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Identification of *Mycobacterium kansasii* by DNA Hybridization

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A DNA probe specific for *Mycobacterium kansasii* was obtained from a plasmid clone library of *Eco*RI-digested genomic DNA. The probe specifically identified culture-confirmed isolates of *M. kansasii* and isolates in cultures of environmental water samples. In an attempt to distinguish between isolates of *M. kansasii*, we used two methods to demonstrate restriction fragment length polymorphisms in the genomic DNA. Both of these methods failed to detect any differences between the isolates. These isolates included the type strain TMC 1201, environmental isolates, and clinical isolates from Australia and the Solomon Islands. This result suggests that the genome of *M. kansasii* is highly conserved and that genetic divergence within this species in insignificant.

Mycobacterium kansasii is a photochromogenic acid-fast bacillus which has been identified as an agent of disease in nearly all parts of the world (16). It characteristically produces a chronic lung infection that closely resembles pulmonary tuberculosis, but with milder symptoms, and rarely infects healthy individuals (16). Infections with *M. kansasii* are more frequent in individuals with an underlying condition such as chronic obstructive pulmonary disease (1, 7) and occur at an increased frequency in certain high-risk groups, such as workers with pneumoconioses (3, 8). Disseminated disease has also been observed in association with AIDS (12, 13). Currently, the identification of *M. kansasii* relies upon a variety of biochemical tests culminating in the ability of the bacterium to produce pigment when it is exposed to light. Because a number of mycobacterial species exhibit a biochemical profile similar to that of *M. kansasii*, photochromogenicity is relied upon as a final identification, and the interpretation of this test can be unreliable. DNA hybridization techniques offer the potential to provide a more specific identification of *M. kansasii* and enable detailed epidemiological studies by demonstrating DNA restriction fragment length polymorphisms (RFLPs). Recently, we identified a cluster of infections in a small coastal country town, Portland, Victoria, Australia, which is situated 200 miles (322 km) southwest of Melbourne. There have been only 13 cases of *M. kansasii* infections in the state of Victoria since 1984, and 6 of these occurred in Portland. We aimed to apply DNA techniques to *M. kansasii* to assist in the investigation of this increased incidence, and this report describes the production of a DNA probe specific for *M. kansasii* and the application of RFLP analysis to the study this outbreak of *M. kansasii* infections.

MATERIALS AND METHODS

Isolation and identification of *M. kansasii*. Mycobacteria were cultured from centrifuged pellets of 1-liter environmental water samples collected from various sites in Portland and from clinical specimens, including sputum, bronchial washings, and tissue biopsies. Samples were inoculated into BACTEC 12B culture vials (Becton Dickinson, Sydney, Australia) and incubated at 35°C for up to 6 weeks. Vials showing a rising growth index were examined for acid-fast bacilli by Ziehl-Neillson staining and were subcultured onto Lowenstein-Jensen medium. Acid-fast organisms growing on Lowenstein-Jensen medium were tested for temperature preference, nitrate-reducing capacity, urease production, aryl sulfatase activity, tellurite-reducing ability, Tween hydrolytic capacity, and photochromogenicity.

Extraction of mycobacterial DNA. Cultures of mycobacterial prototype strains *M. kansasii* TMC 1201, *M. gastri*, *M. marinum*, *M. phlei*, *M. flavescens*, *M. intracellulare*, *M. africanum*, *M. asiaticum*, *M. avium*, *M. malmoense*, *M. scrofulaceum*, *M. simiae* ATCC 25275, *M. szulgai* NCTC 10831, and all clinical isolates of *M. kansasii* were inoculated into 50 ml of Middlebrook 7H9 medium and incubated at 37°C with constant shaking until the midlogarithmic phase of growth was reached. To render the bacteria susceptible to lysis, the amino acid analog D-cycloserine was added to a final concentration of 1 mg/ml and the cultures were incubated for 48 to 72 h at 37°C. The bacteria were then pelleted and washed once with STE (10 mM NaCl, 10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA). The pellets were resuspended in 5 ml of lysis solution containing 15% sucrose, 50 mM Tris hydrochloride (pH 8.5), 50 mM EDTA, and 1 mg of lysozyme per ml. After incubation for 30 min at 37°C, sodium dodecyl sulfate (SDS) was added to a final concentration of 4%, proteinase K was added to 100 μg/ml, and the total volume was increased to 10 ml. The solution was then incubated for 30 min at 37°C and for 5 min at 70°C. Lysates were then extracted with an equal volume of phenol-chloroform, and the DNA was precipitated with ethanol. DNA pellets were resuspended in 0.4 ml of TE (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA) and extracted once with phenol-chloroform followed by an extraction with chloroform, and the DNA was stored as a precipitate in ethanol at −20°C until use.

Cloning of *M. kansasii* DNA. A clone library of the *M. kansasii* genome was established in plasmid pUC13 in order to isolate a specific fragment for use as a species-specific DNA probe. Briefly, DNA was extracted from *M. kansasii* TMC 1201, digested with *Eco*RI, and size fractionated through Sepharose CL-4B (Pharmacia, Uppsala, Sweden). Column fractions containing fragments of 2 kb or greater were pooled and ligated into *Eco*RI-digested alkaline phosphatase-treated pUC13. The ligated DNA was transformed

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into *E. coli* by electroporation, and transformants were selected on nutrient agar containing 40 μg of ampicillin per ml followed by subculturing onto nutrient agar containing 40 μg of ampicillin per ml, 2 mM isopropyl-β-D-thiogalactopyranoside, and 0.04% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Recombinant plasmids were extracted from *Escherichia coli* by alkaline lysis by using a Miniprep Kit Plus (Pharmacia) according to the instructions of the manufacturer.

DNA probe synthesis. All DNA probes used in this study were labeled with digoxigenin by using the nonradioactive DNA labeling and detection kit of Boehringer (Mannheim, Germany) according to the instructions of the manufacturer.

Dot blot hybridization. Before dot blot hybridization, DNA samples were quantified spectrophotometrically and the concentrations were adjusted to ensure that equal amounts of each DNA sample were applied to the blot. Samples of 100 pg of DNA in 40 μl of TE were denatured by boiling them for 5 min followed by chilling on ice. After the addition of 40 μl of 20 × SSC (3 M NaCl, 0.3 M sodium citrate), samples were filtered through a nitrocellulose membrane (Hybond C-extra; Amersham, Buckinghamshire, United Kingdom) by using a dot blot filtration manifold. DNA was fixed to the nitrocellulose by baking it for 2 h at 80°C, and the filter was prehybridized for 2 h at 42°C in hybridization solution containing 50% formamide, 0.1% *N*-lauroylsarcosine, 0.02% SDS, 200 μg of herring sperm DNA per ml, and 5% blocking reagent (Boehringer). The heat-denatured DNA probe in hybridization solution was then added and the filter was incubated overnight. After hybridization, the filter was washed twice for 5 min at room temperature with 2 × SSC–0.1% SDS and twice for 30 min at the hybridization temperature with 0.1 × SSC–0.1% SDS. The presence of the digoxigenin-labeled DNA probe was detected with alkaline phosphatase-conjugated antibody as described in the nonradioactive detection kit (Boehringer).

Southern blot hybridization. Samples containing approximately 1 μg of mycobacterial DNA were digested with 10 U of each restriction enzyme at 37°C for 5 h under the conditions specified by the manufacturers. DNA fragments were separated by electrophoresis through 0.7% agarose gels at 60 V for 2 h (10-cm gel) or 35 V for 16 h (20-cm gel) in 40 mM Tris acetate–2 mM EDTA. The DNA was denatured by soaking the gels for 1 h in 0.5 M NaOH–1.5 M NaCl and was neutralized for 1 h in 1 M Tris base–1.5 M NaCl. Fragments were transferred to nylon filters (Gene Screen Plus; Dupont, Boston, Mass.) by using a vacuum transfer apparatus (Hybaid, London, United Kingdom) at 40-cm H_2O vacuum for 1 h with 20 × SSC. DNA was fixed to the nylon membrane by overlaying the membrane onto filter paper soaked with 0.4 M NaOH for 1 min and was neutralized on 0.2 M Tris (pH 7.5)–2 × SSC for 5 min. Prehybridization and hybridization were performed as described above for dot blot hybridization, except that the concentration of SDS in the hybridization solution was increased to 1%. Nonradioactive detection was done as described by the manufacturer (Boerhringer), except that 1% casein was used in the blocking step and as a conjugate diluent.

RESULTS

Isolation of an *M. kansasii*-specific probe. The genome of *M. kansasii* shares the greatest degree of DNA homology with *M. gastri*, exhibiting an estimated 62% relatedness (6). Consequently, we screened the library of cloned fragments from *M. kansasii* with whole chromosomal DNA probes of *M. kansasii* and *M. gastri* to identify a species-specific DNA fragment. Of 54 clones screened by this method, 6 showed a stronger hybridization signal with the *M. kansasii* probe, while they exhibited a weaker hybridization with the *M. gastri* probe (Fig. 1). These six plasmids were isolated, purified, labeled, and used as probes in hybridization experiments with DNA from various mycobacteria (data not shown). This analysis revealed that one clone, pMK1-9 (indicated in Fig. 1), showed a high degree of specificity for *M. kansasii* and was used in all subsequent dot blot hybridizations. The plasmid contained a single *Eco*RI fragment of 2.2 kb. The specificity of pMK1-9 in comparison with that of a whole chromosomal probe under different stringency conditions for various mycobacterial species and clinical *M. kansasii* isolates is shown in Fig. 2. Probing with a whole chromosomal probe (Fig. 2A) resulted in a large amount of cross-reaction with other mycobacteria, particularly *M. africanum*, *M. gastri*, *M. intracellulare*, and *M. marinum*, even when conditions of high stringency were used. In comparison, when clone pMK1-9 was used as a probe (Fig. 2B), only a small amount of cross-hybridization between these species was seen, although temperatures of 60 to 65°C for hybridization and washing were required to counteract the low-stringency cross-reaction with *M. marinum*.

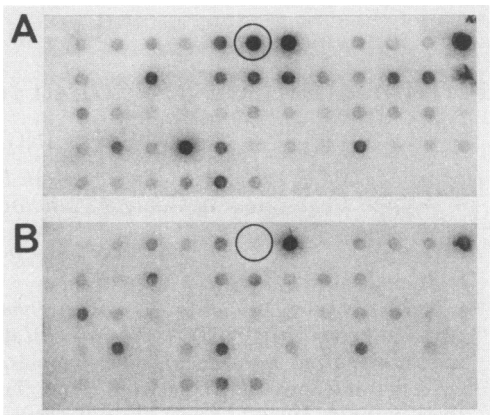

FIG. 1. Dot blot hybridization of 54 plasmid clones from the genomic library of *M. kansasii*. Duplicate filters were hybridized with whole chromosomal probes of *M. kansasii* (A) and *M. gastri* (B). The most specific clone selected from the library, pMK1-9, is circled.

Identification of *M. kansasii* by dot blot hybridization. As part of our investigation into an increase in the incidence of *M. kansasii* infections in Portland, water samples were collected from a variety of sources and were cultured for mycobacteria. DNA was extracted from seven cultures which grew mycobacteria and were dot blotted together with DNA from 13 clinical isolates of *M. kansasii*. The result of probing this filter with plasmid pMK1-9 is shown in Fig. 3. Of the seven cultured water samples, two appeared to be strongly positive, with another two samples showing a small amount of reaction. The identities of the two cultures positive by hybridization agreed with the standard laboratory identification, except that the bacteria isolated from water were nitrate reduction negative, whereas all clinical isolates were nitrate reduction positive. The two weakly reacting isolates from water were identified as *M. phlei*. This result is consistent with the dot blot hybridization shown in Fig. 2B, in which there was a weak signal with *M. phlei* (sample 9a) at both 42 and 65°C. Of the 13 clinical isolates, all

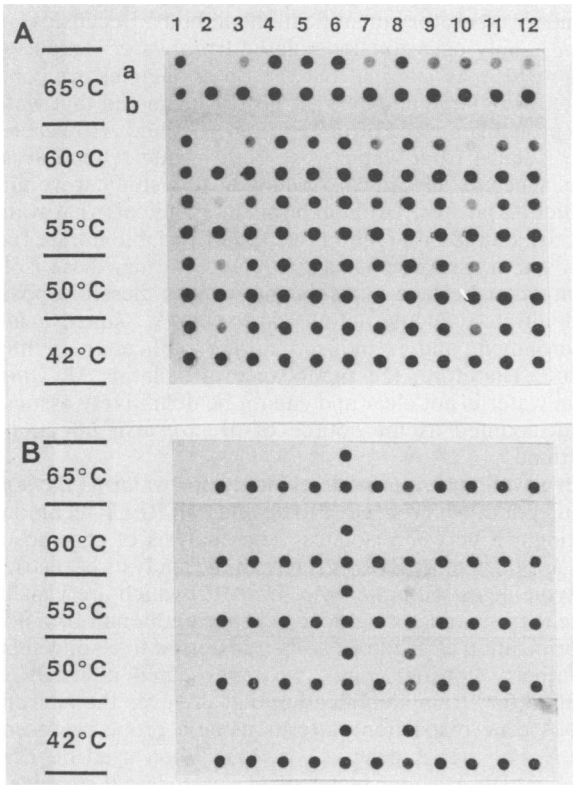

FIG. 2. Comparison of the whole chromosomal probe (A) and the cloned pMK1-9 probe (B) for various mycobacterial DNAs under various stringency conditions. The temperatures shown were used for both hybridization and washing. (Rows a) Lanes: 1, *M. africanum*; 2, *M. asiaticum*; 3, *M. avium*; 4, *M. gastri*; 5, *M. intracellulare*; 6, *M. kansasii*; 7, *M. malmoense*; 8, *M. marinum*; 9, *M. phlei*; 10, *M. scrofulaceum*; 11, *M. simiae*; 12, *M. szulgai*. (Rows b) Lanes: 1, *M. bovis* BCG; 2 to 12, *M. kansasii* isolates from clinical specimens.

except one sample appeared to be positive by DNA hybridization; one sample was clearly negative (Fig. 3, sample 16). This isolate was identified from a clinical specimen in 1984 and was identified before a review of the methodology for photochromogenicity testing. On repeat of the laboratory identification, this isolate appeared to be scotochromogenic, suggesting that it was *M. flavescens*.

Southern blot hybridization analysis of *M. kansasii* isolates. In an attempt to distinguish between isolates of *M. kansasii* and thereby provide evidence to link the isolates from water

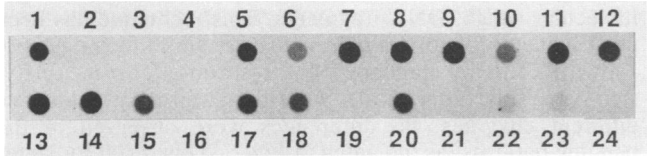

FIG. 3. Dot blot hybridization of DNA from clinical isolates of *M. kansasii* and mycobacteria cultured from water samples by using clone pMK1-9 as the probe. Samples added as controls were as follows: 1, *M. kansasii* (sample 1), *M. gastri* (sample 2), *M. marinum* (sample 3), and *M. flavescens* (sample 4). Clinical isolates of *M. kansasii* were derived from cases in Portland (samples 5 to 10), other isolates from Victoria (samples 11 to 16), and isolates from the Solomon Islands (sample 17). Water samples (samples 18 to 24) were obtained from the Portland area.

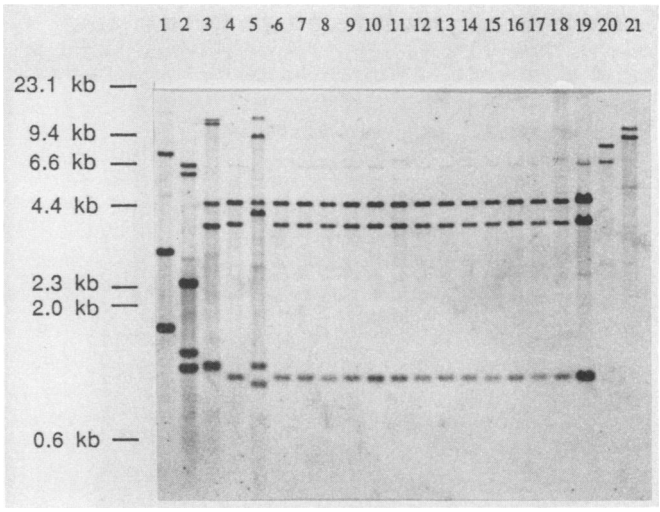

FIG. 4. Southern blot analysis of *Eco*RI-digested mycobacterial DNA with a probe for the rRNA genes. Lanes: 1, *M. intracellulare*; 2, *M. phlei*; 3, *M. flavescens*; 4, *M. kansasii*; 5, clinical isolate negative with probe pMK1-9; 12 to 17, *M. kansasii* isolates from Portland; 6 to 10, *M. kansasii* isolates from Victoria; 11, *M. kansasii* isolate from the Solomon Islands; 18 and 19, *M. kansasii* isolates from water samples; 20 and 21, weakly positive samples with probe pMK1-9. The migration of DNA molecular mass standards (in kilobases) are indicated.

with clinical disease in Portland, we used two methods of DNA RFLP analysis. First, we investigated the rRNA gene restriction patterns, which have been used previously to distinguish between isolates of a variety of bacterial genera (2, 5, 15). Briefly, this procedure involves digestion of chromosomal DNA with restriction enzymes and Southern blot hybridization by using plasmid pKK3535 as the probe. This plasmid contains the rRNA genes of *E. coli* representing 5S RNA, 16S RNA, 23S RNA, and $tRNA_2^{Glu}$ (4). The results of this analysis on the clinical isolates and water samples are shown in Fig. 4. The patterns obtained for *M. kansasii* isolates that were positive by hybridization were identical (Fig. 4, lanes 6 to 19). In addition, the results provide further evidence that the isolate initially considered to be *M. kansasii* that was negative with the pMK1-9 probe is not *M. kansasii*, since the restriction pattern of its RNA genes was different from those of all other isolates (Fig. 4, lane 5).

Second, we applied a more discriminative technique that has been used previously to distinguish between isolates of *M. tuberculosis* (10, 14). This technique involves digestion of the chromosomal DNA with restriction enzymes that recognize 4-base sequences and Southern blotting by using a whole chromosomal probe to detect high-molecular-weight DNA fragments. The result of digesting DNA from *M. kansasii* isolates with the enzyme *Hin*fI and Southern blotting is shown in Fig. 5. No differences between the *M. kansasii* isolates were seen. Similar results were obtained after digestion with the enzymes *Alu*I, *Nde*II, *Rsa*I, and *Dde*I (data not shown).

DISCUSSION

The standard laboratory identification of *M. kansasii* involves a variety of biochemical tests, culminating in a test for the ability of the bacteria to produce pigmentation in the absence of light. Although these tests are adequate for most

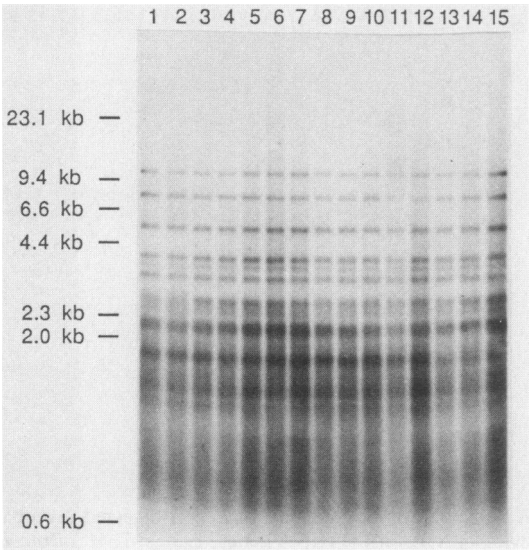

FIG. 5. Southern blot analysis of *M. kansasii* isolates after digestion of chromosomal DNA with restriction enzyme *Hin*fI by using a whole chromosomal probe. Lanes: 1, *M. kansasii* TMC 1201; 2 to 6, *M. kansasii* isolates from Victoria; 7, an *M. kansasii* isolate from the Solomon Islands; 8 to 13, *M. kansasii* isolates from Portland; 14 and 15, *M. kansasii* isolates from water samples. The migration of DNA molecular mass standards (in kilobases) are indicated.

purposes, they require that a pure culture of the organism be obtained and cannot distinguish between strains isolated from geographically distinct regions. We decided to develop a specific DNA probe to identify *M. kansasii* in cultures of water samples and a method to demonstrate RFLPs in genomic DNA to facilitate our epidemiological investigation into an increased incidence of infection in Portland.

Our strategy for selecting an *M. kansasii*-specific DNA clone was based on probing a genomic clone library of *M. kansasii* with labeled homologous DNA and labeled genomic DNA from *M. gastri*. This mycobacterial species shares the greatest degree of genomic homology with *M. kansasii* (6). By comparing the results of these two hybridizations, several *M. kansasii*-specific DNA clones were identified, and the most specific clone, pMK1-9, was selected for further study. Although this probe appeared to hybridize to *M. marinum* under low-stringency conditions, it was specific at higher temperatures and was significantly more specific than a whole-chromosome probe (Fig. 2). Interestingly, when this probe was used on clinical isolates of *M. kansasii*, all but one were positive (Fig. 3). On first identification of the isolate that was negative by hybridization, it appeared to be typical after biochemical testing and pigmentation analysis. When the DNA hybridization result was known, this strain was subcultured and reidentified. Biochemically, the results were the same, but close examination of this culture after incubation revealed that it produced pigment in the presence or absence of light (scotochromogen), indicating that it was probably *M. flavescens*. This finding highlights the usefulness of having such a probe available when the identification of a bacterial strain is dependent upon the testing of a single phenotypic characteristic.

Analysis of seven cultures of water samples by DNA hybridization with the pMK1-9 probe revealed two positive cultures (Fig. 3, samples 18 and 20) which were confirmed by standard laboratory identification methods. Because *M. kansasii* is only occasionally isolated from water samples (11), this result may suggest that the water samples are contaminated with large numbers of this organism and that water is the probable source of infection in Portland. However, the two isolates from water were both nitrate reduction negative, whereas all clinical isolates in this study were nitrate reduction positive. Although this may indicate that water is not the source of infection, we found variable nitrate reduction activity, and on successive subculturing, these isolates from water became nitrate positive. It is therefore possible that nitrate-reducing activity is low in *M. kansasii* in the environment and is induced during replication in human tissue. Therefore, the significance of isolating *M. kansasii* from water is not clear and cannot be definitively associated or discounted as the source of *M. kansasii* infections in Portland.

In an attempt to provide evidence for water as the source of infection, we proceeded to develop an RFLP technique to distinguish between isolates. The analysis of chromosomal DNA by detecting RFLPs refers to the analysis of restriction enzyme digestion patterns of DNA (9) which are visualized by either staining of agarose gels with ethidium bromide or hybridization after the DNA is transferred to a solid support (Southern blotting). First, we investigated a widely used method for strain identification that involves the analysis of rRNA gene restriction patterns using a probe representing the *E. coli* rRNA genes (2, 5, 15). When used on *Eco*RI-digested DNA from isolates of *M. kansasii*, all samples that were positive with the pMK1-9 probe showed identical banding patterns (Fig. 4). This technique was thus unable to distinguish between isolates from geographically distinct regions, including Australian isolates of the prototype strain which originated in the United States. However, this technique does provide additional evidence to indicate that the isolate that was negative with probe pMK1-9 was not *M. kansasii*. Additionally, this technique could be used as a confirmatory test for weakly false-positive reactions after hybridization with the pMK1-9 probe. This was demonstrated with two water samples that showed weakly positive reactions (Fig. 3, samples 22 and 23) that were subsequently shown to have banding patterns vastly different from those of the *M. kansasii* isolates by ribotyping (Fig. 4, lanes 20 and 21).

As a second approach to distinguishing isolates by DNA fingerprinting, we used a technique that has been shown to be more discriminatory than standard restriction enzyme analysis (10, 14). This method involves the digestion of chromosomal DNA with restriction enzymes that recognize 4-base sequences, followed by electrophoresis and Southern blotting with a whole chromosomal probe to identify high-molecular-weight DNA fragments. This technique has been used successfully for *M. tuberculosis* strains which have not shown RFLPs by standard DNA restriction enzyme analysis. After analyzing all *M. kansasii* strains by using five different enzymes, no differences were observed in the banding patterns, as indicated in Fig. 5. This result indicates a high degree of sequence conservation with *M. kansasii* strains and suggests that genetic divergence within this species is insignificant. The extent of this conservation could be further addressed by nucleotide sequence analysis of selected regions within the genome, since restriction enzyme analysis detects changes only within restriction enzyme sites. It is clear that such an approach would have to be pursued to enable individual strains to be distinguished and

allow sources of *M. kansasii* infection to be identified conclusively.

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