JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1993, p. 3216–3222
0095-1137/93/123216-07$02.00/0

Vol. 31, No. 12

Identification of Mycobacteria from Animals by Restriction Enzyme Analysis and Direct DNA Cycle Sequencing of Polymerase Chain Reaction-Amplified 16S rRNA Gene Sequences

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Received 7 June 1993/Returned for modification 5 August 1993/Accepted 22 September 1993

Two methods, based on analysis of the polymerase chain reaction-amplified 16S rRNA gene by restriction enzyme analysis (REA) or direct cycle sequencing, were developed for rapid identification of mycobacteria isolated from animals and were compared to traditional phenotypic typing. BACTEC 7H12 cultures of the specimens were examined for "cording," and specific polymerase chain reaction amplification was performed to identify the presence of tubercle complex mycobacteria. Combined results of separate REAs with *Hha*I, *Msp*I, *Mbo*I, and *Tha*I differentiated 12 of 15 mycobacterial species tested. *Hha*I, *Msp*I, and *Tha*I restriction enzyme profiles differentiated *Actinobacillus* species from mycobacterial species. *Mycobacterium bovis* could not be differentiated from *M. bovis* BCG or *Mycobacterium tuberculosis*. Similarly, *Mycobacterium avium* and *Mycobacterium paratuberculosis* could not be distinguished from each other by REA but were differentiated by cycle sequencing. Compared with traditional typing, both methods allowed rapid and more accurate identification of acid-fast organisms recovered from 21 specimens of bovine and badger origin. Two groups of isolates were not typed definitively by either molecular method. One group of four isolates may constitute a new species phylogenetically very closely related to *Mycobacterium simiae*. The remaining unidentified isolates (three badger and one bovine) had identical restriction enzyme profiles and shared 100% nucleotide identity over the sequenced signature region. This nucleotide sequence most closely resembled the data base sequence of *Mycobacterium senegalense*.

Intradermal tuberculin tests are currently used for diagnosing bovine tuberculosis. These tests, however, have limited specificity. Mycobacteria, both saprophytes and opportunistic pathogens, are ubiquitous in the environment and may complicate the immunodiagnosis of *Mycobacterium bovis* infection in cattle. For example, *Mycobacterium cookii*, isolated from sphagnum mosses, can sensitize cattle to the bovine tuberculin used in the field diagnostic test (14). Culture of mycobacteria from postmortem specimens of tuberculin-reacting cattle is therefore often employed to support diagnosis.

Some laboratories may limit identification of mycobacteria to colony and organism morphology, together with growth characteristics on solid and in liquid media. Biochemical typing is commonly employed for species identification but is time-consuming and is unable to differentiate some closely related species (9, 11). Other methods, such as thin-layer and capillary gas-liquid chromatography, can be used, but supplementary biochemical tests may be required to identify some species (12), and application of pyrolysis gas chromatography-mass spectrometry has permitted recognition only of groups of mycobacterial species (10).

Nucleic acid technologies offer perhaps the best potential for rapid and definitive identification of mycobacteria, as well as enabling determination of evolutionary relationships between and within different species. Typing methods based on the polymerase chain reaction (PCR) amplification of specific genes such as the 16S rRNA gene, a gene present in all prokaryotes, and the 65-kDa gene combined with molecular analyses of the PCR products have been evaluated for differentiation and identification of mycobacterial species (1, 17, 19, 21). These methods, however, have not been utilized for the identification of mycobacteria isolated from veterinary specimens.

For practical identification of *Mycobacterium* species and discrimination from *Actinobacillus* species isolated from clinical specimens in a veterinary mycobacteriology laboratory, we have investigated PCR amplification of the 16S rRNA gene and its subsequent analysis by restriction endonuclease (RE) digestion. We have also developed a novel typing method for mycobacterial species based on direct cycle sequencing of a region within the highly conserved 16S rRNA gene which shows intertypic variability. A comparison of both molecular methods and traditional phenotypic typing was carried out.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The following strains were obtained from the National Collection of Type Cultures, Colindale, London, United Kingdom: *Mycobacterium avium* NCTC 8559, *M. bovis* NCTC 5693, *M. bovis* BCG NCTC 5692, *M. fortuitum* NCTC 10394, *M. gordonae* NCTC 10267, *M. intracellulare* NCTC 10425, *M. kansasii* NCTC 10268, *M. marinum* NCTC 10009, *M. scrofulaceum* NCTC 10803, *M. smegmatis* NCTC 333, *M. terrae* NCTC 10856, *M. tuberculosis* NCTC 7416, *M. vaccae* NCTC 10916, *M. xenopi* NCTC 10042, *Actinobacillus lignieresii* NCTC 4976 and *Actinobacillus* sp. NCTC 10801. *Mycobacterium paratuberculosis* was isolated in our laboratory from a sheep with Johne's disease. Field isolates used included

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TABLE 1. Identification of acid-fast isolates recovered from bovine and badger specimens

Specimen	Status of animal[a]	Growth characteristics		Identification of isolate by[b]:			
		LJ slope[c]	BACTEC culture[d]	Traditional tests	PCR[e]	REA	DNA sequencing
A	+	−	Cords and ATYP	*M. terrae*	−	*M. nonchromogenicum*	*M. nonchromogenicum*
B	Inconclusive	−	ATYP	*M. avium/M. intracellulare*	−	*M. avium/M. paratuberculosis*	*M. paratuberculosis*
C	Contact with TB	−	ATYP	MAIS	−	*M. simiae**	*M. simiae**
D	+	−	ATYP	*M. terrae*	−	*M. nonchromogenicum*	*M. nonchromogenicum*
E	+	−	Cords	*M. kansasii*	−	*M. kansasii/M. gastri/M. szulgai*	*M. kansasii/M. gastri*
F	−	−	ATYP	*M. fortuitum*	−	*M. nonchromogenicum*	*M. nonchromogenicum*
G	Contact with TB	−	ATYP	*M. gordonae*	−	*M. simiae**	*M. intracellulare* 16*
H	Contact with TB	−	ATYP	*M. kansasii*	−	*M. kansasii/M. gastri/M. szulgai*	*M. kansasii/M. gastri*
I	−	−	ATYP	*M. fortuitum*	−	Not identifiable	*M. senegalense**
J	Contact with TB	−	ATYP	MAIS	−	*M. simiae**	*M. simiae**
K	+	−	Cords and ATYP	*M. avium/M. intracellulare*	−	*M. avium/M. paratuberculosis* and *M. nonchromogenicum*	Not identifiable
L	+	+	Cords		+	Tubercle complex	Tubercle complex
M	−	NA	ATYP	*M. avium/M. intracellulare*	−	*M. avium/M. paratuberculosis*	*M. avium*
N	+	+	ATYP	*M. terrae*	−	*M. nonchromogenicum*	*M. nonchromogenicum*
O	+	−	ATYP	*M. terrae*	−	*M. nonchromogenicum*	*M. nonchromogenicum*
P	−	NA	Cords		+	*M. avium/M. paratuberculosis*	*M. avium*
Q	Inconclusive	−	ATYP	*M. avium/M. intracellulare*	−	*M. simiae**	*M. simiae**
R	+	−	Cords		+	Tubercle complex	Tubercle complex
S	NA	−	Cords	MAIS	−	Not identifiable	*M. senegalense**
T	NA	−	Cords	NA	−	Not identifiable	*M. senegalense**
U	NA	−	Cords and ATYP	MAIS	−	Not identifiable	*M. senegalense**

[a] Skin test or classification in the field: +, positive in the single intradermal comparative cervical tuberculin test; −, negative result. TB, tuberculosis; NA, not applicable.
[b] *, most closely resembling.
[c] Colonies growing on LJ agar with pyruvate. +, growth; −, no growth.
[d] Growth observed in 7H12 liquid medium with the BACTEC culture system. ATYP, acid-fast staining organisms with morphology not typical of *M. bovis*.
[e] Detection of TB complex.

acid-fast organisms isolated from bovine lymph glands and lung specimens and from badger feces. Specimens were from cattle which had been either routinely slaughtered or slaughtered after being diagnosed as potentially infected by use of the single intradermal comparative cervical tuberculin test. The status of each animal from which the isolates were recovered is shown in Table 1. Mycobacterial type strains were cultured on Lowenstein-Jensen (LJ) slopes with pyruvate and subcultured in 7H9 Middlebrook broth medium (Difco Laboratories). Field strains were isolated in BACTEC 7H12 medium (Becton Dickinson Diagnostic Systems), subcultured first on LJ medium, and then subcultured in 7H9 Middlebrook broth medium. *Actinobacillus* species were grown in glucose broth. Acid-fast isolates recovered from animals were given the same code numbers as the specimens from which they were derived.

Traditional identification. Fifteen bovine field isolates and three badger isolates were identified by traditional typing methods (8) by the Public Health Laboratory Service Mycobacterium Reference Unit, Cardiff, United Kingdom.

PCR conditions. PCR was carried out under conditions designed to minimize potential contamination: separate work stations with restricted staff access and dedicated equipment, consumables, and laboratory wear were designated for different aspects of PCR. Work surfaces were swabbed with 1 M HCl before and after use. Positive displacement micropipettes and aerosol-resistant tips for other micropipettes were used for PCR.

Bacterial cultures in broth medium were heated at 100°C for 15 min to release DNA. PCR was carried out in 100-μl reaction volumes containing 2.5 U of *Taq* polymerase (Promega), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.75 mM $MgCl_2$, 0.1% Triton X-100, deoxyribonucleotides (each at a final concentration of 150 μM) (Pharmacia), 60 pmol each of two primers, and 15 μl of heat-treated bacterial culture. PCR amplification of a 123-bp fragment within the tubercle complex-specific insertion sequence IS*6110* was performed with specified primers (4). Primers pA (5′-AGA GTT TGA TCC TGG CTC AG; nucleotides 8 to 28 of the 16S rRNA gene of *Escherichia coli*) and pH (5′-AAG GAG GTG ATC CAG CCG CA; nucleotides 1542 to 1522 of the 16S rRNA gene of *E. coli*) (3) based on conserved eubacterial 16S rRNA sequences were utilized for PCR amplification of almost the complete 16S rRNA gene. PCR mixtures were overlaid with light mineral oil (Sigma Chemical Company, Ltd.), and the amplifications were carried out in a thermocycler (LEP Scientific). IS*6110* amplification mixtures were subjected to 4 min of denaturation at 94°C and 30 cycles of 94°C for 1 min, 68°C for 2 min, and 72°C for 2 min, followed by a final extension period of 72°C for 7 min and refrigeration. 16S rRNA amplification mixtures underwent 4 min of denaturation at 94°C and 30 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min, followed by a final extension period of 72°C for 7 min and refrigeration. Negative controls for both the sample preparation procedure and PCR reagents were included to ensure that contamination had not occurred.

PCR product analysis. 16S rRNA PCR products (3 μl) were visualized on 1% agarose gels stained with ethidium bromide (0.5 μg/ml). The remaining products were purified

by using the Magic PCR preps DNA purification system (Promega) as specified by the manufacturer.

IS*6110* PCR products (15 μl aliquots) were analyzed by electrophoresis on 2% agarose gels. DNA was blotted onto Hybond-N+ positively charged nylon membrane by the alkali blotting method as specified by the manufacturer (Amersham International plc). Blots were prehybridized at 45°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0])–0.25% (wt/vol) Marvel for 1 to 2 h and hybridized in the same solution containing a 5′ end-labelled oligonucleotide (5′-GGG CAG GGT TCG CC) specific for the 123-bp PCR product (4) for 1.5 h. Fifty nanograms of oligonucleotide was 5′ end labelled with [τ-^{33}P]ATP (Amersham International plc) in a 20-μl reaction volume containing 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 5 to 10 U of T4 polynucleotide kinase (Promega) at 37°C for 30 min. Blots were washed in 10× SSC for 5 min at room temperature and then in 6× SSC at 45°C for 30 min and exposed to Hyperfilm beta max (Amersham International plc) at room temperature overnight in a cassette containing an intensifying screen.

RE digests. *Alu*I, *Hae*III, *Hha*I, *Mbo*I, *Mnl*I, *Msp*I, *Rsa*I, *Taq*I, and *Tha*I restriction maps were generated for the 16S rRNA nucleotide sequences of 18 mycobacterial type strains in the EMBL sequence data bank with DNASIS version 7 software (Pharmacia Biosystems, GMBH). *Hha*I, *Msp*I, *Mbo*I, and *Tha*I were subsequently selected because they clearly differentiated type strains by resolution of digests on agarose gels. Aliquots of purified PCR products were digested separately with each of the four restriction endonucleases (GIBCO BRL) according to the manufacturer's specifications. *Hha*I, *Msp*I, and *Tha*I digests were resolved on 2% agarose type 1 (Sigma Chemical Company, Ltd.)–2% NuSieve GTG agarose (FMC Bioproducts) gels containing ethidium bromide (0.5 μg/ml) beside *Hae*III-digested ϕX174 (replicative form) molecular weight standard (GIBCO BRL). *Mbo*I digests were resolved on 1.5% agarose type 1–1.5% NuSieve agarose gels. Gels were viewed on a Chromato-vue transilluminator (model TM-15; UVP, Inc., San Gabriel, Calif.) and photographed with a Polaroid MP4 Land camera with Polaroid 55 positive/negative film. An image of each negative was transferred to a SUN IPX workstation by using a Kodak CCD (1,024 by 1,024 pixels) high-sensitivity camera, and the images were analyzed with whole band analysis software (Millipore UK, Ltd.). Bands below 150, 245, 350, and 400 bp were not considered for *Hha*I, *Msp*I, *Tha*I, and *Mbo*I digests, respectively, since these bands were considered either to be of no further discriminatory value or too small to resolve clearly on the type of gel used.

Cycle sequencing. Direct nucleotide sequencing of the purified 16S rRNA PCR products was carried out with a CircumVent thermal cycle dideoxy DNA sequencing kit (New England Biolabs, Ltd.). The oligonucleotide primer MSHA (5′-CAC CAA CAA GCT GAT AGG C; nucleotides 250 to 268 of the 16S rRNA gene of *E. coli*) was 5′ end labelled with [τ-^{33}P]ATP as specified by New England Biolabs. Twenty nanograms of PCR product and 10.5 pmol of the 5′ end-labelled primer were used for each sequencing reaction, which was performed exactly as described by the manufacturer. If secondary structure or sequence ambiguities were observed when primer MSHA was used, the opposite strand was also sequenced with primer MSHB (5′-GGG ATA AGC CTG GGA AAC T; nucleotides 147 to 165 of the 16S rRNA gene of *E. coli*). Primers MSHA and MSHB flank an identified species-specific signature region within the 16S rRNA gene. Direct nucleotide sequencing of 16S rRNA PCR products of isolates G, J, and Q was also carried out through another variable region of the 16S rRNA gene of potential value for confirmation of mycobacterial species with two additional primers: MSHD (5′-GGA AGG CAG CAG TGG GGA AT; spanning nucleotides 313 to 331 of aligned 16S rRNA sequences of mycobacterial species [20]) and MSHE (5′-GCG ACA AAC CAC CTA CGA G; nucleotides 539 to 557 of aligned 16S rRNA sequences of mycobacterial species [20]), which was targeted to the opposite strand. Thermocycler parameters were as follows: 20 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Stop-loading dye solution was added to each tube beneath the mineral oil, and the samples were stored at −20°C before electrophoresis. Immediately prior to electrophoresis, the mineral oil was removed by pipetting and the samples were heated at 100°C for 3 min. Samples were kept on ice during gel loading.

Polyacrylamide gel electrophoresis. Sequencing reactions were resolved on 6% polyacrylamide–7 M urea gels cast in a sequencing gel assembly (Hoefer Scientific Instruments, San Francisco, Calif.) in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1.25 mM EDTA). Gels were fixed in 10% methanol–10% glacial acetic acid, transferred onto Whatman 3 MM filter paper (Whatman LabSales, Ltd.), and dried under a vacuum at 80°C before exposure to Hyperfilm beta max (Amersham International plc) at room temperature in a cassette with an intensifying screen. Autoradiographs were viewed on a light box (Jencons Scientific, Ltd.), and nucleotide sequences were read manually and analyzed by DNASIS version 7 software. DNA sequence similarity searches were performed with the EMBL and GenBank sequence data banks. An acid-fast isolate was definitively identified only when its respective nucleotide sequence shared 100% nucleotide identity with a data base reference sequence.

RESULTS

Differentiation of mycobacterial species by REA of the 16S rRNA gene. (i) Discriminatory value of the REs. The combined results of separate REAs of 16S rRNA gene PCR products with each of four enzymes (*Hha*I, *Msp*I, *Mbo*I, and *Tha*I) enabled differentiation of 12 of the 15 mycobacterial species considered (14 type cultures and the field isolate of *M. paratuberculosis*). *M. bovis* BCG NCTC 5692, *M. bovis* NCTC 5693, and *M. tuberculosis* NCTC 7416 had identical RE profiles with all four enzymes. *M. avium* NCTC 8559 and the field isolate of *M. paratuberculosis* were also indistinguishable from each other when these enzymes were used. A combination of *Hha*I, *Msp*I, and *Mbo*I differentiated 11 of 15 mycobacterial species, with *Tha*I required to differentiate *M. kansasii* from *M. scrofulaceum*. Similarly, a combination of *Hha*I, *Msp*I, and *Tha*I allowed identification of 11 of 15 species, with *Mbo*I required to differentiate *M. intracellulare* from *M. scrofulaceum*. The observed RE profiles of the *Actinobacillus* species were in accordance with the predicted profiles and, with the exception of the *Mbo*I profile, could clearly be differentiated from those of the mycobacterial species. *Hha*I proved to be the most discriminating enzyme (Fig. 1), generating 7 different profiles for the 15 mycobacterial species considered. *Msp*I (Fig. 2) and *Tha*I both produced five patterns, and *Mbo*I generated four profiles.

(ii) Restriction profile anomalies. Some anomalies were apparent between the predicted and observed band sizes of the digests. From 16S rRNA sequence data, *Hha*I was predicted to generate doublets (approximately 403 and 410

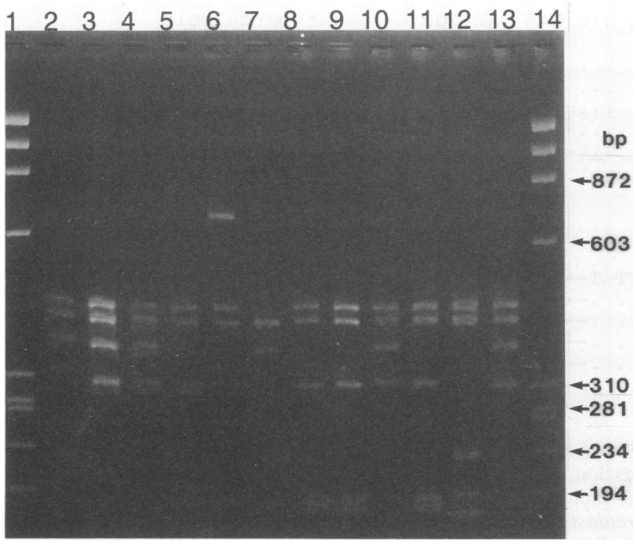

FIG. 1. REA (with *Hha*I) of PCR-amplified 16S rRNA genes from mycobacterial species. The specimens used included *M. smegmatis* NCTC 333 (lane 2), the *M. paratuberculosis* N.I. field isolate (lane 3), *M. marinum* NCTC 10009 (lane 4), *M. bovis* NCTC 5693 (lane 5), *M. gordonae* NCTC 10267 (lane 6), *M. vaccae* NCTC 10916 (lane 7), *M. scrofulaceum* NCTC 10803 (lane 8), *M. kansasii* NCTC 10268 (lane 9), *M. terrae* NCTC 10856 (lane 10), *M. intracellulare* NCTC 10425 (lane 11), *M. fortuitum* NCTC 10394 (lane 12), and *M. avium* NCTC 8559 (lane 13). Fragments of φX174 (replicative form) digested with *Hae*III were included as size standards (lanes 1 and 14).

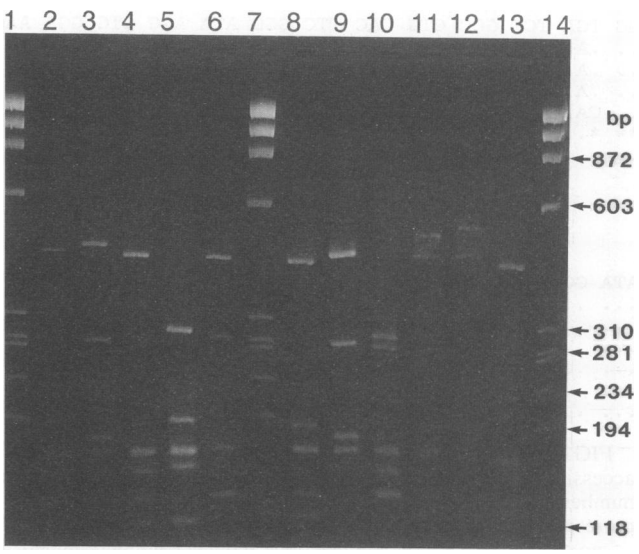

FIG. 2. REA (with *Msp*I) of PCR-amplified 16S rRNA genes from mycobacterial species. Specimens included *M. avium* NCTC 8559 (lane 2), *M. fortuitum* NCTC 10394 (lane 3), *M. intracellulare* NCTC 10425 (lane 4), *M. terrae* NCTC 10856 (lane 5), *M. kansasii* NCTC 10268 (lane 6), *M. scrofulaceum* NCTC 10803 (lane 8), *M. vaccae* NCTC 10916 (lane 9), *M. gordonae* NCTC 10267 (lane 10), *M. bovis* NCTC 5693 (lane 11), *M. marinum* NCTC 10009 (lane 12), and the *M. paratuberculosis* N.I. field isolate (lane 13). Fragments of φX174 (replicative form) digested with *Hae*III were included as size standards (lanes 1, 7, and 14).

bp) for tubercle complex species and for *M. smegmatis*, *M. scrofulaceum*, *M. paratuberculosis*, *M. avium*, *M. kansasii*, *M. intracellulare*, and *M. fortuitum*. However, the upper band of the doublet was calculated by image analysis of the resolved *Hha*I digests (Fig. 1) to range between 436 and 452 bp and the lower band was calculated to range between 411 and 419 bp. Similarly, *Msp*I was predicted to produce a fragment of approximately 247 bp for *M. gordonae*, *M. fortuitum*, and *M. smegmatis*; however, the observed band size for each species was found to range between 277 and 282 bp (Fig. 2). The REs *Hha*I and *Tha*I generated different patterns for *M. xenopi* NCTC 10042 than those predicted (data not shown). For each enzyme, the actual RE profile differed from the predicted pattern in one band only over the size range considered. In the case of *Hha*I, a band of approximately 418 bp (lower band of the aforementioned doublet) replaced the predicted band of 333 bp, and for *Tha*I, one observed band of 540 to 542 bp replaced the predicted band of 466 bp. Band size discrepancies were also noted for *M. vaccae*: with *Hha*I, predicted bands of 406 and 374 bp appeared to comigrate (Fig. 1, lane 7), and with *Msp*I, the predicted band of 204 bp was replaced with an observed band of 281 bp (Fig. 2, lane 9). The loss of a restriction site adjacent to the 204-bp fragment which generates a fragment of 80 bp may account for this.

Identification of acid-fast field isolates. (i) Culture characteristics. "Cording," characteristic of virulent strains of tubercle complex mycobacteria (13), was observed in BACTEC 7H12 cultures of 6 of 18 bovine tissue specimens (A, E, K, L, P, and R) and from three badger feces specimens (S, T, and U) (Table 1). Solid media inoculated with the same specimen preparations produced colonies for specimens L and N only, with morphology characteristic of *M. bovis*. LJ slope cultures were not available for specimens M and P. Acid-fast organisms not showing typical cording were additionally observed in BACTEC 7H12 cultures of specimens A through D, inclusive, F through K, inclusive, M through O, inclusive, Q, and U. These were subcultured onto LJ slopes before phenotypic typing.

(ii) Identification by traditional procedures. The traditional identifications of the atypical acid-fast isolates recovered from bovine and badger specimens are shown in Table 1. Acid-fast organisms from specimens E, S, T, and U, which exhibited cording in BACTEC 7H12 culture, were also typed. Isolate E was designated *M. kansasii*. Isolate S initially could not be classified but was subsequently identified as belonging to the *M. avium-intracellulare-scrofulaceum* (MAIS) complex. Isolate U was identified as *M. fortuitum* and subsequently reclassified as MAIS complex, and isolate T could not be identified.

Detection of tubercle complex mycobacteria in cultures. Tubercle complex-specific PCR amplification was performed on all subcultures. Tubercle complex-specific DNA was detected in subcultures of specimens L, P, and R, which displayed cording in their respective BACTEC 7H12 cultures. REA and DNA sequence analysis of the 16S rRNA PCR product confirmed the presence of tubercle complex-specific DNA in specimens L and R, but REA suggested *M. avium* or *M. paratuberculosis*, and DNA sequencing identified *M. avium* in specimen P. It would appear that the BACTEC 7H12 culture of specimen P was a mixed culture of *M. avium* and tubercle complex mycobacteria, with *M. avium* appearing as the predominant isolate in the Middlebrook 7H9 subculture used for 16S rRNA gene amplification.

REA and DNA sequencing of acid-fast isolates from animals. Comparison of the RE profiles of field isolates with

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GG TGA TCT GCC CTG CAC TTC GGG ATA AGC CTG GGA AAC TGG GTC TAA TAC CGG    M. tuberculosis
.. .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...    Isolate C
.. .A. ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ...    Isolate G
.. .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...    M. simiae
.. CA. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...    M. intracellulare serovar 16
** *.. ... ... ... ... .CT ... ... ... ... ... ... ... ... ... ... ...    Isolate I
.. ... ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ...    M. senegalense
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ATA GG-ACCA CGG GAT GCA TG--TCT-TGT GGT GGA AAG C GC TT T---AG CG        M. tuberculosis
... ..-...T TTT .GC ... ..--C..-.T. ... ... ..- - .. .. .---T. ..        Isolate C
... ..-...T TTT .GC ... ..--C..-.T. ... ... ..- - .. .. .---T. ..        Isolate G
... ..-.... .TT .GC ... ..--C..-... ... ... ..- - .. .. .---T. ..        M. simiae
... ..-...T TTA ..C ... ..--...-.T. ... ... ..- - .. .. .---T. ..        M. intracellulare serovar 16
... ..-...G ..C .C. T.. ..--GTG-... ... ... ..- - .. .. .---** **       Isolate I
... ..-.... ..C .C. T.. ..--GTG-... ... ... ..- - .. .. .---T. ..        M.senegalense
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FIG. 3. Alignment of 16S rRNA sequences of unclassified isolates (C, G, and I) and identified mycobacterial species. *M. simiae* (data base accession number X52931), *M. intracellulare* serovar 16 (data base accession number M61683), and *M. senegalense* (data base accession number M25967) shared the greatest percent nucleotide identity with the indicated isolates over the specified region. The first nucleotide corresponds to *E. coli* position 127. Nucleotide differences from *M. tuberculosis* (data base accession number X52917) are shown. Symbols: ·, no change; –, deletion; *, nucleotide sequence not determined. The 16S rRNA sequences of isolates J, Q, S, T, and U are not shown, since they are identical either to that of isolate C or to that of isolate I.

those of type cultures allowed definitive identification of acid-fast isolates in 13 of 18 bovine specimens (see Table 1). Four bovine field isolates (C, G, J, and Q) had profiles which, for all enzymes considered, closely resembled those of *M. simiae*. With reference to sequence data bases, DNA sequencing confirmed that isolates C, J, and Q most closely resembled *M. simiae*, displaying 96.9% sequence identity over 96 bp (*E. coli* 16S rRNA gene nucleotide positions 127 to 231) (Fig. 3). Isolate G differed from isolates C, J, and Q at one position where adenine was substituted for guanine and shared 96.9% sequence identity with *M. intracellulare* serovar 16 over 130 bp (*E. coli* 16S rRNA gene nucleotide positions ~92 to 231). *M. terrae* and *Mycobacterium nonchromogenicum*, which are biochemically indistinguishable, share 91.7% nucleotide identity over the same 96 bp, and *M. fortuitum* and *M. avium*, which are morphologically and biochemically distinct, share only 87.5% nucleotide identity over this region. Direct sequencing of the variable region corresponding to *E. coli* 16S rRNA gene nucleotide positions 429 to 502 with primers MSHD and MSHE revealed that isolates G, J, and Q shared 100% nucleotide identity with each other over 176 bp and differed from *M. simiae* at only one nucleotide, identified as cytosine for isolates G, J, and Q and denoted N for *M. simiae*. Bovine isolate I could not be identified by RE profiles and displayed the same patterns as the three badger isolates (S, T, and U); *Mbo*I and *Tha*I profiles resembled that of *M. nonchromogenicum*, *Msp*I profiles suggested either *M. terrae* or *M. simiae*, and *Hha*I profiles grouped these isolates with *Mycobacterium gastri*, *M. scrofulaceum*, *M. intracellulare*, *M. szulgai*, *M. malmoense*, and the tubercle complex. DNA sequencing demonstrated a 97.8% sequence identity with *Mycobacterium senegalense* over 89 bp (nucleotide positions 130 to 224 with respect to the *E. coli* 16S rRNA gene) (Fig. 3). Isolate I was identified as *M. fortuitum* by traditional testing.

Comparison of molecular and traditional typing procedures. Traditional identification did not strictly agree with the molecular typing of any of the isolates (Table 1). Isolates A, D, N, and O, identified as *M. terrae*, and isolate F, identified as *M. fortuitum* (a fast grower) by traditional typing, were identified as *M. nonchromogenicum* (a slow grower) by both molecular typing methods (the type culture of *M. nonchromogenicum* was not available and therefore the RE profiles of the acid-fast isolates recovered from the specimens had to be compared with RE profiles generated from predicted bands sizes). Four isolates (B, K, M, and Q) were typed as *M. avium* or *M. intracellulare* by traditional procedures. The molecular typing methods identified isolate M as *M. avium*, isolate B as *M. paratuberculosis*, isolate K as a mixed culture of *M. avium* or *M. paratuberculosis* and *M. nonchromogenicum*, and isolate Q as most closely related to *M. simiae* from comparison with sequence data bases. Isolates C and J were identified as MAIS, and isolate G was identified as *M. gordonae* by traditional typing, although RE profiles of isolates C, G, J, and Q were distinguishable from the type cultures of the MAIS complex and from *M. gordonae*. Traditional phenotyping identified isolates E and H as *M. kansasii*. Neither molecular method, however, could differentiate between *M. gastri* and *M. kansasii*; consequently, traditional typing proved to be more discriminatory for these isolates.

Comparison of REA and DNA sequencing. The identifications made by the two molecular typing systems were in close agreement. DNA sequencing was able to differentiate between *M. avium* and *M. paratuberculosis* and between *M. gastri* and *M. szulgai*, whereas REA could not. REA, however, enabled the identification of two species, *M. avium* or *M. paratuberculosis* and *M. nonchromogenicum*, in Middlebrook 7H9 culture of specimen K, whereas the DNA sequence obtained from this culture was unreadable.

DISCUSSION

Two molecular methods, REA and direct cycle sequencing of the 16S rRNA gene, were developed as practical diagnostic procedures for the identification of mycobacterial species. The 16S rRNA gene was chosen because it appears that, with the exception of *M. gordonae* (7) and *M. intracellulare* (2), the unique sequence differences within the 16S rRNA gene can be ascribed to either species or subspecies level. REA of the 65-kDa gene of slowly growing mycobacterial species has been performed (17). However, the 65-kDa protein-encoding gene displays microheterogeneity within mycobacterial species (17) and may be of more value in

epidemiological studies than in simple species identification. Direct sequencing of the complete 16S rRNA gene can differentiate *Mycobacterium* species (19) but would be too laborious to utilize as a diagnostic procedure. The novel technique of direct cycle sequencing of species-specific variable regions within the 16S rRNA gene, however, has potential value for routine identification of mycobacterial species.

To be of practical routine diagnostic value, molecular methodologies require simplification, and this was a major consideration throughout the study. Potential contamination associated with the PCR was minimized, and sample preparation was simplified by a one-step extraction of DNA. Purification of the PCR products was made simpler and safer by using a commercial kit. Direct DNA cycle sequencing proved to be simpler and required smaller amounts of template DNA than other methods of direct sequencing (3, 22), and Vent (exo$^-$) DNA polymerase displays a higher fidelity of nucleotide incorporation than *Taq* polymerase.

Both of the molecular typing methods were more accurate and rapid than traditional typing. REA and direct cycle sequencing methodologies can provide a result within 48 and 72 h, respectively, whereas identification of mycobacterial species by conventional methodologies may require at least 1 month (11). Cording in BACTEC 7H12 media initially appeared to have value as a presumptive indicator of the presence of tubercle complex mycobacteria. However, tubercle complex-specific PCR confirmed the presence of tubercle complex species in only three of nine cultures in which cording was detected. One such culture (specimen E) was identified as *M. kansasii* by traditional typing and by both molecular typing methods. Cording has previously been associated with *M. kansasii* (6, 23), although the microscopic morphology of these cords differs from the serpentine cord formation of *M. bovis*. Cording has also been noted in cultures of *Mycobacterium phlei*, *M. terrae* (18), and *M. fortuitum* (6). Cording, while useful as a presumptive indicator of tubercle complex mycobacteria, should not therefore be considered definitive for *M. bovis*. It was concluded that cording observed in the six tubercle complex-specific PCR-negative cultures was associated with mycobacteria other than tubercle complex (MOTTs). Alternatively, *M. bovis* may have been present in these cultures but growth of MOTTs also present may have been favored by subculture on egg-based media.

Cultures from four specimens (A, K, P, and U) appeared to contain more than one mycobacterial species. This finding has important implications for mycobacterial species identification in clinical specimens by molecular methodologies. The implementation and interpretation of these tests when applied to clinical specimens containing more than one species has not previously been addressed. This study indicated the need to carry out PCR directly on either the initial 7H12 culture or preferably on DNA extracted from the tissue specimen. However, even if PCR amplification of the 16S rRNA gene is performed directly on the latter, all of the species present may not be detected if one species is predominant. In veterinary mycobacteriology laboratories, both tubercle complex and 16S rRNA-specific PCR amplification would therefore need to be performed. PCR amplification that was performed on tissues with a mixed population of mycobacteria from tuberculin-reacting cattle and that used the latter primers might possibly and falsely imply MOTT involvement in a nonspecific reaction to the skin test only if *M. bovis* is a minority species. Nonspecific sensitization of cattle to bovine tuberculin may be caused by exposure to the MAIS complex, *M. tuberculosis*, *M. paratuberculosis*, *M. kansasii*, and other environmental mycobacteria (14, 15). Interestingly, in this study, *M. nonchromogenicum* and *M. kansasii* were isolated from specimens of cattle recorded positive in the skin test. These species may have been responsible for the positive skin test, but it is also possible that the cattle may have been harboring *M. bovis* in sites that are not routinely examined for the presence of *M. bovis*.

REA of the PCR-amplified 16S rRNA sequences proved to be technically simpler than direct sequencing, but manual analysis of the data was laborious. Accurate quantitative analysis of RE profiles, however, could be automated. Image analysis of the RE profiles revealed that precise determination of band sizes necessitates the presence of molecular weight markers in every lane of the gel to accommodate for "frowning" and "smiling." Plikaytis and colleagues included two molecular weight standards in each gel lane in their study (17), but this may be insufficient if frowning or smiling occur in localized regions of the gel.

Anomalies apparent between observed and predicted RE profiles indicated the need to generate a reference data bank of actual profiles for known mycobacterial species and subspecies. Actual *Hha*I, *Msp*I, *Tha*I, and *Mbo*I RE profiles were determined for 15 mycobacterial species and for *Actinobacillus* species, since these acid-fast organisms may also be recovered from animals.

Direct cycle sequencing was more discriminatory than REA and required significantly less DNA, and analysis of the sequence information was simpler. Cycle sequencing, however, could not identify species present in a mixed culture such as that of specimen K. Again, there is a need to establish a data bank of reference sequences for identified species.

Although 8 of 21 MOTTs could not be definitely identified by either molecular method, these isolates were believed to represent only two species or subspecies for which no reference sequence was available. Three isolates (C, J, and Q) were genetically most closely related to *M. simiae* by both methods and may represent a new species. Isolate G differed from isolates C, J, and Q at one nucleotide, therefore possibly representing a subspecies (2a). Identification of new species or subspecies by molecular methods obviously requires augmentation by other tests, since 16S rRNA sequence information cannot be the only criterion on which species definition is based (5, 16). Four other isolates, most closely related to *M. senegalense* by DNA sequencing, were thought to belong to the *M. fortuitum* complex (2a), whereas traditional typing classified three of these isolates as belonging to the MAIS complex. Telenti and colleagues were also unable to identify some isolates by REA of the 65-kDa protein gene because the profiles were not present in the reference algorithm (21).

There is an obvious need for accurate identification of mycobacterial isolates from cattle being tested for tuberculosis, particularly during the final stages of eradication programs, since some MOTTs have the potential to induce positive reactions to the intradermal skin test. This study indicated that while the accuracy of traditional typing may be adequate for the confirmation of tubercle complex mycobacteria, it is insufficient for the reliable identification of MOTTs. As practical diagnostic tests, both 16S rRNA molecular methods, particularly direct cycle sequencing, offer more accurate and rapid typing systems than traditional testing and could ultimately obviate the need for culture.

ACKNOWLEDGMENTS

We thank the mycobacteriology laboratory staff, particularly R. Hill, for their assistance and gratefully acknowledge P. Jenkins (Public Health Laboratory Service) for identifying the isolates by traditional typing and E. C. Böttger for his helpful information.

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