Mycobacterium avium* subsp. *paratuberculosis* or *M. avium* subsp. *avium*. The aim of this study was to improve molecular methods for the species-specific identification of *M. bovis*, *M. avium* subsp. *avium*, and *M. avium* subsp. *paratuberculosis* in mycobacterial infections of deer. DNA banding patterns were assessed prior to and after Hpy188I restriction of f57–upstream (us)-p34 duplex amplicons. The duplex f57–us-p34 PCR differentiated *M. bovis* from *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* infections, whereas the restriction step differentiated single *M. avium* subsp. *paratuberculosis* or *M. avium* subsp. *avium* infections from mixed *M. avium* subsp. *paratuberculosis*/*M. avium* subsp. *avium* infections. The endonuclease Hpy188I cleaves DNA between nucleotides N and G in the unique TCNGA sequence. This restriction site was found at position 168 upstream of the us-p34 initiation codon in all *M. avium* subsp. *avium* strains tested, regardless of their origin and the results of IS901 PCR. In contrast, the restriction site was abrogated in all *M. avium* subsp. *paratuberculosis* strains tested, independent of their origin, Mycobactin J dependency, and IS900 PCR results. Consequently, a two-step strategy, i.e., duplex us-p34–f57 PCR and Hpy188I restriction, allowed us to exclude *M. bovis* infection and to identify single (*M. avium* subsp. *paratuberculosis* or *M. avium* subsp. *avium*) or mixed (*M. avium* subsp. *paratuberculosis*/*M. avium* subsp. *avium*) infections in wild red deer in Belgium. Accordingly, we propose to integrate, in a functional molecular definition of *M. avium* subsp. *paratuberculosis*, the absence of the Hpy188I restriction site from the us-p34 amplicon.

The most significant mycobacterial diseases of free-living, captive, and farmed red deer (*Cervus elaphus*) are bovine tuberculosis, caused by *Mycobacterium bovis*, Johne's disease (paratuberculosis), caused by *Mycobacterium avium* subsp. *paratuberculosis*, and avian tuberculosis, caused principally by *M. avium* subsp. *avium* (20).

Differential diagnoses of infections by *M. bovis*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *avium* are essential because the epidemiology, zoonotic concern, and prevention and control methods are different for each of these mycobacterial infections (20). In deer, tuberculous lesions are not pathognomonic of *M. bovis* infection and are actually indistinguishable from lesions due to *M. avium* subsp. *paratuberculosis* or *M. avium* subsp. *avium* infection because of their gross and microscopic similarities. Moreover, the actual differential di-

agnosis of these mycobacterial infections is difficult due to similarities in the clinical expression and pathology associated with these infections. Antigenic similarity between *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, and *M. bovis* is also responsible for the lack of sensitivity and specificity of diagnostic tests (i.e., current serological and laboratory cell-mediated diagnostic tests as well as skin tests) (21).

The isolation of mycobacteria requires a decontamination treatment of the sample. Depending on the nature and the time of the decontamination treatment, mycobacteria present in the sample may also be killed. As recently demonstrated for *M. avium* subsp. *paratuberculosis*, routine protocols in the laboratory significantly decrease the number of organisms isolated per sample (for fecal, tissue, and milk samples), stressing the importance of the interpretation of negative culture results (12, 32). Conversely, atypical mycobacteria may still be grown from treated specimens (10).

Mycobacterial species are traditionally differentiated on the basis of their phenotypic characteristics. However, since these do not allow the precise identification of all species, recent molecular biology methods offer new opportunities to differentiate, identify, and type mycobacterial species (31). Several molecular targets have been used to allow species-specific

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TABLE 1. Primer selection for PCR identification and sequencing of *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *avium*, and *M. tuberculosis* complex

Primer name and use	Orientation ^a	Sequence (5' → 3') ^b	Position ^c	Concn (nM)	Temp (°C), duration of annealing (s), no. of cycles
Duplex identification of <i>M. avium</i> subsp. <i>avium</i> , <i>M. avium</i> subsp. <i>paratuberculosis</i> , and <i>M. tuberculosis</i> complex					
Myc1	R	GAGTAGGTCATGGCTCCTCC	811–830	330	56, 60, 40
Myc3 ^d	F	CATGCASYGAATTAGAACGT	574–593		
f57a	F	GGTCGCGTCATTGAGAATC	179–197	132	
f57b	R	TCTCAGACAGTGGCAGGTG	599–617		
Identification of <i>M. avium</i> subsp. <i>paratuberculosis</i>					
IS900 S1	F	GGGTGATCTGGACAATGACGGTTA	202–226	165	68, 60, 40
IS900 R3 (2)	R	AGCGCGGCACGGCTCTTGT	751–770		
Identification of <i>M. avium</i> subsp. <i>avium</i>					
IS901 1/2 SO	F	GCAACGGTTGTGTGCTTGAAGAAT	76–100	165	67, 60, 40
IS901 1/2 RO	R	GCGCACGCATGATGAGTGGACTTAC	264–288		

^a F, forward; R, reverse.

^b Oligonucleotide primers were designed by using the GCG software from the University of Wisconsin through the use of the Belgian EMBnet Node facility.

^c The positions of the Myc, f57, IS900, and IS901 primers were defined according to the 34-kDa antigen gene of *M. avium* subsp. *paratuberculosis* (GenBank accession number AF411607), the f57 DNA fragment of *M. avium* subsp. *paratuberculosis* (GenBank accession number X70277), the *M. avium* subsp. *paratuberculosis* insertion sequence IS900 putative transposase (p43) gene (GenBank accession number no. AF416985), and the *M. avium* subsp. *avium* insertion sequence IS901 open reading frame (GenBank accession number no. X59272), respectively.

^d Primer Myc3 was degenerated as follows, according to the mismatches between the us-p34 sequences of *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, and *M. bovis*: S is G or C, and Y is C or T.

identification, including IS6110 and 16S rRNA for the *Mycobacterium tuberculosis* complex (i.e., *M. bovis*, *M. tuberculosis*, *Mycobacterium africanum*, and *Mycobacterium microti*), IS900 and the f57 sequence for *M. avium* subsp. *paratuberculosis* (3, 4, 24, 34, 36), and IS901 for *M. avium* subsp. *avium* (14, 29). A duplex PCR has been developed to allow the differential identification of *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *avium*, and members of the *Mycobacterium tuberculosis* complex (4). However, the assay does not allow the differentiation of single from mixed infections.

The aim of this study was to further improve molecular methods for the species-specific identification of *M. bovis*, *M. avium* subsp. *avium*, and *M. avium* subsp. *paratuberculosis* in order to diagnose and differentiate single from mixed mycobacterial infections in red deer. During the period of 1999–2002, severe emaciation and mortalities, presumably related to mycobacterial infections, were reported for both adult and young wild red deer in the southeastern part of Belgium. Thirty-three carcasses (or parts of them, after scavenging had occurred) of deer that were found dead were brought to the local veterinary investigation center, and samples (mainly fecal material, as well as mesenteric lymph nodes) were shipped to our laboratories for bacteriological and molecular analysis.

MATERIALS AND METHODS

Bacterial isolates. Reference strains included two *M. avium* subsp. *avium* strains (ATCC 25291 and DSMZ 44156), three *M. avium* subsp. *paratuberculosis* strains (ATCC 19698, ATCC 43015, known as strain Linda [2], and the vaccinal strain 2E [33]), and one *M. bovis* strain (ATCC 19210). As controls, *M. avium* subsp. *paratuberculosis* strains ($n = 12$) isolated from bovine specimens during routine paratuberculosis diagnostic work between 2000 and 2003 were incorporated into this study. The controls also included *M. bovis* isolates from animal sources (1 from a chimpanzee and 2 from cattle) as well as *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* isolates from human sources (3 *M. avium* subsp. *paratuberculosis* isolates from patients with Crohn's ileal disease and 32 *M. avium* subsp. *avium* isolates from AIDS patients with underlying lung disease).

Deer samples. Feces and/or tissue and organs known as sites of active infection, i.e., mesenteric lymph nodes and ileocecal junctions, were taken from

slaughtered farmed red deer (3 animals) and from wild deer that were found dead (33 animals). In the latter case, the age, body condition (mainly emaciation and obvious soiling with green material around the tail, hindquarters, and hocks), and a description of gross lesions were recorded whenever possible. For some of these animals, organs were not available due to scavenging.

Bacteriology. All samples were subjected to diagnostic laboratory protocols for the isolation of *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, and *M. bovis*. Briefly, after a decontamination treatment with 0.75% hexadecylpyridinium chloride overnight, fecal samples were inoculated and tested for Mycobactin J (BioMérieux, France) dependency on Herrold's egg yolk medium (HEYM) slants with and without Mycobactin J as previously described (11). Tissue samples were inoculated on HEYM slants with Mycobactin J and on Coletos slants (Sanofi Diagnostics Pasteur, France) after a decontamination treatment with 3% oxalic acid for 30 min (38). Samples were incubated at 37°C for 4 to 6 months. The primary identification of *M. avium* subsp. *paratuberculosis* was based on its Mycobactin J dependency, whereas the primary identification of *M. bovis* was based on its susceptibility to thiophen-2-carboxylic acid hydrazide (TCH).

Preparation of mycobacterial DNA. DNA extraction was performed on colonies grown for 4 to 6 months. Colonies were suspended in 200 µl of lysing solution (0.1 M NaOH, 1 M NaCl, and 5% sodium dodecyl sulfate) and heated (100°C) for 20 min. The suspension was then cooled, neutralized with 3 volumes of 0.1 M Tris-HCl (pH 7.4) buffer, and centrifuged (5,000 × g , 5 min). Supernatants were extracted with phenol-chloroform, and DNAs were precipitated with ethanol, collected by centrifugation, dissolved in 50 µl of H₂O, and stored at –20°C. DNAs were quantified by UV spectroscopy and/or fluorometry using Picogreen (Molecular Probes, Leiden, The Netherlands).

PCR amplification. The IS900 amplification protocol was a modified version of a previously described method (34). IS900 and IS901 primer sequences and their respective annealing temperatures are listed in Table 1. The amplification protocol was the same for the IS900 and IS901 PCRs and was performed as follows. The PCR mixture (50 µl) consisted of sample DNA (20 ng), 10 mM Tris-HCl, pH 8.3 (at 25°C), 50 mM KCl, 1.5 mM MgCl₂, a 0.25 mM concentration of each deoxynucleoside triphosphate, 165 nM of each primer, and 0.75 U of AmpliTaq DNA polymerase (Perkin-Elmer Applied Biosystems, Foster City, Calif.). After an initial denaturation step (3 min at 94°C), 40 cycles of amplification were performed as follows: denaturation at 94°C for 1 min, annealing at the optimal temperature for each primer set (Table 1) for 1 min, and DNA extension at 72°C for 1 min 30 s. A final extension was performed at 72°C for 15 min.

The duplex us-p34–f57 amplification protocol was a modified version of a previously described protocol, with the original myc3 primer being degenerated and named myc32 (4). The myc1–myc32 (Fig. 1) and f57a–f57b (Fig. 2) primer sets, specific for the us-p34 and f57 target genes, respectively, were used as listed

FIG. 1. Multiple nucleotide sequence alignment of *M. avium* subsp. *avium* (Maa), *M. avium* subsp. *paratuberculosis* (Map) and *M. bovis* (Mbo) *us-p34* genes. Gaps between sequences are indicated by dots. Vertical bars indicate identity across sequences. The start codon (ATG) for the p34 open reading frame is shown in bold. Primer positions are indicated by boxes. The horizontal arrows indicate directions of transcription. The Hpy188I restriction site is indicated by a bolded nucleotide. The single nucleotide substitution between *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* within the restriction site is highlighted with an arrow and a bold nucleotide. Nucleotides shown in bold italics in the myc3 sequence were degenerated in the corresponding primer (see Table 1).

(Table 1). Briefly, the expected size of *us-p34* amplicons was 257 bp for *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* and 178 bp for *M. bovis* and other members of the *M. tuberculosis* complex, whereas the expected size of *M. avium* subsp. *paratuberculosis*-specific *f57* amplicons was 439 bp (Fig. 3 and 4). Accordingly, the banding patterns allowed us to discriminate between these three mycobacterial species as previously described (4). Each PCR mixture (50 μ l) consisted of a DNA sample (20 ng), 10 mM Tris-HCl, pH 8.3 (at 25°C), 50 mM KCl, 1.5 mM MgCl₂, a 0.25 mM concentration of each deoxynucleoside triphosphate, the myc1 and myc32 primers, the *f57a* and *f57b* primers, and 0.75 U of AmpliTaq DNA polymerase (Perkin-Elmer Applied Biosystems). The amplification protocol was the same as that used for IS900 and IS901 PCR.

Identification of *M. bovis* by 16S rRNA PCR was performed with an Amplicor *Mycobacterium tuberculosis* detection kit (Amplicor MTB; Roche Diagnostic Systems, Somerville, N.J.) according to the manufacturer's instructions.

IS900, IS901, 16S rRNA, and duplex *us-p34*-*f57* amplifications were carried out in a DNA 2400 thermocycler (Perkin-Elmer Applied Biosystems). Except for 16S rRNA amplicons, which were detected by hybridization according to the manufacturer's instructions, all of the amplified DNA fragments were run in a 2% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator.

PCR controls. A strict procedure was followed to avoid cross-contamination between samples or carryover of PCR products. For any series of reactions, contamination at the DNA level was ruled out by performing PCR analysis

FIG. 2. Nucleotide sequence of *M. avium* subsp. *paratuberculosis* *f57* segment (29a). Primer positions are indicated by boxes. The horizontal arrows indicate directions of transcription. Hpy188I restriction sites are shown in bold.

FIG. 3. Banding patterns of undigested us-p34–f57 amplicons. (A) Theoretical banding patterns before Hpy188I digestion (▼, Hpy188I restriction site). (B) Amplified DNA fragments from *M. bovis* (lane 3), *M. avium* subsp. *paratuberculosis* (lane 4), *M. avium* subsp. *avium* (lane 5), a mixture of *M. avium* subsp. *avium* and *M. bovis* (lane 6), a mixture of *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* (lane 7), and a mixture of *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, and *M. bovis* (lane 8) were separated by electrophoresis in a 2% agarose gel. The lengths (in base pairs) of the amplified fragments and of the 123-bp DNA ladder (lane 1) and 50-bp DNA ladder (lane 2) (molecular weight markers) are indicated on the right and left sides of the gel, respectively. Hatched boxes represent the p34 gene.

without a DNA template and on a negative control for DNA extraction. For positive controls, DNA samples from reference and control strains of *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, and *M. bovis* were included in each test. For each field sample, PCR inhibition was assessed. Briefly, 10^4 copies of a pCR-XL-TOPO plasmid carrying the 16S rRNA gene of *Staphylococcus aureus* was amplified alone and mixed with the sample. Amplification was carried out as described previously (15). PCR inhibition was considered relevant when no amplification or markedly decreased amplification of 10^4 plasmid copies of *S. aureus* from the mixture was observed.

Hpy188I restriction. Duplex us-p34–f57 amplification generates a banding pattern that does not allow discrimination between single *M. avium* subsp. *paratuberculosis* and mixed *M. avium* subsp. *paratuberculosis*/*M. avium* subsp. *avium* infections (Fig. 3). Recently, a novel endonuclease, Hpy188I, which recognizes the unique TCNGA sequence and cleaves the DNA between nucleotides N and G, was purified from *Helicobacter pylori* (37). In our previous work (4), the TCNGA sequence was found in *M. avium* subsp. *avium*, whereas a C-to-G single-nucleotide substitution (underlined hereafter) was identified in *M. avium* subsp. *paratuberculosis* within the TCNGA sequence, at position 168 upstream of the us-p34 initiation codon (Fig. 1). In a prevalidation step, 10 *M. avium* subsp. *paratuberculosis* and 10 *M. avium* subsp. *avium* strains were subjected to sequence analysis. Amplicons were sequenced in both orientations with a Big Dye Terminator kit (ABI PRISM; Applied Biosystems, Nieuwerkerk, The Netherlands), using an automated ABI 3100 sequencer (Applied Biosystems). The sequences were compared against those in GenBank for identity-based species identification using the BLAST program of the National Center for Biotechnol-

ogy Information database. The C-to-G single-nucleotide substitution was found in all of the *M. avium* subsp. *paratuberculosis* and none of the *M. avium* subsp. *avium* isolates. In a next step, the validity of Hpy188I restriction was assessed with DNA samples from reference strains (three *M. avium* subsp. *paratuberculosis*, two *M. avium* subsp. *avium*, and one *M. bovis* strain) and with one reconstituted mixed *M. avium* subsp. *avium*/*M. avium* subsp. *paratuberculosis* DNA sample. Finally, Hpy188I restriction analysis was performed on control and field samples according to the manufacturer's protocol, except that amplicons were digested for 2 h (Table 2).

RESULTS

Ziehl-Neelsen staining and bacteriology of deer samples.

The results of Ziehl-Neelsen staining and bacteriology of deer samples are depicted in Table 3. A large number of acid-fast bacilli were seen after staining of feces or tissue smears for all but six samples. However, mycobacteria were grown from the six Ziehl-Neelsen stain-negative samples. A total of 36 strains (33 from wild deer and 3 from farmed deer) were isolated and further characterized. Cultures on HEYM with Mycobactin J were obtained for all but one sample. Interestingly, 12 isolates assigned to the *M. avium* subsp. *paratuberculosis* infection

FIG. 4. Hpy188I digestion profiles of duplex *us-p34-f57* amplicons. (A) Theoretical banding patterns after Hpy188I digestion. (B) Banding patterns of *M. bovis* (lane 3), *M. avium* subsp. *paratuberculosis* (lane 4), *M. avium* subsp. *avium* (lane 5), a mixture of *M. avium* subsp. *avium* and *M. bovis* (lane 6), a mixture of *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* (lane 7), and a mixture of *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, and *M. bovis* (lane 8). The lengths (in base pairs) of the amplified fragments and of the 123-bp DNA ladder (lane 1) and the 50-bp DNA ladder (lane 2) (molecular weight markers) are indicated on the right and left sides of the gel, respectively.

group based on molecular techniques were classified as Mycobactin J independent, 4 were classified as Mycobactin J dependent, and 11 were not actually tested for their Mycobactin J dependency. All isolates tested for Mycobactin J dependency and assigned to the *M. avium* subsp. *avium* infection group or the mixed *M. avium* subsp. *paratuberculosis*/*M. avium* subsp. *avium* infection group were classified as Mycobactin J independent. *M. bovis* infection was excluded based on negative TCH test results (data not shown).

IS900, IS901, and 16S rRNA PCR. The IS900 and IS901 PCR results obtained with reference and control strains from different origins are depicted in Table 2. The IS901 and IS900 PCR results were negative for the four *M. bovis* strains. The 2 *M. avium* subsp. *avium* reference strains of avian origin were positive for IS901, whereas the 31 human and 1 bovine *M. avium* subsp. *avium* strain were consistently negative, as were the 18 *M. avium* subsp. *paratuberculosis* strains. The bovine and human *M. avium* subsp. *paratuberculosis* reference strains as well as bovine and human *M. avium* subsp. *paratuberculosis* strains isolated from clinical samples were all IS900 positive. The IS900/IS901 amplification results obtained for deer isolates are depicted in Table 3. Three patterns of IS900/IS901 results were observed: IS900 negative and IS901 positive ($n = 4$), IS900 positive and IS901 negative ($n = 27$), and IS900 positive and IS901 positive ($n = 5$). These results are compat-

ible with three patterns of infection, namely, infection with *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, or a combination of *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium*, respectively. 16S rRNA PCR yielded positive results with the four *M. bovis* strains and negative results with all the other strains (data not shown).

Duplex *us-p34-f57* PCR and Hpy188I restriction of duplex *us-p34-f57* amplicons (PCR-restriction fragment length polymorphism [PCR-RFLP] analysis). (i) **Duplex *us-p34-f57* PCR.** As illustrated with *M. avium* subsp. *avium* ($n = 2$), *M. avium* subsp. *paratuberculosis* ($n = 3$), and *M. bovis* ($n = 1$) reference strains, the banding pattern generated by duplex amplification can distinctly identify each species. However, duplex amplification cannot discriminate *M. avium* subsp. *paratuberculosis* alone from a mixture of *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* (Fig. 3). Likewise, a mixture of *M. avium* subsp. *paratuberculosis* and *M. bovis* cannot be distinguished from a mixture of *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *avium*, and *M. bovis*.

(ii) **Hpy188I restriction.** Hpy188I restriction of duplex *us-p34-f57* PCR amplicons obtained from the *M. avium* subsp. *avium* ($n = 2$), *M. avium* subsp. *paratuberculosis* ($n = 3$), and *M. bovis* ($n = 1$) reference strains as well as from a reconstituted mixed *M. avium* subsp. *avium*/*M. avium* subsp. *paratuberculosis* DNA sample gave the expected banding patterns.

TABLE 2. Multigenotypic identification of reference and control strains of *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *avium*, and *M. bovis*

Strain	Source ^a	Origin ^b	Total no. of strains tested	PCR result				Identification after Hpy188I digestion
				IS900	IS901	Duplex		
						us-p34	f57	
<i>M. avium</i> subsp. <i>avium</i>								
Reference strains								
ATCC 25291	ATCC	Avian	1	—	+	+	—	<i>M. avium</i> subsp. <i>avium</i>
DSMZ 44156	DSMZ	Avian	1	—	+	+	—	<i>M. avium</i> subsp. <i>avium</i>
Clinical strains (<i>n</i> = 32)								
	PIB	Human	30	—	—	+	—	<i>M. avium</i> subsp. <i>avium</i>
	ITM	Human AIDS	1	—	—	+	—	<i>M. avium</i> subsp. <i>avium</i>
	ITM	Bovine	1	—	—	+	—	<i>M. avium</i> subsp. <i>avium</i>
<i>M. avium</i> subsp. <i>paratuberculosis</i>								
Reference strains								
ATCC 43015 (Linda)	ATCC	Human	1	+	—	+	+	<i>M. avium</i> subsp. <i>paratuberculosis</i>
ATCC 19698	ATCC	Bovine	1	+	—	+	+	<i>M. avium</i> subsp. <i>paratuberculosis</i>
2E	NVI	Bovine	1	+	—	+	+	<i>M. avium</i> subsp. <i>paratuberculosis</i>
Clinical strains (<i>n</i> = 15)								
	VAR	Bovine	2	+	—	+	+	<i>M. avium</i> subsp. <i>paratuberculosis</i>
	VAR	Bovine	6	+	—	+	+	<i>M. avium</i> subsp. <i>paratuberculosis</i>
	ARSIA	Bovine	2	+	—	+	+	<i>M. avium</i> subsp. <i>paratuberculosis</i>
	ITM	Bovine	1	+	—	+	+	<i>M. avium</i> subsp. <i>paratuberculosis</i>
	PIB	Bovine	1	+	—	+	+	<i>M. avium</i> subsp. <i>paratuberculosis</i>
	HUMF	Human CD	3	+	—	+	+	<i>M. avium</i> subsp. <i>paratuberculosis</i>
<i>M. bovis</i>								
Reference strain								
ATCC 19210			1	—	—	+	—	<i>M. bovis</i>
Clinical strains (<i>n</i> = 3)								
	VAR	Primate	1	—	—	+	—	<i>M. bovis</i>
	VAR	Bovine	2	—	—	+	—	<i>M. bovis</i>

^a ATCC, American Type Culture Collection; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen. PIB, Pasteur Institute of Brussels, Belgium (M. Fauville-Dufaux); ITM, Institute of Tropical Medicine, Antwerp, Belgium (F. Portaels); NVI, National Veterinary Institute, Oslo, Norway (F. Saxegaard [33]); ARSIA, Association Régionale de Santé et d'Identification Animales, Walloon Region, Belgium (J. Bughin); HUMF, Histology Unit of the Medical Faculty, Université Catholique de Louvain (C. Coetsier); VAR, Veterinary and Agrochemical Research Centre, Brussels, Belgium.

^b CD, Crohn's disease.

Sequence analysis of the amplicons prior to digestion confirmed the restriction patterns. Digestion of the TCCGA site in the 257-bp amplicons from *M. avium* subsp. *avium* yielded 79-bp and 178-bp fragments, whereas the C-to-G substitution in *M. avium* subsp. *paratuberculosis* suppressed the restriction site (Fig. 4). No restriction site was present in the 178-bp us-p34 amplicons from *M. bovis*. The digestion of *M. avium* subsp. *paratuberculosis*-specific f57 amplicons generated several fragments, of which only two (255 bp and 144 bp) were seen on the gel (Fig. 4). Accordingly, the digestion of duplex us-p34–f57 amplicons generated specific banding patterns for *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *avium*, and *M. bovis* alone as well as for any combination of two of these species (Fig. 4). The band size similarity (178 bp) between *M. bovis* us-p34 amplicons and a digested fragment of *M. avium* subsp. *avium* us-p34 amplicons could hamper the detection of mixed *M. bovis*, *M. avium* subsp. *avium*, and *M. avium* subsp. *paratuberculosis* infections. However, the presence of *M. bovis* would have been assessed by the duplex PCR in the first step of the PCR-RFLP analysis, so the hypothetical mixed *M. bovis*, *M. avium* subsp. *avium*, and *M. avium* subsp. *paratuberculosis* infection would also have been identified (Fig. 3).

(iii) Validation with control strains. In the following step, the PCR-RFLP method was tested on a series of strains iso-

lated from human and animal clinical cases, including 32 *M. avium* subsp. *avium*, 15 *M. avium* subsp. *paratuberculosis*, and 3 *M. bovis* isolates. All of the DNA samples and the reconstituted mixture displayed the expected banding patterns according to their species. The molecular results were also in accordance with the IS900 PCR results, but no validation based on the IS901 PCR results could be made because all of the *M. avium* subsp. *avium* control strains were negative for IS901 (Table 2).

(iv) Analysis of mycobacterial colonies from deer samples.

In a final step, the PCR-RFLP method was used to test 36 strains isolated from wild red deer found dead in the field (*n* = 33) and from slaughtered farmed deer (*n* = 3) (Table 3). Prior to enzymatic digestion, the duplex us-p34–f57 PCR generated only two patterns, with a us-p34-positive result for every sample (*n* = 36) and an f57 result that was either negative (*n* = 4) or positive (*n* = 32). No pattern consistent with the presence of *M. bovis* was seen, confirming the bacteriological identification results and the negative 16S rRNA PCR results. The molecular results were also in accordance with the IS900 and IS901 PCR results. For these last tests, it is worth noting that all of the *M. avium* subsp. *avium* deer isolates were IS901 positive. No PCR inhibition was observed.

TABLE 3. Two-step multigenotypic identification of *M. avium* subspecies isolated from red deer ($n = 36$)^a

Cervine field strains (<i>n</i>)	No. of cultures ^b	Growth in culture medium ^d			Ziehl-Neelsen staining result	PCR result				Identification after Hpy188I digestion ^c
		HEYM	HEYM + mycobactin J	Coletos medium		IS900	IS901	Duplex PCR		
								us-p34	f57	
<i>M. avium</i> subsp. <i>avium</i> (4)	2	ND	+	+	+	—	+	+	—	<i>M. avium</i> subsp. <i>avium</i>
	1	+	+	+	+	—	+	+	—	<i>M. avium</i> subsp. <i>avium</i>
	1	ND	+	ND	+	—	+	+	—	<i>M. avium</i> subsp. <i>avium</i>
<i>M. avium</i> subsp. <i>paratuberculosis</i> Single infections (27)	4	—	+	—	+	+	—	+	+	<i>M. avium</i> subsp. <i>paratuberculosis</i>
	2	+	+	+	+	+	—	—	+	<i>M. avium</i> subsp. <i>paratuberculosis</i>
	10	ND	+	+	+	+	—	+	+	<i>M. avium</i> subsp. <i>paratuberculosis</i>
	6	ND	+	ND	+	+	—	+	+	<i>M. avium</i> subsp. <i>paratuberculosis</i>
	5 ^c	ND	+	ND	—	+	—	+	+	<i>M. avium</i> subsp. <i>paratuberculosis</i>
Mixed infections (5)	1	+	+	+	—	+	+	+	+	<i>M. avium</i> subsp. <i>paratuberculosis</i> / <i>M. avium</i> subsp. <i>avium</i>
	3	ND	+	+	+	+	+	+	+	<i>M. avium</i> subsp. <i>paratuberculosis</i> / <i>M. avium</i> subsp. <i>avium</i>
	1	—	—	+	+	+	+	+	+	<i>M. avium</i> subsp. <i>paratuberculosis</i> / <i>M. avium</i> subsp. <i>avium</i>

^a The two-step identification of *M. avium* subspecies included duplex u-p34–f57 PCR as the first step and the Hpy188I restriction of duplex us-p34–f57 amplicons as the second step (independent of the IS900/IS901 PCR results).

^b Since only one culture was tested per animal, the number of cultures represents the number of deer tested.

^c Three samples were obtained from farmed deer.

^d ND, not done.

Multigenotypic molecular identification of deer strains.

Taken together, the results of IS900, IS901, and 16S rRNA amplification, the duplex us-p34–f57 PCR, and the duplex us-p34–f57 PCR-RFLP method identified three patterns of mycobacterial infections in deer field samples: 4 (11%) samples were identified as *M. avium* subsp. *avium*, 27 (75%) were identified as *M. avium* subsp. *paratuberculosis*, and 5 (14%) were identified as a mixture of *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium*, suggesting a coinfection with both mycobacteria.

DISCUSSION

Over 3 million reindeer (*Rangifer tarandus*) and 5 million deer belonging to other gregarious species are currently herded, held in fences, or farmed worldwide. *M. avium* subsp. *paratuberculosis*, *M. bovis*, and *M. avium* subsp. *avium* infections appear to be the most significant mycobacterial infections in free-living, captive, and farmed deer (18, 19). They have been reported in New Zealand (7, 20), North America (6, 22, 28, 30, 35), and Europe (11, 16, 17, 23, 27). Bacterial isolation is considered the “gold standard” and the most sensitive method for the diagnosis of mycobacterial diseases (19, 20). However, it is a very slow and labor-intensive procedure, and

results are not available for several weeks (if not months) after inoculation, especially for samples containing small numbers of mycobacteria. Moreover, due to decontamination procedures, false-negative results may be obtained (12, 32). The identification of *M. avium* subsp. *paratuberculosis* is characterized by the organism's requirement for the iron-chelating growth factor Mycobactin J for in vitro growth. Recently, it was stressed that the diagnosis of paratuberculosis relying on Mycobactin J dependency also needed to be interpreted with caution (26). Therefore, as also confirmed in our study, subspecies differentiation between *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* cannot rely solely on this criterion.

Likewise, indirect tests also have limitations. Although *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* differ markedly in their virulence, particularly for cattle, they are antigenically and genetically very similar organisms (1). Serological tests and skin tests used for the diagnosis of paratuberculosis therefore have a low specificity. Moreover, cross reactions occur between *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, and *M. bovis* (21). Thus, the differential diagnosis of mycobacterial infections remains difficult with currently available tests (20).

Accordingly, a wide panel of molecular tests have therefore

been proposed to improve the identification of mycobacteria. Among these, IS900- and IS901-based PCR assays have largely been used for the detection of *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium*, respectively (14, 24, 29, 36). Both markers, however, have limitations. Indeed, current data confirm the lack of the IS901 insertion sequence in human and bovine clinical *M. avium* subsp. *avium* strains, as reported previously for *M. avium* subsp. *avium* strains of various origins, including deer (14, 29). Interestingly, in our study, all of the *M. avium* subsp. *avium* strains isolated from deer were IS901 positive. Regarding IS900, recent data suggest that IS900-like elements are present in mycobacterial species other than *M. avium* subsp. *paratuberculosis*, generating false-positive results (5, 8, 13, 26, 34). Accordingly, false-negative and false-positive results cannot be ruled out with both insertion sequences, and IS900 or IS901 PCR results alone should therefore be interpreted with caution and compared to those obtained with other specific DNA markers. Moreover, as recently highlighted, because DNA is often not extracted from single colonies, it is possible that the extracted DNA contains a low level of DNA from another mycobacterial strain that is masked by the differential growth of the predominant mycobacteria in the sample. This was the most likely explanation for IS901-positive results obtained with DNA extracted from a sample containing *M. avium* subsp. *paratuberculosis*, as recently reported (26).

In our study, IS900-positive results were obtained with 89% (32/36) of samples from deer, whereas 25% of the samples (9/36) were IS901 positive. Among the latter, 4/9 were IS900 negative and 5/9 were IS900 positive. Accordingly, the combined IS900 and IS901 results suggested either single *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* infections, or in some cases, a mixed *M. avium* subsp. *paratuberculosis*/*M. avium* subsp. *avium* infection. However, due to the fact that DNAs were not extracted from single colonies and due to the potential limitations of the IS900/901 PCR, the definitive identification of the IS900/901 pattern in deer samples was still pending.

Since the us-p34-f57 duplex PCR (4) does not allow us to discriminate between *M. avium* subsp. *paratuberculosis* infection alone and a mixed *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* infection, Hpy188I restriction was carried out on duplex amplicons. This two-step strategy, i.e., integration of the pre- and postrestriction results, allowed the identification of single (*M. avium* subsp. *paratuberculosis* or *M. avium* subsp. *avium*) and mixed (*M. avium* subsp. *paratuberculosis*/*M. avium* subsp. *avium*) infections. The results of the banding patterns confirmed the IS900 and IS901 PCR results supporting the mixed infection hypothesis.

At this point, the TCNGA sequence had been found in all *M. avium* subsp. *avium* strains tested, regardless of their origin, Mycobactin J dependency, and IS901 PCR results, whereas the C-to-G single-nucleotide substitution within the TCNGA sequence, at position 168 upstream of the us-p34 initiation codon, was identified in all *M. avium* subsp. *paratuberculosis* strains tested. Consequently, all *M. avium* subsp. *avium* strains were restricted by Hpy188I, whereas none of the *M. avium* subsp. *paratuberculosis* strains were restricted. No restriction site was present in the 178-bp us-p34 amplicons from *M. bovis*.

These results suggest that Hpy188I restriction performed on duplex amplicons will allow a clear discrimination between *M.*

avium subsp. *avium* and *M. avium* subsp. *paratuberculosis* strains, regardless of the Mycobactin J and IS900/901 PCR results. Moreover, this technique will also assess the possible contamination of *M. avium* subsp. *paratuberculosis* DNA by *M. avium* subsp. *avium* DNA and hence will help to further explain unexpected IS900/901 results, as recently documented (26), allowing validation of further molecular typing studies. We therefore propose to integrate the absence of the Hpy188I restriction site at position 168 upstream of the us-p34 initiation codon in a functional molecular definition of *M. avium* subsp. *paratuberculosis*.

Taken together, our molecular results strengthen the hypothesis that besides well-documented *M. avium* subsp. *paratuberculosis* infections, *M. avium* subsp. *avium* infections were the cause of macroscopic tuberculous lesions and eventually of death in a substantial number of wild red deer in Belgium. Moreover, we cannot rule out that mixed *M. avium* subsp. *paratuberculosis*/*M. avium* subsp. *avium* infections may result in more aggressive acute clinical outbreaks, particularly in young animals.

As recently reemphasized, there are very limited data, if any, on the proportions of *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* subclinically infected deer that show no macroscopic evidence of infection, so that background information is missing (20). In our opinion, because of its ubiquitous nature, the issue becomes the identification of factors contributing to the implication of *M. avium* subsp. *avium*, alone or in association with *M. avium* subsp. *paratuberculosis*, in infection and eventually in clinical diseases in wild red deer. Factors likely to affect the susceptibility of deer to mycobacterial infection include age, environment, population density, exposure, and genetics (9, 18, 20).

Clinical outbreaks due to *M. avium* subsp. *avium* were reported for groups of deer kept in feedlots or indoors in winter, where they are exposed to food or water contaminated by bird feces (19). The hypothesis of a great exposition of these animals to *M. avium* subsp. *avium* is also strengthened, for farmed red deer, by the widespread reactivity to *M. avium* subsp. *avium* in skin tests and lymphocyte transformation assays (20). As reported for *M. bovis* infections of white-tailed deer (*Odocoileus virginianus*) (25) and *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* infections of red deer (20), supplemental feeding or winter feeding may combine several risk factors, such as population density, exposure, and cross-contamination by infected vectors and contaminated water, soil, or food. High-density and winter feeding practices could at least partly explain the unusual pattern of mycobacterial infections in Belgian wild deer by enhancing exposure to *M. avium* subsp. *avium* so that a "clinical threshold" is reached and pathology is seen.

In conclusion, *M. avium* subsp. *avium*, besides or in association with *M. avium* subsp. *paratuberculosis*, causes clinical disease and eventually death in wild red deer in Belgium. Hence, the diagnosis of mycobacteriosis in deer is difficult, and current immunological tests do not allow a clear discrimination between both infections. Mixed infections may further complicate the picture and even challenge the definite bacteriological diagnosis of mycobacterial infections in deer. Therefore, the identification of mycobacterial infections requires a combination of phenotypic and molecular markers.

Our studies suggest that a combination of classical bacteriological isolation and identification methods, IS900/IS901- and 16S rRNA-based PCRs, and duplex us-p34-f57 PCR followed by Hpy188I restriction is a useful tool for the identification of mycobacterial infection in deer, particularly to discriminate between single *M. avium* subsp. *paratuberculosis* or *M. avium* subsp. *avium* infections, mixed *M. avium* subsp. *paratuberculosis*/*M. avium* subsp. *avium* infections, and *M. bovis* infections.

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