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# Rapid Detection and Identification of *Mycobacterium avium* by Amplification of 16S rRNA Sequences

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**An assay that is based on the amplification of 16S rRNA sequences and that was initially developed to detect *Mycobacterium paratuberculosis* in cattle was used to test 20 serotypes of the *Mycobacterium avium* complex (MAC) and atypical mycobacterial species not belonging to MAC. Only serotypes 1 to 6 and 8 to 11, designated *M. avium*, were detected by the assay, indicating that it can be used for the rapid detection and identification of *M. avium*. The results of the assay for clinical samples from animals suspected of having mycobacterial infections indicated that it can also be used directly on clinical samples.**

Mycobacteria are widespread in nature and remain an important cause of infections in humans and animals worldwide (17, 18, 25). Disease is most often associated with *Mycobacterium tuberculosis* and other bacteria of the *M. tuberculosis* complex (*M. bovis*, *M. africanum*, and *M. microti*). There is, however, an increasing incidence of opportunistic infections caused by other mycobacterial species that otherwise grow in diverse biotopes such as water, soil, and dust (5). These infections are most commonly caused by mycobacteria belonging to the *M. avium* complex (MAC) (2).

MAC includes *M. intracellulare*, *M. avium*, *M. paratuberculosis*, and mycobacteria from wood pigeons. *M. avium* and *M. intracellulare* are phenotypically indistinguishable, but 16S rRNA sequence analysis, which is considered to be a reliable phylogenetic tool (24), has confirmed that some serotypes initially designated *M. intracellulare* (serotypes 4 to 6 and 8 to 11) are in fact *M. avium* (3). *M. paratuberculosis*, the causative agent of a chronic proliferative enteritis in ruminants, has also been included in MAC. It could not be distinguished from *M. avium* by DNA homology studies (14), and its 16S rRNA sequence is 99.9% identical to that of *M. avium* (13).

*M. avium* serotypes 1, 2, and 3 and *M. avium* subsp. *silvaticum*, which are isolated from wood pigeons (8), are the main cause of avian tuberculosis. In the United States, serotypes 1 and 2 of *M. avium* are the most frequently isolated mycobacteria from nontuberculous lesions in cattle, swine, and birds. Serotype 8 has been isolated from swine, and these infections can cause economic losses (17). In The Netherlands, *M. avium* serotypes 1, 2 and 3 are most frequently isolated (15) from birds.

In humans, *M. avium* complex infections are uncommon and usually occur as opportunistic infections in children and immunosuppressed patients. They are now a major cause of systemic bacterial infections in patients with AIDS (1), with serotypes 1, 4, and 8 occurring most frequently in the United States (19). In addition, *M. avium* infections are increasingly detected in patients without predisposing conditions (12).

Accurate and rapid differential diagnosis of infections caused by MAC or the *M. tuberculosis* complex in animals and humans is important, because the two infections require different therapies or management. Culturing is reliable but time-consuming. Several weeks are required before the diagnosis is completed (23), even with the use of commercially available DNA probes (Gen-Probe) to identify the cultured bacteria *M. avium*, *M. intracellulare*, and bacteria belonging to the *M. tuberculosis* complex (4, 11). There is a need for an assay that can more rapidly detect *M. avium* infections (2, 10). Recently, we developed a rapid test that is based on the amplification of 16S rRNA sequences of *M. paratuberculosis* and *M. avium* (20).

We used this assay on 20 serotypes of MAC, several other atypical mycobacteria, and clinical samples from animals with mycobacterial infections to study the potential of the test for the diagnosis of *M. avium* infections. Primers S (5′-GTCGAACGGAAAGGCCT-3′) and P (5′-CCGAGAAA ACCCGGAC-3′) were based on sequences from the 16S rRNA genes of *M. paratuberculosis* and *M. avium* (20) and were used to amplify a fragment of 413 bp. Genomic DNAs were extracted from MAC serotypes 1 to 20, two strains from wood pigeons, *M. bovis* BCG, and several other atypical mycobacterial species (Table 1) (20). The polymerase chain reaction (PCR) was carried out in a total volume of 100 μl containing 200 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.1 g of gelatin per liter, 200 μM (each) deoxynucleoside triphosphates 1 μM (each) both primers, 2.5 U of *Taq* polymerase (Promega), and 400 ng of genomic DNA. After 35 cycles (1 min at 95°C, 2 min at 58°C, and 3 min at 72°C), a 10-μl sample was analyzed by electrophoresis. A strong amplification product was detected in the *M. avium* strains. Only a weak amplification product was detected in the *M. intracellulare* strains and *M. bovis* (Fig. 1A). Three microliters of amplified DNA was spotted onto Zetaprobe (Bio-Rad Laboratories, Richmond, Calif.), which was hybridized at 72°C for 2 h with a $^{32}P$-labeled internal probe (5′-CTCAAGACGCATGTCTTCTGGTGGAAAGCT TTTGCGGT-3′) in 0.5 M $NaOH_2HPO_4$ (pH 7.2) with 70 g of sodium dodecyl sulfate per liter (21). This resulted in a positive hybridization signal for the *M. avium* strains of serotypes 1 to 6 and 8 to 11 and the strains from wood pigeons (Fig. 1B). The *M. intracellulare* strains and *M. bovis* were negative after hybridization with the specific probe (Fig. 1B). Furthermore, to test the specificity of the assay, DNAs from several mycobacterial species not belonging to

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TABLE 1. Sources and origins of mycobacteria used in the PCR

| MAC strain | Serotype | Source | Origin |
|---|---|---|---|
| D4 | | Type strain | Weybridge[a] |
| D48841 | 1 | Vulturine guinea fowl | Lelystad[b] |
| D48863 | 2 | Chicken | Lelystad |
| D34626 | 3 | Turkey | Lelystad |
| P55 | 4 | Serotyping strain | Paris[c] |
| 5433-1693 | 5 | Serotyping strain | Bilthoven[d] |
| 148/70 | 6 | Human | Lelystad |
| V450 | 7 | Serotyping strain | Bilthoven |
| 23435 | 8 | Serotyping strain | Paris |
| 6450-204 | 9 | Serotyping strain | Bilthoven |
| 290-152 | 10 | Serotyping strain | Bilthoven |
| V455 | 11 | Serotyping strain | Bilthoven |
| P42 | 12 | Serotyping strain | Bilthoven |
| Orchi Rogers | 13 | Serotyping strain | Bilthoven |
| P39 | 14 | Serotyping strain | Bilthoven |
| Simpson | 15 | Serotyping strain | Bilthoven |
| Jandle | 16 | Serotyping strain | Bilthoven |
| P54 | 17 | Serotyping strain | Bilthoven |
| Melnick | 18 | Serotyping strain | Bilthoven |
| Huntly Claude | 19 | Serotyping strain | Bilthoven |
| Findley | 20 | Serotyping strain | Bilthoven |
| *M. avium* subsp. *silvaticum* D91865 | | Tawny owl | Lelystad |
| *M. avium* subsp. *silvaticum* D40353 | | Jackdaw | Lelystad |
| *M. bovis* BCG | | Type strain | Bilthoven |
| *M. gastri* | | Pig | Lelystad |
| *M. gordonae* | | Fish | Lelystad |
| *M. kansasii* | | Cow | Lelystad |
| *M. luciflavum* | | Cow | Lelystad |
| *M. marinum* | | Fish | Lelystad |
| *M. terrae* | | Buzzard | Lelystad |
| *M. triviale* | | Anteater | Lelystad |
| *M. scrofulaceum* | | Cow | Lelystad |
| *M. phlei* R82 | | Laboratory strain | Weybridge |
| *M. fortuitum* | | Pig | Lelystad |
| *M. chelonei* | | Lion | Lelystad |
| *M. smegmatis* | | Laboratory strain | Weybridge |

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[c] Institute Pasteur, Paris, France.
[d] Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands.

MAC or the *M. tuberculosis* complex were tested (Table 1). Although most strains gave an amplification product of the right size after agarose gel electrophoresis (Fig. 2A), these mycobacteria were all negative after hybridization with the internal oligonucleotide probe (Fig. 2B). This is in accordance with 16S rRNA sequence analysis, because *M. intracellulare*, bacteria belonging to the *M. tuberculosis* complex, and other atypical mycobacterial species have different sequences in the area of the probe (3, 13). Thus, the positivity of the assay had a perfect correlation with the presence of *M. avium*.

Clinical and autopsy materials from different animals suspected of having mycobacterial infections were examined. Samples of approximately 500 mg of animal tissue (bone biopsy, liver, spleen) were put in a 1.5-ml screw-cap tube filled with 500 μl of water. To this, 500 μl of 10 mM Tris–1 mM EDTA (pH 8.0) (TE)-saturated phenol-chloroform-isoamyl alcohol (25:24:1) and 200 μl of 0.5-mm-diameter zirconium beads were added. The tube was placed in a Mini-beadbeater (Biospec Products, Bartesvilles, Okla.), which was shaken for 3 min at high speed. After centrifugation at 13,000 × *g* for 15 min, 100 μl of the supernatant was added to 900 μl of guanidine isothiocyanate wash buffer and 40 μl of activated silica (Sigma, St. Louis, Mo.), and the mixture was incubated for 10 min at room temperature. The supernatant was discarded and the pellet was washed twice with 1 ml of 70% ethanol and once with acetone. DNA was eluted in 100 μl of water. One microliter of eluted DNA was used in the PCR described above. For confirmation, the PCR products were spotted and hybridized with an internal probe as described above. The same samples were also tested by a PCR assay which is specific for the *M. tuberculosis* complex (6). This PCR assay was performed (35 cycles, 1 min 95°C; 1 min 60°C; 2 min 72°C) as described above for the *M. avium* PCR with primers (5′-CGTGAGGGCATCGAGGTGGC-3′ and 5′-GCGTAGGCGTCGGTGACAAA-3′) derived from IS*986* (9) for amplification of a fragment of 245 bp. After the PCR, fragments were spotted and hybridized at 55°C with a $^{32}$P-labeled IS*986*-specific probe (5′-TGGGTAGCAGACCTCACCTA-3′). Agarose gel electrophoresis analysis of the PCR samples showed a positive signal with the tissue samples in the *M. avium* PCR but not in the *M. tuberculosis*

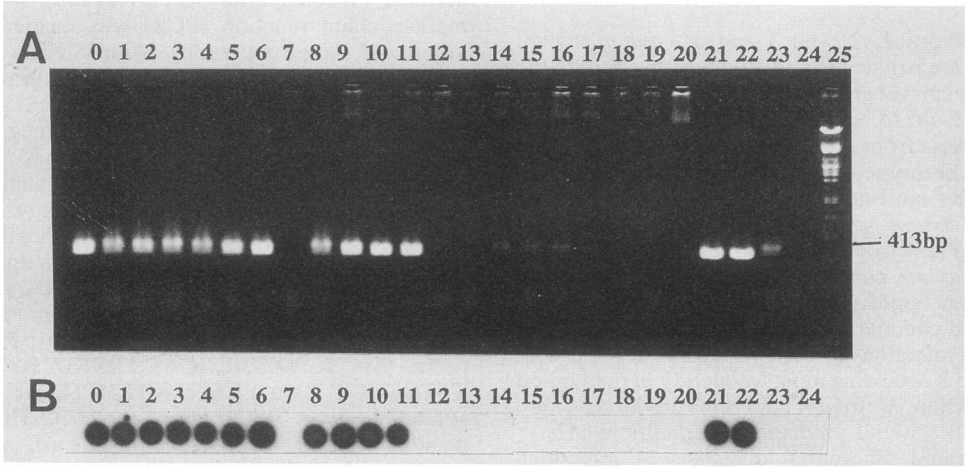


FIG. 1. (A) Analysis of PCR products of various MAC serotypes amplified with MAC-specific PCR primers. The products were separated by electrophoresis on a 1% agarose gel. Lanes 0 to 23, *M. avium* type strain D4, serotypes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, wood pigeon strains D91865 and D40353, *M. bovis* BCG, respectively; lane 24, negative control (without template); lane 25, bacteriophage lambda *Pst*I marker. (B) The same PCR fragments shown in panel A spotted onto nylon membranes and hybridized with a $^{32}$P-labeled *M. avium* internal probe.

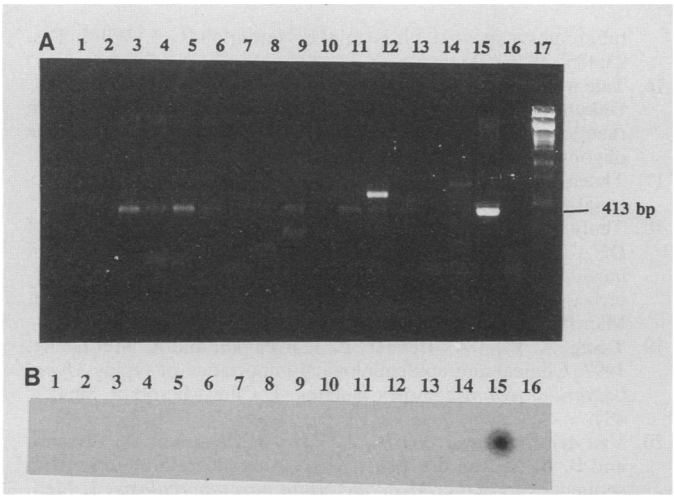


FIG. 2. (A) Analysis of PCR products from DNAs of several mycobacterial species amplified with MAC-specific PCR primers. The products were separated by electrophoresis on a 1% agarose gel. Lanes 1 to 12, *M. gastri*, *M. gordonae*, *M. kansasii*, *M. luciflavum*, *M. marinum*, *M. scrofulaceum*, *M. terrae*, *M. triviale*, *M. phlei*, *M. chelonei*, *M. fortuitum*, *M. smegmatis*; lanes 13 and 14, lymph node and intestinal tissue (ileum) from noninfected cows; lane 15, *M. avium* D4 (positive control); lane 16, no template (negative control); lane 17, bacteriophage lambda *Pst*I marker. (B) The same PCR fragments shown in panel A spotted onto nylon membranes and hybridized with a $^{32}$P-labeled *M. avium* internal probe.

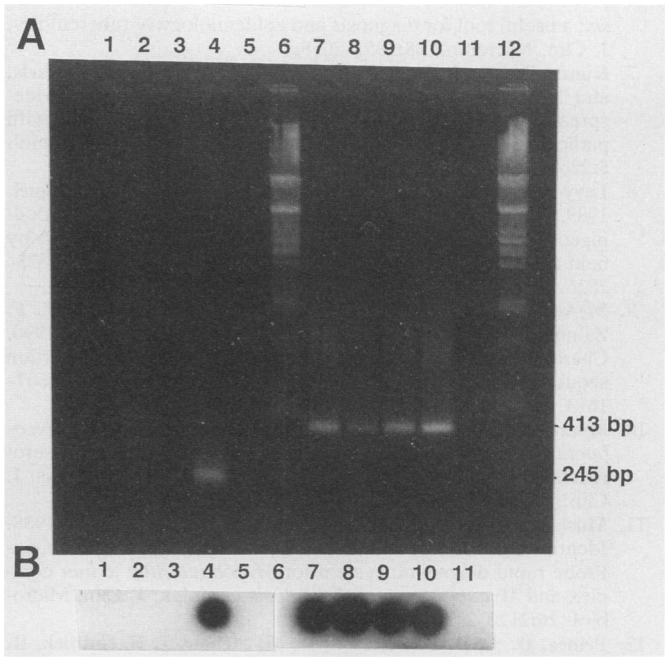


FIG. 3. (A) Agarose gel electrophoresis of PCR fragments amplified from clinical material with *M. tuberculosis*-specific primers (lanes 1 to 3) and with MAC-specific primers (lanes 7 to 9). Lanes 1 and 7, bone biopsy of parrot 1; lanes 2 and 8, liver of parrot 2; lanes 3 and 9, spleen of a mink; lanes 4 and 10, positive controls (with 1 μg of template DNA; lane 4, *M. bovis* BCG; lane 10, *M. avium* D4); lanes 5 and 11, negative controls (without template); lanes 6 and 12, bacteriophage lambda *Pst*I markers. (B) The same PCR fragments shown in panel A spotted onto nylon membranes and hybridized with the $^{32}$P-labeled *M. tuberculosis* and the *M. avium* internal probes.

PCR (Fig. 3A). Hybridization with the *M. avium*-specific probe of the spotted amplified products of clinical and autopsy materials also showed positive results, whereas no positive results were obtained after hybridization with the *M. tuberculosis*-specific probe (Fig. 3B). The clinical and autopsy samples were also cultured onto Middlebrook 7H10 medium, and this resulted in the isolation of *M. avium* serotype 2 in all cases. In addition, to test the specificity of the assay on clinical material, lymph nodes and intestinal tissues from noninfected animals (specific pathogen free) were tested by the assay. The assay was negative with both tissues after agarose gel electrophoresis and after hybridization with the internal probe (Fig. 2).

In summary, we described the use of a rapid and specific test which was initially developed for the detection of *M. paratuberculosis* (20) but which is also suitable for the detection and identification of *M. avium* infections in animals. The assay is specific for *M. avium*, mycobacterial strains from wood pigeons, and *M. paratuberculosis*. The detection limit of this test is $1.3 \times 10^{-13}$ g of DNA, the equivalent of about 20 bacteria (21).

An assay based on the amplification of the mycobacterial *dna-J* gene and species-specific oligonucleotide probes has been described for *M. avium*, but this assay was not tested on different serotypes of *M. avium* (16). Assays based on insertion elements, which have a very high specificity, have proven to be of use in the detection of *M. tuberculosis* (6) and *M. paratuberculosis* (22). An insertion element, IS*901*, specific for *M. avium* has been described (7). However, not all *M. avium* strains, in particular those found in patients with AIDS, contain IS*901* (7). The assay described in this report detects *M. avium*, including serotypes 1, 4, and 8, which are isolated from patients with AIDS (19), and is also suitable for use in testing clinical samples, although only a limited amount of clinical material was tested. A great advantage of performing both the *M. avium* and the *M. tuberculosis* PCRs is the ultimate differential diagnosis of these two infections in humans and animals in only 2 days.

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