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Determination of the Etiology of Presumptive Feline Leprosy by 16S rRNA Gene Analysis

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PCR-amplified 16S rRNA gene sequences were obtained directly from tissue specimens from eight cats with presumptive feline leprosy. Acid-fast bacilli were observed in sections from all eight specimens, but culture for mycobacteria was successful for one specimen only. Analysis of the V2 variable region of each 16S rRNA PCR product identified a sequence with 100% nucleotide identity to the sequences of *Mycobacterium lepraemurium*, *Mycobacterium avium*, and *Mycobacterium paratuberculosis* in four of the specimens from cats with feline leprosy. Separate *M. paratuberculosis*- and *M. avium*-specific PCR amplifications of the four specimens were negative, thus substantiating the identification of *M. lepraemurium* in these specimens from cats with feline leprosy. Further sequence analysis of the V3 variable region of one of the four specimens provided conclusive evidence of the presence of *M. lepraemurium*. This is the first report of the definitive identification of *M. lepraemurium* in cats with feline leprosy by molecular biology-based analyses. *M. avium*, which is rarely reported in cats, and *Mycobacterium chitae*, a reported nonpathogenic, rapidly growing mycobacterial species found in the environment, were identified in the specimen from which acid-fast bacilli were cultured. Two of the specimens from cats were infected with a potentially novel species of mycobacteria which had a 16S rRNA gene sequence sharing the closest nucleotide sequence identity with that of *Mycobacterium malmoense*. Molecular biology-based analyses provided for the accurate and rapid diagnosis of mycobacterial infections in cats and circumvented the problems of culture and misdiagnosis of feline leprosy associated with traditional methods.

Cat leprosy has been described as a localized skin infection containing acid-fast bacilli which are nonculturable by standard mycobacteriological methods (5). The disease has also been associated with granulomatous nodules (20, 28, 38). Cat leprosy has a widespread geographic distribution (23, 28). It was first reported in New Zealand in 1962 (35), and in 1979 the number of recorded cases of cat leprosy was reported to be higher in New Zealand than in any other country (35). de Lisle and colleagues (5) suggested, however, that cases of cat leprosy among cats in the large area in the center of the North Island in New Zealand where bovine tuberculosis is endemic may have been misdiagnosed as cat leprosy.

The causative agent of cat leprosy is purported to be *Mycobacterium lepraemurium* (2, 18, 20, 23). This bacillus causes leprosy in rodents, from which cats are believed to contract *M. lepraemurium* (23). *M. lepraemurium* is a slowly growing mycobacterial species which is only culturable with difficulty on enriched medium (14, 22). The basis of ascribing this bacterium as the etiological agent of cat leprosy depends mainly on transmission experiments and delayed-type hypersensitivity skin reactions (20, 35) and not on primary isolation of a mycobacterial agent.

Mycobacteria other than *M. lepraemurium* have been isolated from cats; these include *M. bovis*, *M. fortuitum*, *M. smegmatis*, *M. thermoresistible*, *M. xenopi*, *M. avium* complex, *M. chelonei*, and *M. phlei* (15, 39, 40). Accurate diagnosis of cat leprosy can be complicated since histopathologically this disease may not always be distinguishable from other mycobacterial infections (5). Consequently, culture has been recommended for the definitive diagnosis of feline mycobacterial infections, which are of public health significance when zoonotic mycobacteria are concerned (10, 15, 39).

Molecular biology-based methodologies may offer a faster and more reliable option than bacterial culture for the diagnosis of *M. lepraemurium* infection. PCR amplification and sequencing of the 16S rRNA gene have proven to be of value for the diagnosis of infections caused by mycobacterial species of veterinary origin (12). The 16S rRNA gene is highly conserved because of structural and functional constraints on the rRNA molecule, but regions within the gene are highly variable and species specific for mycobacteria (25). This variability within a conserved gene has been of value in determining phylogenetic relationships between mycobacterial species as well as for species identification (25, 26, 30). *M. paratuberculosis*, *M. avium*, and *M. intracellulare* form a distinct branch on the phylogenetic tree constructed on the basis of 16S rRNA gene sequences (30). The position of *M. lepraemurium* on this tree has yet to be ascertained. However, genetic and serological analyses have indicated that *M. lepraemurium* is closely related to the aforementioned species (9). Identification of mycobacterial species by this method has been shown to be more discriminatory and more rapid than traditional typing methods and obviates the need for culture. In this paper we report the application of the 16S rRNA typing method to specimens from eight cats presumptively diagnosed with cat leprosy.

MATERIALS AND METHODS

Case history. Specimens from seven cats in New Zealand (subsequently referred to as NZ cat leprosy specimens 1 to 7) and one cat in Northern Ireland (subsequently referred to as NI cat leprosy specimen 8) were submitted to the laboratories in the respective countries for microbiological and histopathological examinations. The NZ specimens were lyophilized and comprised six skin biopsy specimens and one swab specimen from a relapsing abscess. The NI specimen was from a submandibular lymph node. The history of each specimen was

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recorded on a standard submission form (see Table 1). In most cases the presumptive diagnosis was recorded as cat leprosy by the clinician.

Bacterial culture. Portions of the NZ cat leprosy specimens were cultured by previously described methods (4). Duplicate sets of media were incubated for 12 weeks at 30°C as well as at 37°C. A portion of the NI cat leprosy tissue specimen was homogenized, decontaminated in 5% (wt/vol) oxalic acid at 37°C for 30 min, and centrifuged at 1,615 × *g* for 10 min. The resulting pellet was inoculated directly onto Lowenstein-Jensen slopes containing pyruvate with a cotton swab. The remaining pellet was washed with sterile 0.85% (wt/vol) saline solution and was resuspended in 1 ml of the same solution before inoculation into BACTEC 7H12 medium (Becton Dickinson Diagnostic Systems, Oxford, United Kingdom). The inoculated media were incubated at 37°C.

Histopathogical examination. Tissues were fixed in 10% (wt/vol) neutral buffered formalin and embedded in paraffin. Sections (5 μm) were stained by the Ziehl-Neelsen (ZN) method (33) and with hematoxylin and eosin (34) and were examined microscopically.

DNA extraction. Genomic DNA was extracted from the cat leprosy specimens for PCR amplification. Each lyophilized NZ cat leprosy tissue specimen was resuspended in 100 μl of sterile deionized water (SDW). DNA was extracted from the resuspensions by the following methods (i) A 1:4 dilution of a resuspension in SDW was heat treated for 15 min at 97°C. (ii) An aliquot (25 μl) of each resuspension was diluted in 500 μl of SDW and heat inactivated at 75°C for 1 h, and the DNA was extracted by the method of Liebana and colleagues (19). (iii) DNA was isolated from aliquots (10 μl) of each resuspension by the procedure chosen by Wards and colleagues (37) as described by them and also with the following modification; after the initial proteinase K digestion, sodium dodecyl sulfate and proteinase K were added (as specified) directly to the suspension.

DNA extraction from finely cut portions of a fresh NI cat leprosy specimen was attempted by the Chelex method of Stein and Raoult (32), omitting the lauryl sulfate and using 5-μl aliquots of undiluted extracted DNA and a 1:50 dilution, in SDW, of the extracted DNA for PCR amplification, and using the QIAamp Tissue Kit (Qiagen Ltd., Surrey, United Kingdom). Extraction by the latter method was performed according to the manufacturer's instructions, eluting the DNA with 100 μl of SDW, preheating the DNA at 70°C, and using the eluent for the second elution. PCR amplification was performed with 20-μl aliquots of extracted DNA. DNA was also extracted from the BACTEC culture of the NI cat leprosy tissue specimen. A 1-ml aliquot of culture was pelleted by centrifugation at 13,000 × *g* for 5 min. The pellet was washed three times in SDW, resuspended in 35 μl of SDW, and heat treated at 100°C for 15 min, and a 30-μl volume was used for PCR amplification.

PCR amplifications. Four separate PCR amplifications were performed with the cat leprosy specimens. These included a 16S rRNA PCR and PCR amplifications specific for the *M. tuberculosis* complex, *M. paratuberculosis*, and *M. avium*, respectively. The purpose of the 16S rRNA PCR was to enable identification of the bacterial species present in the specimens. The *M. tuberculosis*-specific PCR amplification was carried out to determine whether cat leprosy had been misdiagnosed in the cats considered. The PCR amplifications specific for *M. paratuberculosis* and *M. avium* were included to substantiate the identification of *M. lepraemurium* in the relevant specimens.

PCR amplification of a region (approximately 600 bp) of the 16S rRNA gene was performed with DNA prepared from all eight cat leprosy specimens with primers pA (5′-AGA GTT TGA TCC TGG CTC AG) (7) and MSHE (5′-GCG ACA AAC CAC CTA CGA G) (12). Unless otherwise specified, 5 μl of extracted DNA was added to 45 μl of the PCR amplification mixture, with the final 50-μl volume containing 1.25 U of *Taq* DNA polymerase (Promega Corporation, Madison, Wis.), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.1% Triton X-100, deoxyribonucleotides, each at a concentration of 150 μM (Pharmacia, San Francisco, Calif.), 30 pmol of each of the two primers, and 50 μg of 8-methoxypsoralen (Sigma Chemical Company Ltd., Dorset, United Kingdom) per ml, and the mixture was overlaid with light mineral oil (Sigma Chemical Company Ltd.). The PCR mixture was preexposed to 366-nm UV light for 4 min, to eliminate *Taq* DNA polymerase contaminants before the addition of extracted DNA (13). The PCR amplifications were performed in a thermocycler (Perkin-Elmer Corporation, Norwalk, Conn.), and the amplification mixtures were subjected to 4 min of denaturation at 94°C and 40 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. The 16S rRNA-specific amplification was also carried out with 1:10 and 1:100 dilutions, in SDW, of DNA extracted by the methods of Liebana and colleagues (19) and Wards and coworkers (37).

M. tuberculosis complex-, *M. paratuberculosis*-, and *M. avium*-specific PCR amplifications were attempted with heat-treated samples of all seven NZ cat leprosy tissue specimens. *M. paratuberculosis*- and *M. avium*-specific PCR amplifications were also performed with DNA extracted by using the QIAamp Tissue Kit and from BACTEC culture of the NI cat leprosy specimen. PCR controls were used and precautionary measures against contamination were taken as stated previously (12). The PCR mixtures were as described above for the 16S rRNA gene amplifications, with the omission of 8-methoxypsoralen and the following modifications for the specific PCR amplifications.

M. tuberculosis complex-specific PCR amplification mixtures contained 1.75 mM $MgCl_2$, the primers (5′-CCT GCG AGC GTA GGC GTC GG) and (5′-CTC GTC CAG CGC CGC TTC GG) specified by Eisenach and colleagues (8), and 2.5% (vol/vol) formamide to reduce the nonspecific amplification associated with tissue specimens (37). The reaction mixtures were subjected to 4 min of denaturation at 94°C and 40 cycles of 94°C for 1 min, 68°C for 2 min, and 72°C for 2 min, followed by an extension period of 72°C for 7 min and refrigeration, to amplify a 123-bp fragment within the *M. tuberculosis* complex-specific insertion sequence IS*6110*.

Primers P90 (5′-GTT CGG GGC CGT CGC TTA GG) and P91 (5′-GAG GTC GAT CGC CCA CGT GA) (21) were used for the amplification of a 400-bp region of the *M. paratuberculosis*-specific insertion sequence IS*900*. The reaction mixtures were subjected to 4 min of denaturation at 94°C and 40 cycles of 94°C for 1 min, 58°C for 2 min, and 72°C for 2 min, followed by an extension period at 72°C for 7 min and refrigeration. A nested PCR for IS*900* was also carried out with DNA prepared by heat extraction from cat leprosy specimens 1, 3, and 4 by using primers P90 and P91 in the first round of amplification. After 40 cycles, 3-μl aliquots of the PCR products were reamplified with primers (5′-CCG CTA ATT GAG AGA TGC GAT TGG and 5′-AAT CAA CTC CAG CAG CGC GGC CTC G) (36) which allow for the amplification of a 229-bp sequence internal to the first PCR product.

PCR mixtures containing 3.7 mM $MgCl_2$, deoxyribonucleotides each at a concentration of 25 μM, and primers 102 (5′-CTG ATT GAG ATC TGA CGC-3′) and 103 (5′-TTA GCA ATC CGG CGC CCT) (21) were used for the amplification of a 252-bp fragment of the *M. avium*-specific insertion sequence IS*902*. The reaction mixtures were subjected to 3 min of denaturation at 94°C and 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, followed by an extension period at 72°C for 10 min and refrigeration.

PCR product analysis. The PCR products (10 μl) were visualized on 2% (wt/vol) agarose gels stained with ethidium bromide (0.5 μg/μl; Sigma Chemical Company Ltd.), and the specificities of the species-specific PCR products were confirmed with oligonucleotide probes. *M. tuberculosis* complex-, *M. paratuberculosis*-, and *M. avium*-specific PCR products were transferred from agarose gels onto a Hybond N^+ (Amersham International plc, Amersham, United Kingdom) nylon membrane by Southern blotting as directed by the manufacturer. An oligonucleotide (5′-GGG CAG GGT TCG CC) (8) and MaviumP (5′ GGT GGG TAT CGA CGT CGG), specific for the *M. tuberculosis* complex and *M. avium* PCR products, respectively, were hybridized to the corresponding PCR products immobilized on Hybond N^+ membranes. Oligonucleotides were 3′ end labelled with a fluorescein-dUTP tail, and the signal was detected by the enhanced chemiluminescence (ECL 3′) oligolabelling and detection system (Amersham International plc) as directed by the manufacturer. A PCR product yielded by amplification of *M. paratuberculosis* NCTC 8378 with the specified primers (36) was labelled with horseradish peroxidase by the ECL Direct Nucleic Acid labelling kit, hybridized to immobilized products of the *M. paratuberculosis*-specific PCR, and detected by the ECL detection system.

The 16S rRNA PCR products of specimens 5, 6, and 7, visualized as bands of low intensity, were picked with sterile needles, and the DNA was reamplified. These reamplified products and the 16S rRNA PCR products from specimens 1, 2, 3, and 4 were purified with the Wizard PCR Prep DNA Purification System (Promega Corporation). Purified DNA was ligated into the pCRII cloning vector (Invitrogen Corporation) and was subsequently used to transform Epicurian Coli Sure 2 Supercompetent cells (Stratagene, La Jolla, Calif.), as directed by the manufacturer. Ampicillin-resistant transformants were picked and grown overnight in Luria-Bertani broth containing ampicillin (50 μg/ml) at 37°C. Minipreps of clones were prepared from 5-ml cultures by using the Modified Wizard Miniprep Procedure DNA Purification System (Promega Corporation) for use with Applied Biosystems Inc. automated sequencing. The minipreps from each clone were digested with *Eco*RI (Promega Corporation), and the products were visualized on 1% (wt/vol) agarose gels stained with ethidium bromide to select clones containing 600-bp inserts.

The 16S rRNA PCR products of cat leprosy specimens 1, 2, 4, 5, and 7 and clones of the same products of all 7 NZ cat leprosy specimens, together with 16S rRNA PCR products of *M. paratuberculosis* NCTC 8378, *M. avium* NCTC 8559, and *M. bovis* NCTC 5693, were electrophoresed on a 1% (wt/vol) agarose gel, and the DNAs were transferred to a Hybond N^+ membrane by Southern blotting. An *M. paratuberculosis*-, *M. lepraemurium*-, and *M. avium*-specific oligonucleotide, 16SMP (5′-GGA CCT CAA GAC GCA TGT TTT CT), targeted to the V2 variable region of the 16S rRNA gene (16), was 3′ end labelled with a fluorescein-dUTP tail, hybridized to the transferred DNA, and detected as described above.

Sequencing was performed for selected clones by using a variety of primers, T7 promoter (5′-TAA TAC GAC TCA CTA TAG GG), MSH-A (5′-CAC CAA CAA GCT GAT AGG C), MSH-B (5′-GGG ATA AGC CTG GGA AAC T), MSH-D (5′-GGA AGG CAG CAG TGG GGA AT), MSH-E, pA, and M13 reverse primer (5′-AAC AGC TAT GAC CAT G), as described previously (12). The CircumVent Thermal Cycle Dideoxy DNA Sequencing Kit (New England Biolabs) and the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems Inc.) were used. The sequencing reactions produced by the latter kit were purified by phenol-chloroform extraction according to the manufacturer's directions, and the products were resolved on model 373A DNA sequencer (Applied Biosystems Inc.). The sequences produced by this method were edited by using Sequence Navigator Software (Applied Biosystems Inc.). All sequences were analyzed with DNASIS, version 7, software (Pharmacia Biosystems GmbH). DNA sequence similarity searches were performed with the sequences in the EMBL and GenBank sequence data banks.

TABLE 1. case history, histopathology, and presence of acid-fast bacteria in samples from cats putatively with cat leprosy

Cat no.	Age (yr)	Sex[a]	Cat details	Clinical observations	Histopathology	Acid-fast bacilli
1	9	M	Domestic long hair	Multiple cutaneous and subcutaneous lesions on ventrum and dorsum	Poorly organized granulomatous inflammation; bacteria in vacuoles	4+
2	9	M	Chinchilla	Lump on foreleg	Nodular to diffuse infiltrate of macrophages within deep dermis and subcutis; few interspersed neutrophils and lymphocytes	4+
3	3	M	Domestic short hair	Tumor	Extensive granulomatous reaction	4+
4	8	F		Domed appearance on forehead	Sinus filled with fluid; granulomatous tissue	3+
5	1	F	Domestic tortoiseshell	Skin lump on left shoulder	Pyogranulomatous lesion with central necrosis	2+
6	2	M	Siamese		Chronic inflammatory response	1+
7	2	M	Domestic short hair	Multiple skin ulcers and nodules on head and body	Dermal lesions of confluent granulomata comprising activated histiocytes and neutrophils	2+
8	10	M	Domestic short hair	Lesion discovered in neck region at vaccination	Node is a fibrous mosaic of groups of lymphocytes and epithelioid cells with neutrophils; epithelioid cell cytoplasm with faint elongated structures; fibrous capsule	4+

[a] M, male; F, female.

RESULTS

Culture and histopathology findings. Bacteria were not isolated from any of the seven NZ cat leprosy tissue specimens by culture. Numerous acid-fast bacilli, however, were recovered from the NI cat leprosy tissue specimen cultured in BACTEC 7H12 medium, but culture on Lowenstein-Jensen slopes containing pyruvate was unsuccessful. The results of histopathological examination are presented in Table 1. Acid-fast bacilli were observed in all the specimens, although the relative abundance of bacilli varied from few (ZN staining, 1+) to numerous (ZN staining, 4+) between specimens.

Amplifications of 16S rRNA by PCR. PCR products of 16S rRNA of the expected size (approximately 600 bp) were obtained for the heat-treated samples of the seven NZ cat leprosy specimens and for the positive control. Nonspecific bands were also observed for heat-treated samples of specimens 1, 2, and 3. Bands of low intensity were visualized for heat-treated samples of specimens 5, 6, and 7; reamplification of the 600-bp PCR product for each of these samples resulted in higher yields of the products and reduced the occurrence of nonspecific amplification. The 16S rRNA PCR amplification of DNA extracted by the method of Liebana and colleagues (19) was unsuccessful; likewise, except for amplifications with specimen 2, amplifications with undiluted and diluted aliquots of DNA extracted by the method of Wards and colleagues (37) were unsuccessful.

PCR products of 16S rRNA of the expected size were also obtained from the NI cat leprosy tissue specimen and from the BACTEC culture of the same tissue specimen. Bands of strong intensity were obtained by PCR amplification of the latter and of DNA extracted from the tissue with the QIAamp Tissue Kit. A band of low intensity was visualized only for a 1:50 dilution of the Chelex-extracted DNA. This band was reamplified successfully, but further attempts at 16S rRNA PCR amplification of DNA extracted by the Chelex method yielded no products, and the extract was found to inhibit PCR amplification of the positive control.

***M. tuberculosis* complex-, *M. paratuberculosis*-, and *M. avium*-specific PCR amplifications.** Products of the expected size (123 bp) were not observed for any of the heat-extracted samples of the seven NZ cat leprosy specimens following *M. tuberculosis* complex-specific PCR amplification. Nonspecific bands were observed for heat-treated samples of specimens 1, 2, 3, and 4, and visible bands were not apparent for heat-treated samples of specimens 5, 6, and 7 (Fig. 1a). A 123-bp band of strong intensity was obtained for the PCR-positive control (*M. bovis* type culture).

M. paratuberculosis-specific PCR amplification of the heat-extracted samples of the seven NZ specimens and the QIAamp Tissue Kit-extracted DNA from the NI specimen did not yield products of the expected size (400 bp), although a band of strong intensity was observed for the PCR-positive control (*M. paratuberculosis* type culture). Nonspecific bands were obtained for all seven NZ specimens (Fig. 2a). The nested PCR amplification carried out with heat-treated samples of specimens 1, 3, and 4 resulted in no products of the expected size (229 bp), although multiple nonspecific bands were observed. A band of strong intensity was again observed with the PCR-positive control.

PCR amplification with *M. avium*-specific primers resulted in a strong band of the expected size (252 bp) for the NI cat leprosy specimen, DNA extracted from both the BACTEC culture and directly from the tissue with the QIAamp Tissue Kit (not shown in Fig. 3a), and the PCR-positive control (*M. avium* NCTC 8559). Multiple bands were again observed for heat-extracted samples of all seven NZ cat leprosy specimens (Fig. 3a).

Southern blot analyses. *M. tuberculosis* complex-, *M. paratuberculosis*-, and *M. avium*-specific probes were hybridized to the corresponding PCR products of the NZ cat leprosy specimens which had been immobilized on Hybond N^+ membranes. In each case, the probe hybridized to its respective PCR-positive control only. No hybridization with the seven cat leprosy specimens or the negative controls was detected (Fig. 1b, 2b, and 3b). The Southern blot of the 16S rRNA PCR products of the NZ cat leprosy specimens, their respective clones, and the 16S rRNA PCR products of the *M. paratuberculosis*, *M. avium*, and *M. bovis* type cultures was probed with 16SMP, an oligonucleotide specific for *M. lepraemurium*, *M. paratuberculosis*, and *M. avium*. Hybridization was detected with the 16S rRNA PCR products of specimens 4, 5, and 7 (the 16S rRNA PCR product for specimen 3 was not available) and

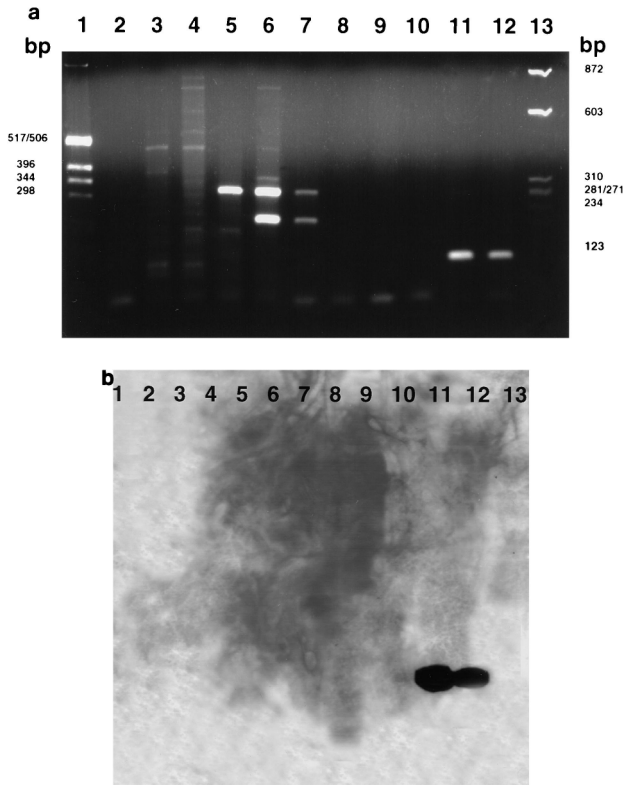

FIG. 1. (a) *M. tuberculosis*-complex specific PCR amplification of NZ cat leprosy specimens. PCR products were visualized on a 2% (wt/vol) agarose gel by ethidium bromide staining. The lanes contain a 1-kb DNA ladder size standard (Gibco BRL; lane 1), PCR mixture negative control (SDW; lane 2), cat leprosy specimens 1 to 4 (lanes 3 to 6, respectively), cat leprosy specimens 4 to 7 (lanes 7 to 10, respectively), PCR positive control (*M. bovis* NCTC 5693; lanes 11 and 12), and fragments of ϕX174 (replicative form) digested with *Hae*III (included as size standards; lane 13). (b) Southern blot of tubercle complex-specific PCR products (the lanes are the same as those described for panel a) probed with a fluorescein-dUTP 3′-end-labelled tubercle complex-specific oligonucleotide contained within the 123-bp amplicon.

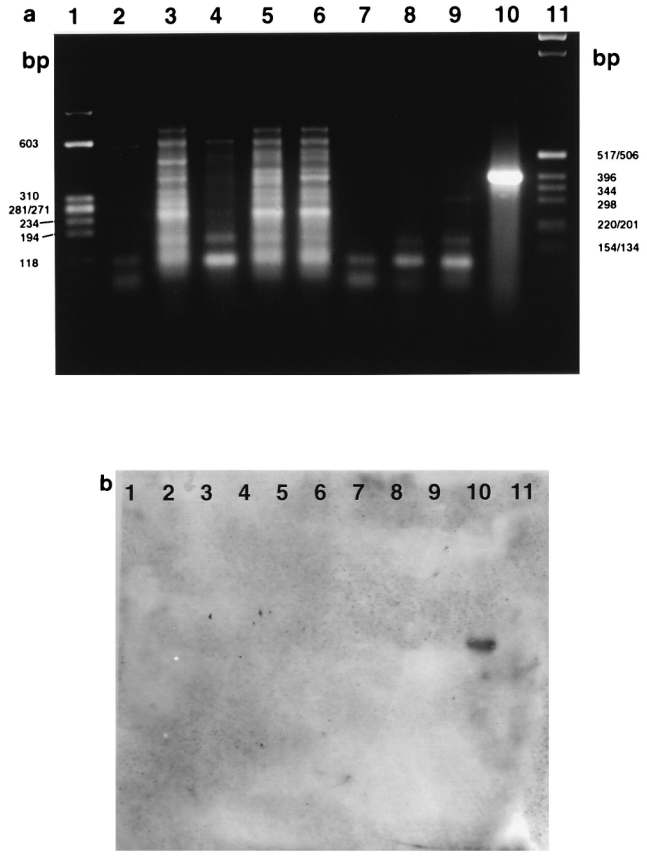

FIG. 2. (a) *M. paratuberculosis*-specific PCR amplification of NZ cat leprosy specimens. PCR products were visualized on a 2% (wt/vol) agarose gel by ethidium bromide staining. The lanes contain fragments of ϕX174 (replicative form) digested with *Hae*III (included as size standards; lane 1), PCR mixture negative control (SDW; lane 2), cat leprosy specimens 1 to 7 (lanes 3 to 9, respectively), PCR positive control. *M. paratuberculosis* NCTC 8378 purified DNA (lane 10), and a 1-kb DNA ladder as a size standard (Gibco BRL; lane 11). (b) Southern blot of *M. paratuberculosis*-specific PCR products (the lanes are the same as those described for panel a) probed with a fluorescein-dUTP 3′-end-labelled *M. paratuberculosis*-specific oligonucleotide contained within the 400-bp amplicon.

clones of the respective products of specimens 3, 4, and 5, with the exception of one clone pertaining to specimen 5 (the clone of cat leprosy specimen 7 containing a *M. lepraemurium* insert was not tested). The probe also hybridized with the *M. paratuberculosis* 16S rRNA PCR product but not with that of *M. bovis.*

Analysis of 16S rRNA sequence. Not all the selected clones contained inserts of the expected size; only those which did were sequenced. The 16S rRNA V2 variable region sequence (16) data were obtained for all the cat leprosy specimens, and the 16S rRNA V3 variable region sequence (16) data were obtained for some of the specimens. Species sharing the most nucleotide sequence identity with the 16S rRNA sequences analyzed, the percent nucleotide sequence identity, the number of nucleotides considered, and the nucleotide positions relative to the nucleotide positions in *Escherichia coli* 16S rRNA and those of aligned mycobacterial 16S rRNA sequences are presented in Table 2.

V2 region sequences with the most nucleotide sequence identity to the sequences of *M. lepraemurium*, *M. paratuberculosis*, and *M. avium* were identified in specimens 3, 4, 5, and 7. The V3 region of the 16S rRNA sequence from specimen 3 shared 100% identity with the equivalent sequence of *M. lepraemurium* only. Sequences with the most nucleotide sequence identity in the V2 and V3 regions with the sequence of *M. malmoense* were identified in tissue specimens 1 and 2 (Fig. 4).

Clones from tissue specimen 5 revealed sequences from two different bacterial species. One sequence had 99.3% nucleotide sequence identity with the sequences of *M. lepraemurium*, *M. paratuberculosis*, and *M. avium* over 142 bp of the V2 region, and the other sequence (Fig. 4) shared the most nucleotide identity over the V2 region with the sequence of *Arthrobacter ilicis*. Sequences (Fig. 4) with highest levels of nucleotide sequence identity with the sequences of *Arthrobacter pascens* and *Arthrobacter ramosus* in the V2 region were isolated from specimens 6 and 7. Two further distinct sequences were detected in specimen 7; one shared 99.3% nucleotide sequence identity with the sequences of *M. lepraemurium*, *M. paratuberculosis*, and *M. avium* over 145 bp, and the other (Fig. 4) had the most nucleotide identity with the sequence of *Micrococcus luteus* in the V2 region.

The DNA sequences of two clones of the Chelex-extracted PCR product from the NI cat leprosy tissue (specimen 8) were determined. Both sequences shared the most nucleotide sequence identity (100 and 99.7% over 251 and 360 bp, respectively, spanning nucleotides 1 to 256 and 1 to 365 of aligned

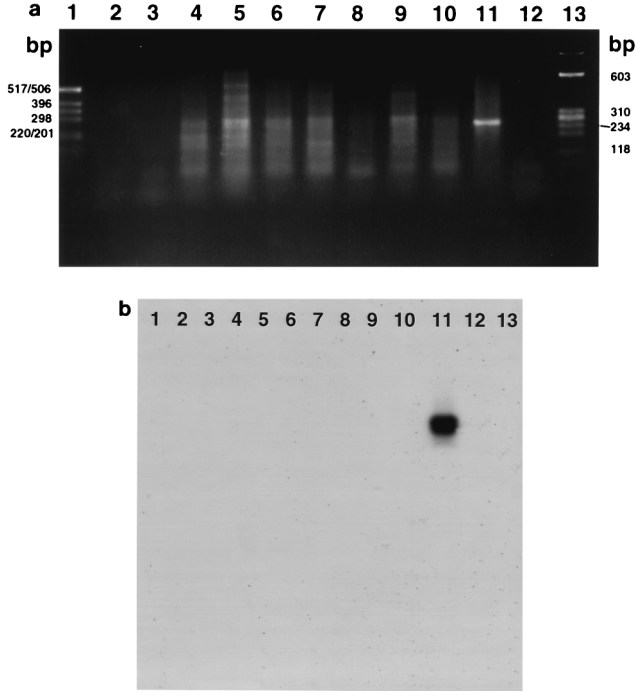

FIG. 3. (a) *M. avium*-specific PCR amplification of NZ cat leprosy specimens. Products were visualized on a 2% (wt/vol) agarose gel by ethidium bromide staining. The lanes contain a 1-kb DNA ladder size standard (Gibco BRL; lane 1), PCR mixture negative control (SDW) (lane 2), PCR sample negative control (SDW; lane 3), cat leprosy specimens 1 to 7 (lanes 4 to 10, respectively), PCR positive control (*M. avium* NCTC 8559 purified DNA; lane 11), PCR negative control (SDW; lane 12), fragments of ϕX174 (replicative form) digested with *Hae*III (included as size standards; lane 13). (b) Southern blot of *M. avium*-specific PCR products (the lanes are the same as those described for panel a) probed with a fluorescein-dUTP 3′-end-labelled *M. avium*-specific oligonucleotide contained within the 252-bp amplicon.

mycobacterial 16S rRNA sequences, respectively) with the sequence of *M. chitae* (mismatches due to the presence of unidentified nucleotides (N) are not considered). Two clones each of the BACTEC culture and the QIAamp Tissue Kit-extracted PCR products of specimen 8 were also sequenced. The sequences of none of the clones were identical to each other. Nucleotide sequence identities ranged from 99.2 to 99.6% over 520 and 521 bp, but all four clones shared the most nucleotide sequence identity (99.6, 99.6, 99.8, and 99.8%) with *M. avium* and *M. paratuberculosis* sequences (17) (mismatches due to the presence of "N" nucleotides are not considered) over 521, 521, 520, and 521 bp, respectively, spanning nucleotides 1 to 552 and 553 of aligned mycobacterial 16S rRNA sequences and encompassing both the V2 and V3 variable regions.

DISCUSSION

There was no evidence of *M. bovis* infection in the seven NZ cat leprosy specimens by either culture or an *M. tuberculosis* complex-specific PCR. These analyses were performed with the NZ specimens since bovine tuberculosis caused by *M. bovis* infection is endemic in some areas of New Zealand and *M. bovis* has been isolated from New Zealand cats with skin lesions which had some histopathological features in common with cat leprosy (5).

M. avium- and *M. paratuberculosis*-specific PCR amplifications were also performed with all eight cat leprosy specimens since sequences with 100% nucleotide sequence identity with the sequence of the V2 region of the 16S rRNA genes of *M. lepraemurium*, *M. avium*, and *M. paratuberculosis* were detected in some of the specimens. The species-specific PCR amplifications enabled the possibility of *M. avium* or *M. paratuberculosis* infection to be discounted for all specimens except the NI cat leprosy specimen (specimen 8). Sequence analysis of the V3 region enabled differentiation of *M. lepraemurium* from *M. paratuberculosis* and *M. avium*; *M. lepraemurium* differs from the other two species at *E. coli* 16S rRNA gene nucleotide position 443 (17). Generally, the V3 region is less discriminatory than the V2 region for the identification of mycobacterial species (25). Some strains of *M. avium* are distinguishable from *M. paratuberculosis* in the V2 region; this is not the case for the V3 region, but the identity of one 16S rRNA sequence isolated from the NI cat leprosy specimen could only be ascribed to *M. avium* and *M. paratuberculosis*, and an *M. avium*-specific PCR was essential for definitive identification. The NI cat leprosy specimen contained vast numbers of acid-fast bacilli, which may also characterize *M. avium* infections (9, 24). It was the only specimen from which acid-fast bacilli were cultured easily by conventional methods, which suggested prior to molecular analyses that the observed acid-fast bacilli were not *M. lepraemurium*. Although *M. chitae* was also identified in the same specimen, *M. avium* was believed to be responsible for the lesion since *M. chitae* is regarded as a nonpathogenic mycobacterial species which is found in the environment (1). The diagnosis was of interest since felines generally are resistant to *M. avium* complex infections (15) and *M. avium* infections of felines are rarely reported (6).

Molecular analyses enabled the definitive identification of *M. lepraemurium* in four of the eight specimens (specimens 3, 4, 5, and 7) from cats which had been presumptively identified as having cat leprosy. A presumptive diagnosis was made on the basis of histopathological examination and clinical observations, which are the normal criteria for the diagnosis of this disease due to difficulties associated with culturing the causative agent (20, 38). Bacterial isolates were not recovered from the seven NZ cat leprosy specimens by culture, which supported conventional diagnosis of these infections as cat leprosy. However, failure to culture acid-fast bacilli does not imply only *M. lepraemurium* infection in such cases, since it is likely that other mycobacterial species which are difficult to culture may cause skin lesions in cats. The molecular procedures used therefore proved to be of great value in allowing for the definitive and rapid identification of *M. lepraemurium*, the purported causative agent of cat leprosy, in the cats from which specimens 3, 4, 5, and 7 were obtained and identifying other mycobacterial species in cats from which specimens 1, 2, and 8 were obtained.

Numerous acid-fast bacilli in macrophages have been associated with cat leprosy (35). In this study, *M. lepraemurium* was detected in four specimens which varied in their relative abundance of acid-fast bacilli from ZN staining scores of 2+ to 4+. The apparent paucity of acid-fast bacilli in two of the specimens may be explained by observations that such microorganisms may be numerous in cats with the lepromatous form of the disease but not in cats with the tuberculous form or in the associated lymph nodes (10). Whether the specimens from cats with cat leprosy analyzed could be categorized as tuberculous or lepromatous, however, is unknown. Brown and colleagues (3) also found very few bacteria in three nontuberculous granulomas in cats in their first report of cat leprosy in New Zealand.

A sequence most closely resembling that of *M. malmoense* was detected in two of the NZ cat leprosy specimens (speci-

TABLE 2. V2 and V3 16S rRNA sequence data for specimens from cats putatively with cat leprosy

Cat	V2 region				V3 region			
	Species identity[a]	% Nucleotide identity	No. of nucleotides	16S rRNA nucleotide positions[b]	Species identity[a]	% Nucleotide identity	No. of nucleotides	16S rRNA nucleotide positions[b]
1	*M. malmoense*, *M. interjectum*	97.2	142	126–276 (E)	*M. szulgai*, *M. avium*, *M. intracellulare*, *M. paratuberculosis*, *M. malmoense*	99.1	223	332–557 (M)
2	*M. malmoense*, *M. interjectum*	97.2	142	126–276 (E)	*M. szulgai*, *M. avium*, *M. intracellulare*, *M. paratuberculosis*, *M. malmoense*	99.1	223	332–557 (M)
3	*M. lepraemurium*, *M. paratuberculosis*, *M. avium*	100	137	123–268 (E)	*M. lepraemurium*	100	242	313–557 (M)
4	*M. lepraemurium*, *M. paratuberculosis*, *M. avium*	100	122	54–178 (M)				
5	*M. lepraemurium, M. paratuberculosis,*	99.3	142	123–273 (E)				
	M. avium and A. ilicis	98.3	121	65–190 (M)				
6	*A. pascens*, *A. ramosus*	91.5	223	2–230 (M)				
7	*A. pascens*, *A. ramosus*;	100	118	60–1799 (M)				
	M. lepraemurium, *M. paratuberculosis*,	99.3	145	123–276 (E)				
	M. avium; and *M. luteus*	89.4	198	1–203 (M)				
8[c]	*M. chitae* and *M. avium*,	100	251	1–256 (M)				
	M. paratuberculosis	99.8	521	1–553 (M)				

[a] The species that the organism most resembles.
[b] E, *E. coli* positions; M, mycobacterial positions.
[c] The V2 and V3 regions were considered together.

mens 1 and 2) with numerous acid-fast bacilli. The sequence does not have 100% nucleotide identity with any of the sequences in the EMBL or GenBank database, but its high degree of nucleotide sequence identity with the *M. malmoense* sequence suggests strongly that it is a mycobacterial species. Furthermore, the presence of a long helix 18 in the V3 region indicates that it is a slow grower (30). A previous 16S rRNA gene sequence analysis of veterinary specimens of bovine origin identified a group of four isolates which could not be typed definitively (12). It was suggested that these isolates may rep-

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GGG TAA TCT GCC CTG CAC TTC GGG ATA AGC CTG GGA AAC TGG GTC TAA TAC CGA   M. malmoense
... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...   Specimens 1 & 2

GAG TAA CCT GCC CTT AAC TCT GGG ATA AGC CTG GGA AAC TGG GTC TAA TAC CGG   A. pascens
... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...   Specimen 5
... ... ... ... ... ... ... ... ... ... ..T ... ... G.. ... ... ... T..   Specimens 6 & 7

GAG TAA CCT GCC CTT AAC TCT GGG ATA AGC CTG GGA AAC TGG GTC TAA TAC CGG   M. luteus
... ... ... ... ... ... ... ... ... ... ..T ... ... G.. ... ... ... T..   Specimen 7


ATA GG-ACCC CGA GGC GCA TGC CTT GGG GTG GAA AGC TTT TGC GGT GTG GGA TGG   M. malmoense
... ..-...T TA. ... ... ... ... .T. ... ... ... ... ... ... ... ... ...   Specimens 1 & 2

ATA TGA CTC CTC ATC GCA TGG TGG GGG GTG GAA AGC TTT TGT GGT TTT GGA TGG   A. pascens
... ... .CA TCT GG. ... ..TCAT. .T. ... ... ... ... ... ... ..* *** ***   Specimen 5
...T... .AT .AT CC. ... ... G.T ..T ..T ... ..A ... AT. ... ... ... ...   Specimens 6 & 7

ATA GGA GCG TCC ACC GCA TGG TGG GTG TTG GAA AGA TTT ATC GGT TTT GGA TGG   M. luteus
...TT.. CAT .GT C.. ... ... G.T .GT G.T ... ... ... ..T ... ... ... ...   Specimen 7


GCC CGC GGC CTA TCA GCT TGT TGG TGG GGT GAC G                             M. malmoense
... ... ... ... ... ... ... ... ... ... ... . .                         Specimens 1 & 2

ACT CGC GGC CTA TCA GCT TGT TGG TGA GGT AAT G                             A. pascens
*** *** *** *** *** *** *** *** *** *** *** *                           Specimen 5
... ... ... ... ... ... ... ... ... ... ... . .                         Specimen 6 & 7

ACT CGC GGC CTA TCA GCT TGT TGG TGA GGT AAT G                             M. luteus
... ... ... ... ... ... ... ... ... ... ... . .                         Specimen 7
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FIG. 4. Alignment of 16S rRNA sequences of unclassified isolates from specimens 1, 2, 5, 6, and 7 with database sequences for *M. malmoense* (EMBL accession no. X52930), *A. pascens* (EMBL accession no. X80740), and *M. luteus* (EMBL accession no. M38242). The nucleotides of each unclassified isolate different from the nucleotides in the related database sequence are indicated. The first nucleotide corresponds to *E. coli* position 126. Symbols: periods, no change; −, deletion; *, nucleotide sequence not determined.

resent a novel mycobacterial species. This was supported by the subsequent identification of a new species of slowly growing mycobacteria, *M. lentiflavum*, in human clinical specimens (31). The sequences of three of the four isolates from bovine specimens share 100% nucleotide sequence identity with the sequence of the type strain of *M. lentiflavum* (ATCC 51985) over the V2 region and, similar to *M. lentiflavum*, grow at 22 and 37°C as yellow smooth colonies. Consequently, it is suggested that the *M. malmoense*-related sequence, detected in the two specimens from cats with cat leprosy, may represent a novel mycobacterial species which is difficult to culture by conventional methodologies and which is capable of causing skin lesions in cats.

More than one bacterial isolate was detected in three of the eight specimens from cats with cat leprosy by 16S rRNA gene analysis. This finding was not unexpected because a range of different bacterial species are normally found in the outermost layers of the skin. In addition, the skin is likely to be contaminated with environmental bacteria. In the specimens containing larger numbers of acid-fast bacilli, the mycobacterial species probably were the predominant species, so that the presence of other bacterial genera remained undetected. In addition, the 16S rRNA primers used are not strictly universal and would not amplify all bacterial species. Specimen 6, which contained very low numbers of acid-fast bacilli, was the only specimen in which mycobacteria were not identified by molecular biology-based analyses; this probably reflects the difficulty of extracting DNA from mycobacterial species. The sequence of one of the nonmycobacterial species detected in specimen 6 had a high degree of nucleotide sequence identity with the sequence of *A. ilicis*, and it is postulated that the isolate probably represented an arthrobacter species. It is possible that this bacterium was a skin contaminant because the principal natural habitat of this genus is soil (11).

Because more than one bacterial species may have been present in the tissue specimens, the 16S rRNA PCR product obtained for each cat leprosy specimen was cloned, and individual clones containing the insert of the appropriate size were sequenced. Nucleotide mismatches between clones were found in regions conserved between mycobacterial species. Hence, clones of cat leprosy specimens 5 and 7 shared 99.3% and not 100% nucleotide sequence identity with the sequence of *M. lepraemurium* over 142 and 145 bp, respectively. Similarly, two 16S rRNA clones from the NI cat leprosy specimen were identified as *M. chitae*, but whereas one shared 100% nucleotide sequence identity over 251 bp with the sequence of the 16S rRNA gene of *M. chitae*, the other shared only 99.7% nucleotide sequence identity over 360 bp. These erroneous nucleotides were attributed to nucleotide misincorporation during PCR amplification resulting from *Taq* infidelity (29), since sequencing data were consistent when different enzymes and different primers were used.

A variety of different nucleic acid extraction procedures that allowed for the successful amplification of the 16S rRNA gene were also investigated in this study. The one-step heat extraction method performed significantly better than the methods described by Liebana and colleagues (19) and Wards and coworkers (37). This might be due partly to the loss of specific DNA incurred in tube changes involved in the last two methods (27). The method with the QIAamp Tissue Kit also performed significantly better than the method with Chelex extraction for the single specimen tested. The bacterial sequence identified in specimen 6, containing smaller numbers of acid-fast bacteria, may not have represented the causative agent, although decontamination of the *Taq* DNA polymerase prior to PCR amplification ensured that the sequence identified was present in the tissue. *M. lepraemurium* may have been present in this specimen, but it was not detected due to the limited sensitivity of extraction.

Further improvements in the sensitivity of extraction procedures and the use of a high-fidelity *Taq* DNA polymerase should alleviate the problems of the 16S rRNA method described here. These minor disadvantages are outweighed, however, by the ability of this method to allow for the definitive and rapid identification of *M. lepraemurium* directly from tissue specimens without a requirement for culture. The study has also highlighted the potential for the 16S rRNA typing method to reduce the incidence of misdiagnosis of feline leprosy; three of eight of the specimens tested were found to contain mycobacterial species other than *M. lepraemurium*. The definitive and rapid diagnosis of infections caused by particular mycobacterial species facilitated by the procedures described here should increase the potential for successful drug therapy and quickly identify possible zoonotic risks.

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