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# Molecular Identification of *Mycobacterium avium* subsp. *silvaticum* by Duplex High-Resolution Melt Analysis and Subspecies-Specific Real-Time PCR

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**Accurate identification of mycobacterial species and subspecies is essential to evaluate their significance and to perform epidemiological studies. The subspecies of *Mycobacterium avium* have different attributes but coincide in their zoonotic potential. Our knowledge about *M. avium* subsp. *silvaticum* is limited, since its identification is uncertain. *Mycobacterium avium* subsp. *avium* and *M. avium* subsp. *silvaticum* can be discriminated from each other based only on phenotypic characteristics, as they have almost identical genome sequences. Here we describe the development of a diagnostic method which enables the molecular identification of *M. avium* subsp. *silvaticum* and discrimination from *M. avium* subsp. *avium* based on genomic differences in a duplex high-resolution melt and *M. avium* subsp. *silvaticum*-specific mismatch real-time PCR. The developed assay was tested on reference strains and 199 field isolates, which were analyzed by phenotypic methods previously. This assay not only identified all 63 *M. avium* subsp. *silvaticum* and 138 *M. avium* subsp. *avium* strains correctly but also enabled the detection of mixed *M. avium* subsp. *avium*-*M. avium* subsp. *silvaticum* cultures. This is the first time that such a large panel of strains has been analyzed, and we also report the first isolation of *M. avium* subsp. *silvaticum* from red fox, red deer, wild boar, cattle, and badger. This assay is reliable, rapid, simple, inexpensive, and robust. It eliminates the long-existing problem of ambiguous phenotypic identification and opens up the possibility for detailed and comprehensive strain studies.**

The species *Mycobacterium avium* is divided into three subspecies according to the currently valid taxonomical classification: *Mycobacterium avium* subsp. *paratuberculosis*, the etiological agent of Johne's disease; *Mycobacterium avium* subsp. *avium*, the pathogen of avian tuberculosis; and *Mycobacterium avium* subsp. *silvaticum* (1). In addition, the designation "*Mycobacterium avium* subsp. *hominissuis*" has been proposed for human/porcine-type *M. avium* isolates (2). *M. avium* subsp. *silvaticum*, previously called "wood pigeon *Mycobacterium*," was assigned to the *Mycobacterium avium* complex (MAC) in 1990 (3). Besides wood pigeons, it was isolated from crane (3), penguin (4), roe deer (5), and hazel hen (5). The zoonotic potential of MAC members is renowned (6), but less is known about *M. avium* subsp. *silvaticum*; hence, the detection and study of this subspecies are hindered by its unreliable and ambiguous identification.

Accurate identification of mycobacterial species and subspecies is essential for evaluation of their significance, pathogenicity, and epidemiology. The four subspecies of *M. avium* differ greatly in their host range, growth potential, and environmental occurrence (4). Three subspecies can be easily differentiated by molecular biological methods thanks to distinct molecular differences. IS*900* is the specific insertion sequence (IS) of *M. avium* subsp. *paratuberculosis*. "*M. avium* subsp. *hominissuis*" and *M. avium* subsp. *avium* both harbor IS*1245* but can be discriminated with IS*901* (absent from "*M. avium* subsp. *hominissuis*" and present in *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum*) (7). The identification of *M. avium* subsp. *silvaticum* is still based only on phenotypic characteristics, namely, the rough colony morphology and slow and mycobactin-dependent growth of *M. avium* subsp. *silvaticum* versus the smooth colony morphology, rapid growth, and mostly mycobactin-independent nature of *M. avium* subsp. *avium* (8). As phenotypic features can differ among isolates of the same subspecies (9), and mycobactin dependence can vanish in subcultures (1), misidentifications can occur, which urges the need for a reliable molecular biological identification method.

Attempts at molecular identification of MAC members were done by use of primary ISs (10). Moss and coworkers (11) assumed that IS*902* would be a specific marker of *M. avium* subsp. *silvaticum* strains, but subsequently, it proved to be identical to IS*901* (12). Other ISs, such as IS*1311* (13) or IS*1612* (9, 14), are also present in both *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum*, which hinder their differentiation by phenotypic characteristics further on.

Despite numerous other attempts, such as fatty acid composition studies (5), restriction fragment length polymorphism (RFLP) analysis (15, 16), large-sequence polymorphism analysis (17), multilocus sequence analysis (4), or multispacer sequence typing (18), some of which had promising results but were tested on only two to five isolates (4, 16, 17) or the reference strain alone

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(18), *M. avium* subsp. *silvaticum* generally remained undifferentiable from *M. avium* subsp. *avium* (19).

Before December 2013, only 19 partial sequences from *M. avium* subsp. *silvaticum* reference strain ATCC 49884$^T$ were available in GenBank, of which the 16S rRNA, 16S-23S rRNA intergenic spacer, *hsp65*, *secA1*, *rpoB*, *ssrA*, and *tuf* sequences were completely identical to the corresponding *M. avium* subsp. *avium* ones. Deposition of *M. avium* subsp. *silvaticum* reference strain ATCC 49884$^T$ whole-genome shotgun sequence data in GenBank enabled detailed comparative studies of the *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum* genomes (GenBank accession number AYOC00000000 and BioProject number PRJNA219418).

With the advent of the high-resolution melt (HRM) technique, single base changes (single nucleotide polymorphisms [SNPs]), which cause only subtle changes in melting temperature ($T_m$), became sufficient for accurate species/subspecies/genotype/serovar differentiation (20).

The aim of our study was to develop a diagnostic method based on genomic differences between *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum* that enables omission of phenotypic identification and offers fast and reliable differentiation of these subspecies from one another for diagnostic laboratories.

## MATERIALS AND METHODS

**Sequence analysis and primer design.** We initially designed primers for IS*1613* (GenBank accession number AJ011837.1) and IS*1612* and then performed GenBank BLAST homology searches (http://www.ncbi.nlm.nih.gov/blast) of the nonredundant nucleotide collection (nr/nt) and whole-genome shotgun contig (wgs) databases. At the time of our first search, only 36 *M. avium* subsp. *silvaticum* nucleotide sequences were found in the nr/nt database, and 3 *M. avium* subsp. *avium* whole genomes were unassembled in the wgs database. Available sequences of *M. avium* subsp. *silvaticum* and *M. avium* subsp. *avium* were aligned with DNASTAR SeqMan Pro software (Lasergene 12; DNASTAR Inc., Madison, WI, USA), and the corresponding sequences were checked for differences.

As the contigs of the complete genome sequence of *M. avium* subsp. *silvaticum* reference strain ATCC 49884$^T$ (GenBank accession number AYOC00000000 and BioProject number PRJNA219418) were deposited in GenBank, a new search was conducted. The 808 *M. avium* subsp. *silvaticum* contigs were aligned with 258 contigs of *M. avium* subsp. *avium* ATCC 25291$^T$ (GenBank accession number ACFI00000000 and BioProject number PRJNA30909), 886 contigs of *M. avium* subsp. *avium* 10-9275, 577 contigs of *M. avium* subsp. *avium* 11-4751 (GenBank accession numbers AYOB00000000 and AYNY00000000 and BioProject numbers PRJNA216926 and PRJNA216924), 772 contigs of *M. avium* subsp. *avium* Env77, and 1,201 contigs of *M. avium* subsp. *avium* DT78 (21). Based on preliminary sequence and alignment studies, out of 2,800 nucleotide position differences, 50 potential sequence variations, such as single or multiple gaps, SNPs, or multiple mismatching (MM) base pairs, were selected. For 12 such sequence sections (including *est*, *aspB*, and *pepB* gene variable positions [4]), MM and HRM primer pairs were designed with DNASTAR Primer Design software (see Data Set S2 in the supplemental material). Primers were checked theoretically by BLAST homology searches in order to ensure specificity.

**Test strain collection and DNA extraction.** The test strain collection used contained the *M. avium* subsp. *avium* ATCC 25291$^T$, *M. avium* subsp. *silvaticum* ATCC 49884$^T$, *M. avium* subsp. *paratuberculosis* ATCC 19851$^T$, and *Mycobacterium bovis* AN5$^T$ reference strains and 202 field isolates. Field isolates were collected and typed at the Bacteriology Laboratory of the Veterinary Diagnostic Directorate (National Food Chain Safety Office [NFCSO]) in Budapest, Hungary, during routine *Mycobacterium* culture for samples from different wild and domestic mammals and birds. All strains were tested to determine whether they belonged to the MAC and whether they harbored or lacked the insertion sequences IS*901*, IS*1245*, and IS*900*, according to methods described previously by Wilton and Cousins (22), Álvarez et al. (23), and Castellanos et al. (24), respectively. *M. avium* subsp. *silvaticum* strains were identified phenotypically by their rough colony morphology, mycobactin dependence, slow growth, and inability to grow on egg medium. The 137 *M. avium* subsp. *avium* strains came from 14 cattle, 47 swine, 49 wild boars, 2 red deer, 8 red foxes, 7 chickens, 1 duck, 1 pigeon, 1 turkey, 1 long-eared owl, 1 tragopan, 1 tauraco, 1 monitor lizard, 1 pochard, 1 mallard, and 1 wigeon. The 62 *M. avium* subsp. *silvaticum* strains (see Data Set S1 in the supplemental material) were isolated from wild boars (44 isolates), red foxes (2), red deer (10), badger (1), and cattle (5). Isolates of *Mycobacterium intracellulare* (1 isolate), "*M. avium* subsp. *hominissuis*" (1 isolate), and *M. saskatchewanense* (1 isolate) were additionally sequenced to ensure correct identification.

Strains were grown on Lowenstein-Jensen and Middlebrook 7H11 (with mycobactin J) slants. The DNA was extracted by sonication at 80°C for 15 min at 80 Hz and boiling at 95°C for 10 min. Nucleic acid concentrations were determined with a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA).

**PCR settings and data analysis.** After conventional PCR amplification with reference strains, MM primers were optimized for real-time PCR by using a Rotor Gene 6000 real-time PCR machine and further tested on the field isolates.

HRM primer pairs were also tested and optimized with reference strains. Amplicons were sequenced by using an ABI Prism 3400 DNA sequencer. HRM primers 5′-CGGCGATCGGAATGGAAATA-3′ and 5′-CGGAACCCTGGTCAAGAT-3′ and *M. avium* subsp. *silvaticum*-specific MM primers 5′-TTCCTGGCCTGCTTCGACC-3′ and 5′-GTTGACCAC CACGGCATTCC-3′ were chosen and used in the PCR master mix at a final concentration of 0.6 μM each. The PCR mixture was further composed of 0.1 μl (5 U/μl) GoTaq G2 Flexi DNA polymerase (Promega, Madison, WI, USA), 0.5 μl of 10 mM deoxynucleoside triphosphate mix (Fermentas, Burlington, Ontario, Canada), 5 μl of 10× PCR buffer, 2.2 μl of a 25 mM $MgCl_2$ solution, 1.25 μl of 20× EvaGreen green fluorescent nucleic acid dye (Biotium, CA, USA), and PCR-grade $H_2O$ to a final volume of 25 μl with 20 ng template DNA per reaction mixture, under the following PCR conditions: denaturation at 95°C for 10 min followed by 40 cycles at 95°C for 20 s, 62°C for 40 s, and 72°C for 30 s. Melt curve conditions were 85°C to 99°C at a ramp rate of 0.5°C/s. HRM analysis was performed with temperatures from 87°C to 99°C with increases of 0.02°C. Data processing was performed with RotorGene Q 2.2.3 software (Qiagen, Venlo, Netherlands) based on the HRM plot and normalized graph.

To evaluate the reliability of identification of *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum* genotypes by HRM analysis, an independent-sample *t* test was used.

Specificity was tested with reference strains *M. bovis* AN5$^T$, *M. avium* subsp. *avium* ATCC 25291$^T$, *M. avium* subsp. *silvaticum* ATCC 49884$^T$, and *M. avium* subsp. *paratuberculosis* ATCC 19851$^T$ and field isolates of *M. intracellulare*, "*M. avium* subsp. *hominissuis*," and *M. saskatchewanense*.

For input template range determination and mixed-culture testing, reference strains *M. avium* subsp. *silvaticum* ATCC 49884$^T$ and *M. avium* subsp. *avium* ATCC 25291$^T$ were used.

**Nucleotide sequence accession numbers.** The sequences of the reference strain and one field isolate obtained by using the MM primers were deposited in GenBank under accession numbers KP792235 and KP792233. The sequences of the reference strains and one field isolate obtained by using the HRM primers were deposited in GenBank under accession numbers KP792236, KP792232, and KP792234.

## RESULTS

The primers designed for IS*1613* gave no amplification products with either *M. avium* subsp. *avium* or *M. avium* subsp. *silvaticum*,

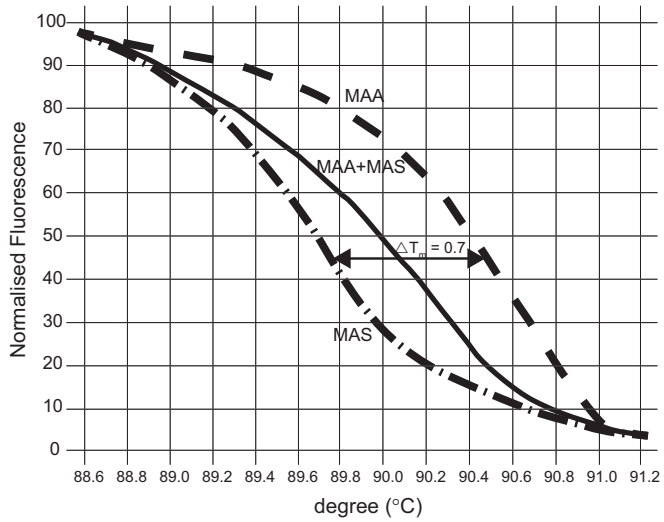


**FIG 1** Normalized HRM graph of *Mycobacterium avium* subsp. *avium* (MAA) and *Mycobacterium avium* subsp. *silvaticum* (MAS). The normalized graph displays the unique melting-curve profiles of *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum* with a temperature shift of 0.7°C and contains the altered midlevel melting curve shape of a mixed *M. avium* subsp. *avium*-*M. avium* subsp. *silvaticum* sample.

while IS*1612* primers generated amplicons with both *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum* reference strains.

Our first attempt at discrimination based on the sequence differences of the few available sequences at that time was unsuccessful, as the seemingly adequate sequence variations proved to be identical in our sequencing tests.

In our second attempt, of the 10 MM primers, only 1 was suitable. It was designed to contain a C/G difference in the *aspB* genes of the *M. avium* subsp. *silvaticum* and *M. avium* subsp. *avium* genomes. PCR identified the *M. avium* subsp. *silvaticum* reference strain correctly, gave a 369-bp-long amplification product with a $T_m$ of 94.9°C, and was specific for *M. avium* subsp. *silvaticum*, as no products appeared with *M. bovis* AN5$^T$, *M. avium* subsp. *avium* ATCC 25291$^T$, *M. avium* subsp. *paratuberculosis* ATCC 19851$^T$, *M. intracellulare*, "*M. avium* subsp. *hominissuis*," or *M. saskatchewanense*. The sequenced products from the *M. avium* subsp. *silvaticum* reference strain and 10 *M. avium* subsp. *silvaticum* field isolates confirmed the presence of the sequence variation (see Data Set S2 in the supplemental material).

Of the 13 HRM primers, 4 were applicable. The most robustly working primer pair with an appropriate $T_m$ was designed to contain a TT/CG difference in a putative membrane protein gene. The PCR products of the *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum* reference strains had unique melting-curve profiles in the normalized graph, with a temperature shift of 0.7°C (Fig. 1). The sequenced HRM products from both reference strains and 10 *M. avium* subsp. *silvaticum* field isolates confirmed the presence of the sequence variations (see Data Set S2 in the supplemental material). By testing mixed *M. avium* subsp. *avium*-*M. avium* subsp. *silvaticum* cultures, an altered midlevel melting-curve shape (Fig. 1) was detected.

The two systems were combined, optimized to run in a single tube as a duplex HRM and *M. avium* subsp. *silvaticum*-specific real-time PCR (Fig. 2; see also Data Set S3 in the supplemental material), and tested on 199 field isolates, which were all members of the MAC, IS*900* negative, and IS*901* and IS*1245* positive.

This method correctly identified the 199 field isolates. All 137 strains previously identified as *M. avium* subsp. *avium* had the *M. avium* subsp. *avium*-specific melting-curve profile with no amplification of the *aspB* gene product. The remaining 62 strains, phenotypically identified as *M. avium* subsp. *silvaticum*, yielded the *M. avium* subsp. *silvaticum*-specific PCR product with $T_m$s of

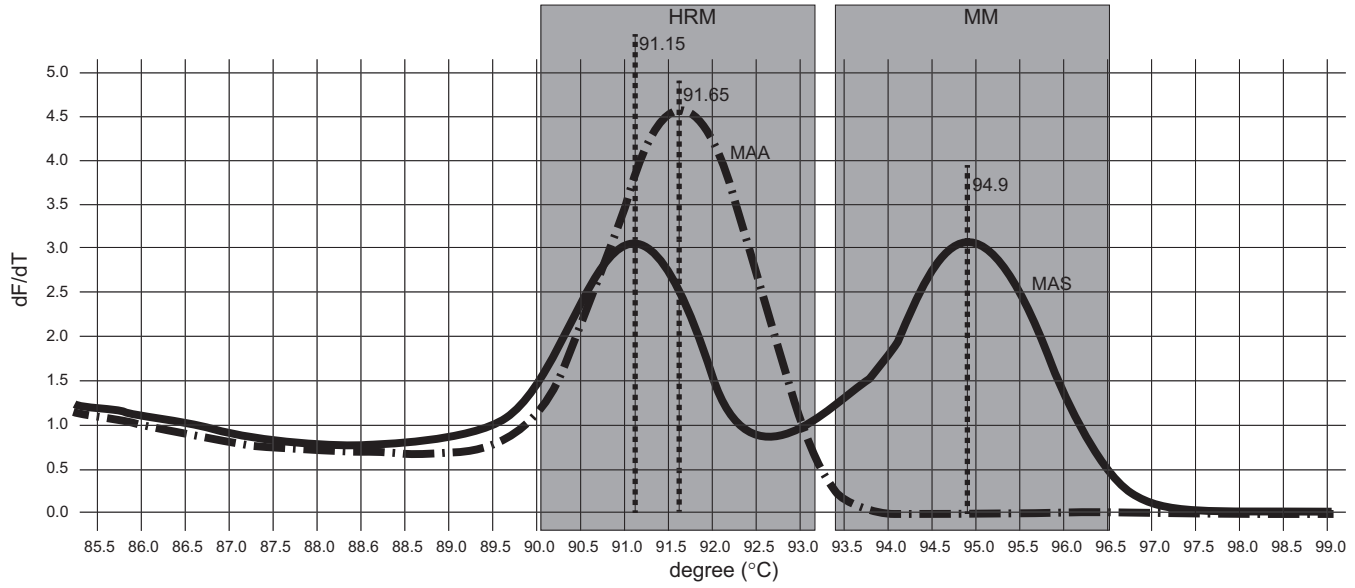


**FIG 2** Melting-curve graph for *Mycobacterium avium* subsp. *silvaticum* (MAS) identification by duplex real-time PCR. The graph displays the melting curves of reference strains *Mycobacterium avium* subsp. *avium* (MAA) ATCC 25291 and *Mycobacterium avium* subsp. *silvaticum* ATCC 49884 in the developed duplex HRM and *M. avium* subsp. *silvaticum*-specific real-time PCR assay. The *M. avium* subsp. *silvaticum*-specific MM amplification product has a $T_m$ of 94.9°C, while the obtained HRM products had $T_m$s of 91.15°C and 91.65°C for the *M. avium* subsp. *silvaticum* and *M. avium* subsp. *avium* genotypes, respectively.

94.65°C to 95.1°C and the *M. avium* subsp. *silvaticum*-specific melting-curve profile in the HRM analysis.

As samples of mixed *M. avium* subsp. *avium*-*M. avium* subsp. *silvaticum* cultures have an altered midlevel melting curve shape (Fig. 1), mixed-culture samples can also be distinguished with our assay.

The $T_m$s and their standard deviations in the HRM assay were 91.17°C ± 0.13°C and 91.63°C ± 0.05°C for *M. avium* subsp. *silvaticum* and *M. avium* subsp. *avium* genotypes, respectively. The independent-sample *t* test confirmed that the results of the HRM analysis were statistically significant ($P < 0.0001$). The input DNA template range extended between 100 ng and 15pg.

Besides the developed molecular identification assay, we report the first isolation of *M. avium* subsp. *silvaticum* from wild boars, red foxes, red deer, badgers, and naturally infected cattle.

## DISCUSSION

Since its first isolation, the existence of *M. avium* subsp. *silvaticum* as a distinct species or subspecies has been debated (25), as no molecular difference from *M. avium* subsp. *avium* could be traced. However, in 1990, it was classified as a *M. avium* subspecies, and its identification is still based on the detection of phenotypic characteristics, which can be subjective and may vary among laboratories, depending strongly on personal experience.

There is a significant lack of data on the pathogenicity and zoonotic potential of *M. avium* subsp. *silvaticum*. Apart from its first description as a pathogenic agent causing tuberculosis in wood pigeon (1), only a few experimental and hypothetical data are available in the literature (26).

MAC members can interact in tuberculin skin tests, thus hampering tuberculosis diagnoses. *M. avium* subsp. *avium* and "*M. avium* subsp. *hominissuis*" can cause severe conditions in immunocompromised people, and *M. avium* subsp. *paratuberculosis* is associated with human Crohn's disease (1). Contrary to the thoroughly studied subspecies *M. avium* subsp. *avium*, "*M. avium* subsp. *hominissuis*," and *M. avium* subsp. *paratuberculosis*, the ecology and zoonotic potential of *M. avium* subsp. *silvaticum* are barely known. Moss et al. (27) and McFadden et al. (28) reported the identification of *M. avium* subsp. *silvaticum* in human samples, but the almost identical *M. avium* subsp. *avium* genome sequence makes their results dubious. However, the significance of this less characterized fourth member of the MAC should not be dismissed without further clarification.

Numerous attempts to differentiate *M. avium* subsp. *silvaticum* strains from *M. avium* subsp. *avium* have been undertaken. Most recently, Chiers and coworkers designed a PCR-RFLP method that targeted the 85B antigen gene (29). Among others, it was tested on *M. avium* subsp. *silvaticum* ATCC 49884$^T$ and *M. avium* subsp. *avium* ITG75/219, but not one *M. avium* subsp. *avium* reference strain was included. By retesting the protocol with *M. avium* subsp. *avium* ATCC 25291$^T$ and *M. avium* subsp. *silvaticum* ATCC 49884$^T$, contrary to their results, we obtained identical RFLP profiles (data not shown) for these subspecies. Our results call the applicability of this method and also the identification of the horse isolate into question.

IS*1613* is supposed to be a *M. avium* subsp. *avium*-specific IS (GenBank accession number AJ011837) (10), but by searching the GenBank wgs database for the 1,734-bp-long sequence, we found only 99% (1,468/1,474) sequence identity with the *M. avium* subsp. *avium* 2285(S)gma2285S.contig.5 wgs sequence (GenBank accession number JAOD01000006.1). Furthermore, IS*1613* could not be aligned with either the *M. avium* subsp. *avium* ATCC 25291$^T$ or the *M. avium* subsp. *silvaticum* ATCC 49884$^T$ wgs database sequence, which supports our negative results.

The results of our IS*1612* PCR are consistent with those reported by Bull et al. (9) but cast doubt on the results of Orrú et al. (14). When we tested their primers, we obtained identical amplification products from both the *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum* reference strains. Although IS*1612* is not present in *M. avium* subsp. *paratuberculosis*, it cannot be used to reliably identify *M. avium* subsp. *silvaticum*, as it is also present in *M. avium* subsp. *avium* strains.

The results of our first attempt at sequence variation-based differentiation of *M. avium* subsp. *silvaticum* from *M. avium* subsp. *avium* raise questions regarding sequences deposited in GenBank. Higgins et al. (30) reported sequence differences in the same gene of the *M. avium* subsp. *silvaticum* reference strain, presumably as a result of a sequencing failure. Partial and sometimes even faulty sequence data are not a suitable basis for the design of identification assays.

In this study, a duplex HRM and *M. avium* subsp. *silvaticum*-specific real-time PCR was developed to discriminate thus-far-undifferentiable *M. avium* subsp. *silvaticum* and *M. avium* subsp. *avium* strains by molecular analysis reliably and easily.

Until the discovery and development of HRM analysis, the SNPs observed in the *est*, *aspB*, and *pepB* genes (4) and at several other points of the genome could have been tested only by sequencing, which is time-consuming and expensive for routine laboratory diagnostic purposes.

As HRM analysis does not require probes, labeled primers, or any post-PCR processing, it is a rapid and cost-effective identification method.

The sequence difference in the putative membrane protein gene targeted by the chosen HRM primer was not described previously. The MM primer targeted the C/G SNP in the *aspB* gene, as described previously by Turenne et al. (4). The *est* and *pepB* gene differences (described in that same study) failed to bring the expected results.

Due to practical considerations, the two systems were combined in a duplex real-time PCR for routine use on both solid and liquid culture isolates in high-throughput laboratories.

Parallel gene scanning strengthens the trustworthiness of the method applied, and as amplification is simultaneous in a single closed tube, its implementation is easy to perform.

The stable $T_m$ differences of the HRM curves and the low standard deviation of the $T_m$ of the *M. avium* subsp. *silvaticum*-specific amplification products contribute to the robustness of the system.

The fact that our assay identified all 199 tested field isolates correctly makes it an accurate and reliable method for the identification of *M. avium* subsp. *silvaticum* and the discrimination of *M. avium* subsp. *silvaticum* from *M. avium* subsp. *avium*.

HRM analysis usually requires preamplification template concentration normalization. The wide input DNA range of this system is a great advantage in rendering DNA quantification redundant, thus making our assay flexible and ready to use for routine diagnostic laboratories.

As *M. avium* subsp. *silvaticum* is usually first identified in liquid cultures, and mixed cultures with other mycobacterial strains can occur, besides *M. avium* subsp. *silvaticum* identification, the

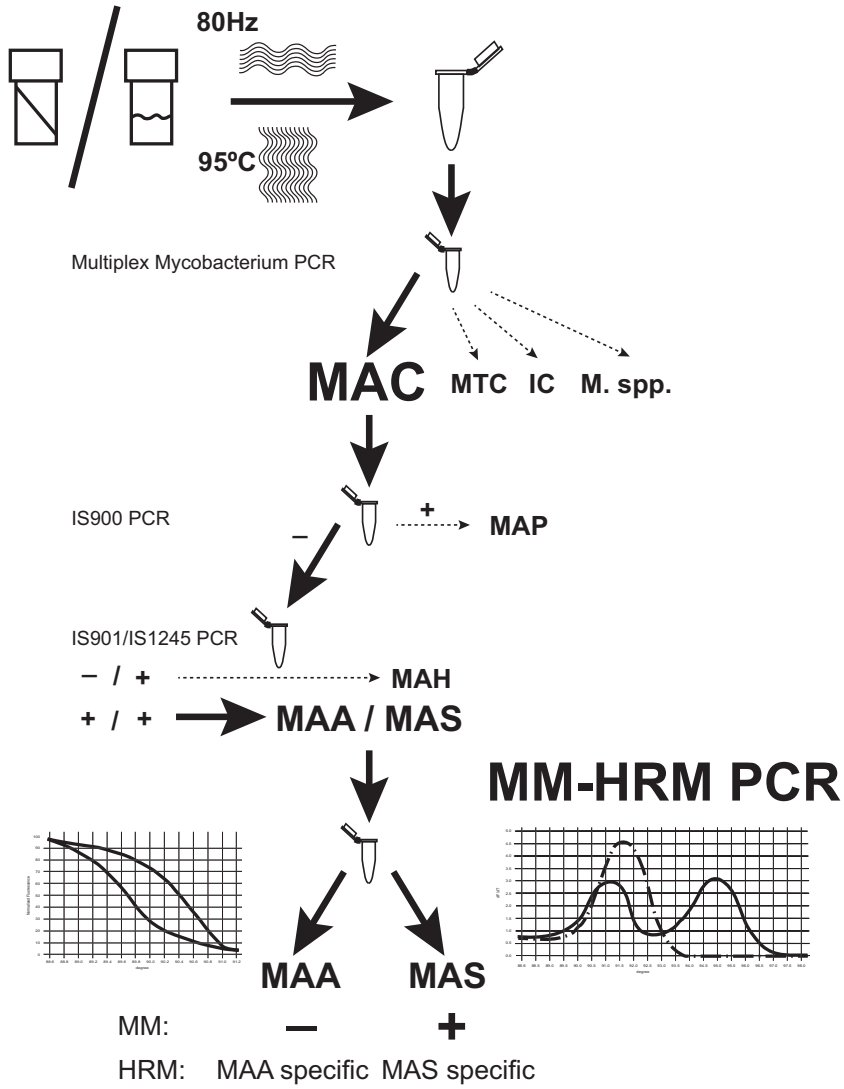


**FIG 3** Flow chart for the recommended mycobacterial strain characterization with special emphasis on *Mycobacterium avium* subspecies identification. The steps of the potential *Mycobacterium avium* complex subspecies-level identification method are presented in the flow chart. Abbreviations: MTC, *Mycobacterium tuberculosis* complex; MAC, *Mycobacterium avium* complex; MI, *Mycobacterium intracellulare*; M., *Mycobacterium*; MAP, *Mycobacterium avium* subsp. *paratuberculosis*; MAH, "*Mycobacterium avium* subsp. *hominissuis*"; MAA, *Mycobacterium avium* subsp. *avium*; MAS, *Mycobacterium avium* subsp. *silvaticum*.

ability of our assay to detect mixed *M. avium* subsp. *avium*-*M. avium* subsp. *silvaticum* cultures enhances its importance.

This is the first time that such a large panel of strains has been analyzed. In previous studies, at most 5 strains were tested, and in some cases, even the same few strains were used across various studies (31). The numerous field isolates better reflect environmental circumstances and are able to reveal potential strain differences.

The test strains originated from 17 different species, which underlines the ubiquitous nature and wide host range of *M. avium* subsp. *avium*. To our knowledge, isolation of *M. avium* subsp. *silvaticum* from wild boars, red foxes, red deer, badgers, and naturally infected cattle has not been reported previously. The infected cattle gave positive reactions by the intradermal tuberculin skin test. However, the sample size from wild animals was limited; macroscopic lesions were found in 35% (20/57) of samples, of which 65% (13/20) resembled tuberculotic lesions in their microscopic structure.

Considering the results of specificity testing, it can be stated that the specificity of the assay is high, but we recommend previous MAC identification and IS*900*, IS*901*, and IS*1245* characterization steps for *M. avium* subsp. *paratuberculosis* and "*M. avium* subsp. *hominissuis*" discrimination (Fig. 3).

Altogether, a reliable, rapid, and robust *M. avium* subsp. *silvaticum* identification method was developed, which eliminates the long-existing problem of the ambiguous phenotypic *M. avium* subsp. *silvaticum* identification and opens up the possibility of detailed and comprehensive *M. avium* subsp. *silvaticum* strain studies.

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