

Pseudoepidemic Due to a Unique Strain of *Mycobacterium szulgai*: Genotypic, Phenotypic, and Epidemiological Analysis

Qing Zhang,¹ Randa Kennon,² Maureen A. Koza,^{3,4} Kristina Hulten,³ and Jill E. Clarridge III^{1,2,5*}

Department of Pathology,¹ Department of Molecular Virology and Microbiology,⁵ and Department of Medicine,³ Baylor College of Medicine, and Pathology and Laboratory Medicine Services² and Infection Control,⁴ Veterans Affairs Medical Center, Houston, Texas

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In mid-1999, we noted multiple isolations at the Veterans Affairs Medical Center (VAMC) Houston Tex. of an unusual nonpigmented *Mycobacterium* species. Since, on the basis of 16S rRNA gene sequence analysis, the strains were identical to the *Mycobacterium szulgai* type strain and since *M. szulgai* has been reported only rarely as a commensal or environmental isolate, we were concerned about laboratory contamination, nosocomial spread, or even the possibility that this could be a novel organism associated with disease. Our investigation found that from 1999 to 2000, 37 strains of *M. szulgai* were isolated from patients at the VAMC (the base rate for the previous 10 years had been <1 isolation per year). We compared the phenotypic properties and genetic relatedness of these 37 strains (31 of which were nonpigmented) as well as eight stock strains and the *M. szulgai* type strain. All strains were similar in cellular fatty acid patterns, growth rates, and biochemical characteristics. However, we found three genogroups by gene sequence analysis. Genogroup I comprised the *M. szulgai* type strain, all the tested nonpigmented strains (27 of the 31 strains were tested), two pigmented strains isolated in 1999 and 2000, and five pigmented stock strains. Genogroup II comprised five pigmented strains: three were isolated from 1999 to 2000 and two were stock strains. The single strain (isolated in 1996) in genogroup III was pigmented and was the only strain associated with disease. Whereas the randomly amplified polymorphic DNA (RAPD) patterns of all nonpigmented strains were identical, indicating that they came from a common source (the pseudoepidemic strain), the RAPD patterns of the other strains were varied. In our investigation for a possible source, we found that there were no common reagents, specimen-processing or patient locations, or procedures linking the 31 pseudoepidemic strains. However, a nonpigmented *M. szulgai* strain with a gene sequence and RAPD pattern identical to those of the pseudoepidemic strain was recovered from a water storage tank serving the hospital. We concluded that the strains most likely originated from hospital water, which transiently inoculated our patients. Although no disease was associated with this cluster of isolates, the event was costly because identification was problematic and we could not easily discount the isolations, since most of the patients were immunocompromised and were candidates for opportunistic infection.

Mycobacterium szulgai is a rarely isolated mycobacterial species that has been associated with pulmonary disease in immunocompromised patients (1, 2, 4, 7, 8). *M. szulgai* is said to be unique in that it is a scotochromogen (produces pigment in both light and darkness) at 37°C but a photochromogen (forms pigment only after exposure to light) at 25°C (10, 11). Its key characteristics are its slow growth and its positive test results for nitrate reduction and urease (10, 11).

Early in 1999, we noticed that a number of isolates from clinical specimens at the Veterans Affairs Medical Center (VAMC) in Houston, Texas, although identified as *M. szulgai* by both cellular fatty acid (CFA) analysis and 16S rRNA gene sequencing, were not typical in phenotypic characteristics because they were nonpigmented. Since these nonpigmented strains were all isolated from paucibacillary specimens (e.g., from BACTEC 12B broth medium only), we were concerned that the laboratory was introducing a contaminant during processing or that a hospital procedure was introducing the or-

ganism (9, 13, 14). We also considered the possibility that this was a novel organism associated with disease. Our genotypic, phenotypic, and epidemiologic investigations of these clinical strains as well as stock strains and one type strain are the subject of this report.

MATERIALS AND METHODS

Phenotypic and genotypic microbiological characterization of strains. A total of 47 strains were examined in this study (Tables 1 and 2): 38 strains were isolated during this outbreak, including 1 from a hospital water tank; 8 were stock strains, including 5 from the Houston Department of Health; and 1 was the *M. szulgai* type strain (ATCC 35799). The BACTEC 460 apparatus, Löwenstein-Jensen solid medium, and 12B bottles (Becton Dickinson Diagnostic Instrument Systems, Cockeysville, Md.) were used for the initial isolations.

After initial isolation, the strains were frozen at –70°C. For the growth characteristic study and biochemical tests, all isolates were subcultured on 7H11 medium, incubated at 37 and 25°C for 4 weeks, and checked for microbial growth daily for 7 days and every 3 days thereafter. After 2 weeks of incubation, urease and nitrate reduction tests were performed by standard methods (10). All pigmented strains were tested for photoactivation of pigment production at 25°C. All nonpigmented strains were exposed to light for 2 h and observed for pigmentation.

16S rRNA gene sequence analysis was performed. The nucleotide sequence of about 500 bp of the 16S rRNA gene was determined (MicroSeq 16S rRNA gene kit 500; PE Applied Biosystems, Foster City, Calif.). This sequence was compared to those of the 52 mycobacterial type strains in the MicroSeq database by

* Corresponding author. Mailing address: Pathology and Laboratory Medicine Services (113), VA Medical Center and Baylor College of Medicine, 2002 Holcombe Blvd., Houston, TX 77030. Phone: (713) 794-7336. Fax: (713) 794-7657. E-mail: jillc@bcm.tmc.edu.

TABLE 1. Characteristics of *M. szulgai* study isolates and associated patients

Patient no.	VAMC accession no.	Specimen source	Hospital unit(s)	Date of positive culture (mo/day/yr)	Risk factor(s) ^a	RAPD-PCR gel pattern ^b	16S rRNA geno-group cluster	Pigment
1	00TB0087	Sputum	3A	01/16/00	MSOF, CA	C	ND ^c	Buff
2	00TB0146	Sputum	4A	01/27/00	CA	C	I	Buff
3	00TB0180	Sputum	4A, 3C	02/02/00	CHF, PE	C	I	Buff
4	00TB0296	Sputum	4A	02/19/00	CA	C	I	Buff
5	00TB0479	Urine	4A	03/26/00	CML	C	I	Buff
6	00TB0524	Urine	3A	04/04/00	DM	C	I	Buff
7	00TB0525	Sputum	3A	04/04/00	RA	C	I	Buff
8	00TB0556	Sputum	3A	04/08/00	COPD	C	I	Buff
9	00TB0999-2	Sputum	3B	06/25/00	MAC, CLL	C	I	Buff
10	00TB1093	Bronchial wash	3B	07/10/00	SCCA	C	ND	Buff
11	00TB1375	Sputum	3A	08/25/00	DM, COPD	C	ND	Buff
12	00TB1423	Sputum	3B	09/02/00	None	C	I	Buff
13	00TB1448-2	Stool	3B	09/09/00	HIV	C	I	Buff
14	00TB1517	Urine	4A	09/18/00	MSOF	C	I	Buff
15	00TB1538	Bronchial wash	3C	09/20/00	CA	C	ND	Buff
16	00TB1571	Sputum	4A, 5K	09/24/00	CA	C	I	Buff
17	00TB1594	Bronchial wash	3B, 3D	09/27/00	SCI	C	I	Buff
18	00TB1744	Sputum	1A	10/25/00	SCI	C	I	Buff
19	00TB1796	Sputum	3A	11/06/00	None	C	I	Buff
20	98TB2561	Sputum	3A	12/25/98	COPD	C	I	Buff
21	99TB0239	Sputum	4D	02/05/99	Treated TB	C	I	Buff
22	99TB0446	Sputum	4A	03/04/99	COPD	C	I	Buff
23	99TB0950	Sputum	3B	05/28/99	ESRD	C	I	Buff
24	99TB1239-1	Sputum	4D	07/20/99	CVA	C	I	Buff
25	99TB1327	Sputum	3B	08/03/99	CA	C	I	Buff
26	99TB1390	Sputum	4A	08/12/99	HIV	C	I	Buff
27	99TB1544	Sputum	3A	09/19/99	CA	C	I	Buff
28	99TB1592	Sputum	3A	09/29/99	HIV	C	I	Buff
29	99TB1866	Sputum	4A	11/12/99	CA	C	ND	Buff
30	99TB1918	Sputum	4A	11/19/99	HIV	C	I	Buff
31	99TB2036-2	Sputum	3A	12/09/99	HIV	C	I	Buff
32	00TB0011	Sputum	4B	01/05/00	Myelofibrosis	A-3	I	Yellow
36	00TB1638	Sputum	Outpatient	10/04/00	COPD	B	I	Yellow
37	99TB1239-2	Sputum	4D	07/20/99	CVA	A-2	II	Yellow
35	00TB1577	Sputum	3B	09/25/00	TB	A-2	II	Yellow
34	00TB1448-1	Stool	3B	09/09/00	HIV	ND	II	Yellow
33	00TB0544	Sputum	Outpatient	04/06/00	HIV	ND	ND	Yellow
38	None	Hospital water (main tank)		12/12/00		C	I	Buff

^a Abbreviations: MSOF, multisystem organ failure; CA, carcinoma; CHF, congestive heart failure; PE, pleural effusion; CML, chronic myelogenous leukemia; DM, diabetes mellitus; RA, rheumatoid arthritis; COPD, chronic obstructive pulmonary disease; MAC, *Mycobacterium avium* complex; CLL, chronic lymphocytic leukemia; SCCA, squamous cell carcinoma; HIV, human immunodeficiency virus; SCI, spinal cord injury; TB, tuberculosis; ESRD, end-stage renal disease; CVA, cerebrovascular accident.

^b RAPD-PCR gel patterns refer to the results shown in Fig. 2 as follows: pigmented *M. szulgai* strain patterns A-2 (lanes 6 to 9), A-3 (lane 10), and B (lane 11); pseudoepidemic strain pattern C (lanes 12 to 20).

^c ND, not done.

MicroSeq analysis software (PE Applied Biosystems) and to the sequences in the GenBank database. Neighbor-joining analysis was performed (MicroSeq analysis software).

The CFA were analyzed by the Hewlett-Packard HP 5890 II microbial identification system (MIDI, Inc., Newark, Del.). We performed randomly amplified polymorphic DNA (RAPD) analysis. In brief, the isolates were suspended in 0.9% sterile saline to a turbidity that matched that of a 3.0 McFarland standard and were stored at -20°C. Before they were used, the isolates were thawed and boiled for 10 min. Several primers were tested for the arbitrary primed PCR, and a 10-mer (5'-TGGTTCGCGGC) was chosen for the analysis based on its higher yield in number of bands. Fifty-microliter reaction mixtures containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 3 mM MgCl₂, 60 pmol of primer, 2.5 U of *Taq* DNA polymerase (Promega, Madison, Wis.), and 1 μl of template were run in a thermocycler (MJ Research, Watertown, Mass.) with the following program: 94°C for 5 min; 40 cycles at 94°C for 30 s, 37°C for 1 min, and 72°C for 1 min; and 72°C for 10 min.

Epidemiological investigation. To determine the prevalence of isolates, all positive mycobacterial cultures obtained at the VAMC between 1995 and 2000 were reviewed. To determine if a particular person, laboratory procedure, or reagent was associated with the outbreak, the names of all personnel involved, the procedures employed, and the specimen type, receiving date, processing batch, and lot number for each reagent used were reviewed. The following reagents used in processing were cultured: phosphate buffer (BBL, Sparks, Md.), water (Baxter Healthcare Corporation, Deerfield, Ill.), *N*-acetyl-L-cysteine-NaOH (used for specimen digestion; Alpha-Tec Systems, Vancouver, Wash.), and the antimicrobial supplement PANTA (Becton Dickinson Diagnostic Instrument Systems). The BACTEC 460 apparatus, including the sampling needles, was inspected.

The hospital water system was also investigated. The hospital receives water from two tanks, both of which are filled with city water. The first tank is the main tank for the hospital water supply, while the second tank is a reservoir. Fifteen thousand gallons of water from the second tank flows into the main tank daily.

TABLE 2. Characteristics of stock strains of *M. szulgai*

Stock strain	Specimen source ^a	Hospital unit	Date of positive culture (mo/day/yr)	Risk factor ^b	RAPD-PCR gel pattern ^c	16S rRNA geno-group cluster	Pigment
97TB2917	Sputum	4B	12/23/97	DM	ND	I	Yellow
CH2	CHDH				A-1	I	Yellow
CH3	CHDH				A-1	I	Yellow
CH4	CHDH				A-1	I	Yellow
Type strain	ATCC 35799				A-4	I	Yellow
CH1	CHDH				ND	II	Yellow
CH5	CHDH				A-2	II	Yellow
96TB1204	Sputum	4A	5/9/96	None	Unique	III	Yellow
97TB1869	Sputum	4B	7/30/97	Lung cancer	ND	ND	Yellow

^a CHDH, city of Houston Department of Health.

^b DM, diabetes mellitus.

^c RAPD-PCR gel patterns refer to the results shown in Fig. 2, as follows: pigmented *M. szulgai* strain patterns A-1 (lanes 3 to 5), A-2 (lanes 6 to 9), and A-4, (lane 2).

^d ND, not done.

Four liters of water from each tank was collected. The water was concentrated by one of two methods. Either it was centrifuged for 30 min at 4,000 × *g* or it was filtered through a 0.45-μm-pore-size membrane. The pellet or the filter wash was obtained and decontaminated with 1.5% NaOH; the specimen was then inoculated on 7H11 plates (BBL) and BACTEC 12B vials (Becton Dickinson). Tap water and ice water from four patient wards were cultured similarly.

Patient records were reviewed for evidence of clinical diseases and therapeutic intervention. The nursing units involved, patient room numbers, admittance and discharge dates, and patient risk factors were recorded and reviewed.

RESULTS

Phenotypic and genotypic microbiological characterization.

Forty-seven strains (Tables 1 and 2) were studied for their phenotypic characteristics. Thirty-eight strains were isolated at the VAMC during this outbreak, including one from the hospital water tank, eight were stock strains, including five from the Houston Department of Health, and one was the *M. szulgai* type strain. All nonpigmented strains and seven of nine pigmented strains were isolated from BACTEC 12B broth only.

All strains showed certain similar characteristics and conformed to the description of *M. szulgai* (4, 10). All strains were slow growing. Both the pigmented and nonpigmented strains isolated from patient specimens required 3 to 4 weeks of culture to become positive. Ten days after subculture, pinpoint colonies (0.1 mm diameter) were seen. At 2, 3, and 4 weeks, the colony sizes were 0.2, 0.5 to 1, and 1 to 2 mm, respectively. Urease and nitrate reduction test results were positive. The CFA patterns of all tested strains (21 nonpigmented and 4 pigmented) were similar, with C_{16:0} (42%) and C_{18:1ω9c} (30%) predominating, but other fatty acids (3 to 6%) (C_{14:0}, C_{16:1ω7c}, and C_{16:1ω5c}) were also detected. All isolates were identified as *M. szulgai* by the CFA analysis software. All colonies were moist; however, for any one strain, some colonies were irregular while others were smooth and entire.

However, some strains were yellow at 37°C and were photochromogens at 25°C whereas others were nonpigmented (buff) at both 37 and 25°C (Table 1). The buff strains had no pigmentation after 2 h (not even when left for 2 days with exposure to light).

A total of 40 isolates were sequenced, including 32 VAMC outbreak isolates, seven stock strains, and the type strain. There were three genotypic clusters, as shown in the dendrogram (Fig. 1) and as listed in Tables 1 and 2. Cluster I included all of the 27 nonpigmented (pseudoeidemic) strains analyzed, including the isolate from the hospital water tank. Cluster II

included two pigmented strains isolated from 1999 to 2000, four stock strains, and the *M. szulgai* type strain. In cluster II, there were five pigmented strains, three of which were isolated from 1999 to 2000 and two of which were stock strains. Cluster

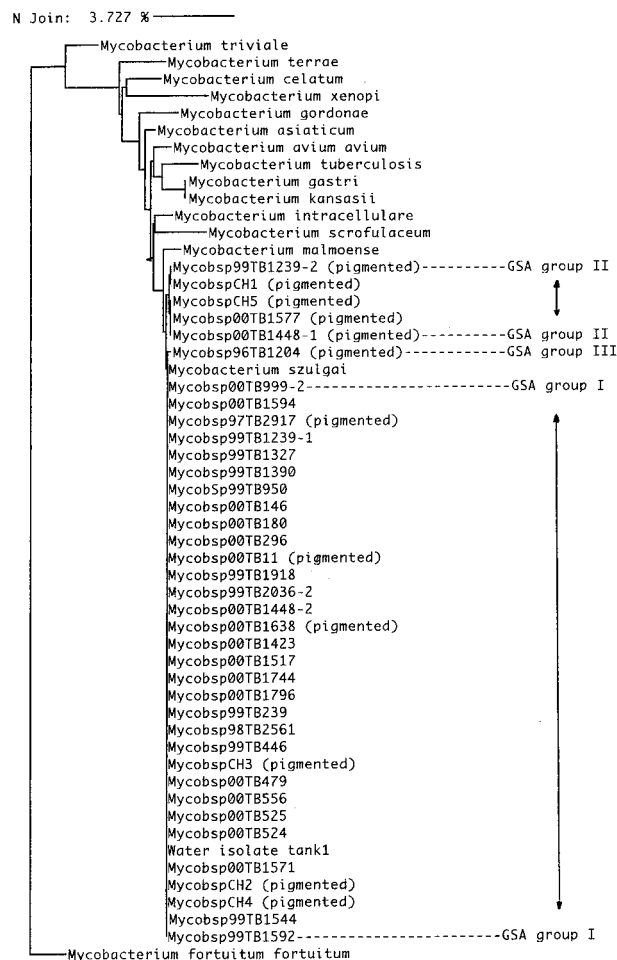


FIG. 1. Dendrogram of sequence data for 27 nonpigmented *M. szulgai*, 12 pigmented *M. szulgai*, and some mycobacterial type strains in the MicroSeq database, with *Mycobacterium fortuitum* subsp. *fortuitum* as an out-group. GSA, gene sequence analysis. The dendrogram was generated by the neighbor joining (N Join) method, with the horizontal line at the top representing a 3.727% genetic difference.

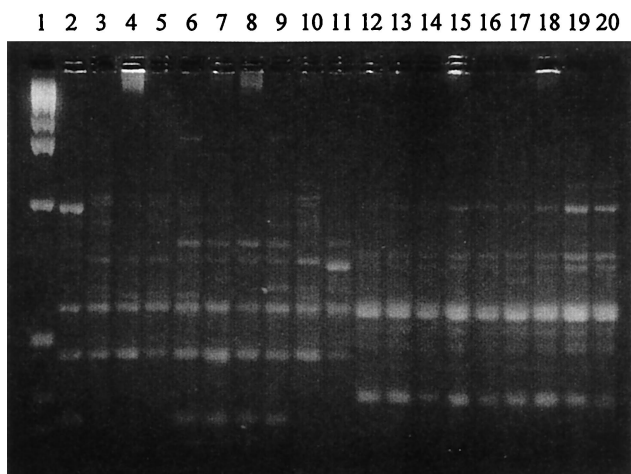


FIG. 2. RAPD-PCR patterns of nine nonpigmented *M. szulgai* strains, nine pigmented *M. szulgai* strains, and one ATCC 35799 strain. Lane 1, 1-kb DNA ladder (Life Technologies, Inc.); lane 2, strain ATCC 35799; lanes 3 to 5, 10, and 11, pigmented *M. szulgai* strains in 16S rRNA gene sequence analysis group I; lanes 6, 8, and 9, pigmented *M. szulgai* strains in 16S rRNA gene sequence analysis group II; lane 7, pigmented *M. szulgai* strain in 16S rRNA gene sequence analysis group III; lanes 12 to 20, nonpigmented *M. szulgai* strains.

II differed from cluster I by only 0.19%; the difference was a T-to-C base pair mutation at position 462. The single strain (isolated in 1996) in genogroup cluster III was pigmented and was the only strain associated with disease.

All the nonpigmented (pseudoe epidemic) strains tested, including the isolate from the water tank, had identical RAPD patterns (pattern C), which differed from those of the type strain and the other tested strains (Fig. 2). The RAPD patterns of the pigmented strains were varied although the strains that had the same 16S rRNA genetic clusters were more alike in their RAPD patterns.

Epidemiological investigation. From 1999 to 2000, three medical technologists alternately processed specimens at the VAMC. No technologist was specifically associated with the occurrence of the unusual nonpigmented (pseudoe epidemic) strains. Throughout the study period, (1995 to 2000), the procedures used were standard and had not changed (10). No organism was cultured from any reagents, and no specific lot number of any reagent correlated with the pseudoe epidemic isolates. The BACTEC instrument had been functioning properly, and BACTEC needles were changed at every use.

Of the 31 nonpigmented (pseudoe epidemic) patient isolates, 24 were obtained from sputum, three were from bronchial wash, three were from urine, and one was from stool (Table 1). There were 30 different collection and processing dates. Only two of the specimens were consecutive and were processed on the same day; however, one was a urine sample and the other was a sputum sample. We concluded that contamination due to laboratory error was unlikely.

From 1995 through 2000, approximately 2,697 specimens per year were processed for mycobacterial culture in the VAMC microbiology laboratory. Pigmented *M. szulgai* strains were isolated from 0 to 5 patients each year from 1995 to 2000. The unusual nonpigmented (pseudoe epidemic) strains were isolated from 1 patient in December 1998, 11 patients in 1999, and 19

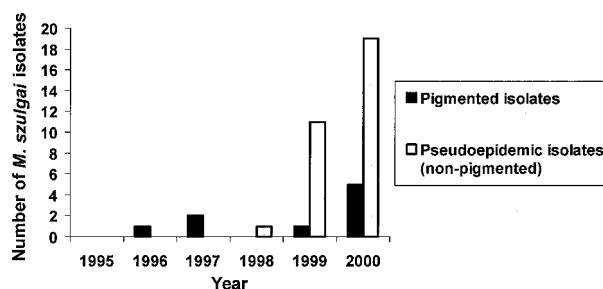


FIG. 3. Numbers of *M. szulgai* isolates collected at the Houston VAMC from 1995 to 2000.

patients in 2000 (Fig. 3). Figure 4 shows that the number of pseudoe epidemic *M. szulgai* isolates increased, while the total number of acid-fast bacillus (AFB) cultures processed during 1999 (Fig. 4A) and 2000 (Fig. 4B) did not. There was a statistically significant increase of *M. szulgai* isolates collected in September 2000 as compared with the numbers collected in other months ($P < 0.01$) and a lesser increase in August 1999, possibly indicating better growth of the organism in warm weather.

Some of the characteristics of patients from whom *M. szulgai* was recovered are shown in Table 1. Almost all of the patients had an underlying disease. However, *M. szulgai* was not thought to be causing disease in any of these patients. Although pseudoe epidemic strains were isolated from patients from a variety of hospital rooms and nursing units, there were more isolates of *M. szulgai* found in patients from one of the

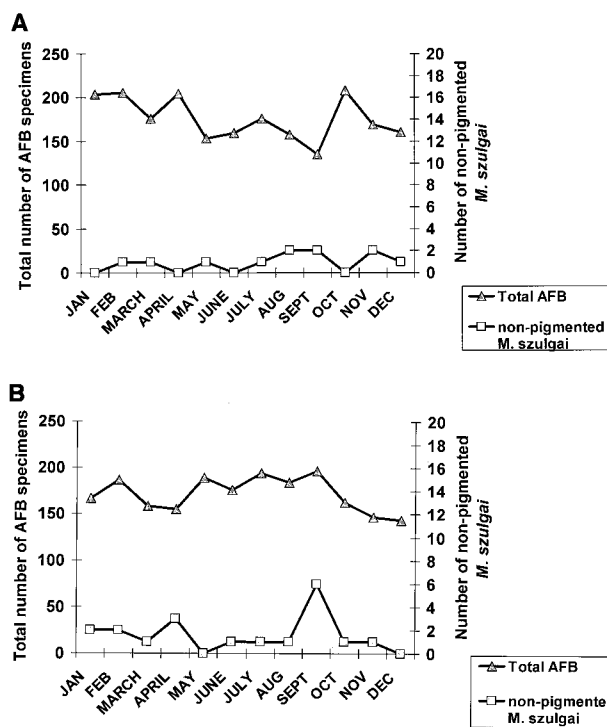


FIG. 4. Seasonal changes in the numbers of nonpigmented *M. szulgai* isolates collected and of total AFB cultures processed in 1999 (A) and 2000 (B).

nursing units (unit 4A) in 2000 than in patients from the other nursing units ($P < 0.001$).

We examined the tap water and ice water from this ward and several other wards as well as water from the hospital water storage tanks. No *M. szulgai* strain was found in any cultures from the nursing units. However, the pseudoepidemic strain was recovered from the second hospital water storage tank.

DISCUSSION

Many species of nontuberculous mycobacteria, such as *Mycobacterium gordonae*, *M. xenopi*, *M. kansasii*, and *M. abscessus*, have been reported to cause pseudo-infection (5, 9, 13, 14). The isolates causing contamination have been traced to a variety of sources, such as PANTA PLUS (Becton-Dickinson Instrument Systems), carryover from one positive specimen to the next by the BACTEC apparatus, the hospital water and ice supplies, bronchoscopes, and a variety of laboratory handling errors (9, 13, 14). To our knowledge, there has been no previous report of *M. szulgai* pseudo-infection.

Although *M. szulgai* is an uncommon cause of human diseases, it has been most commonly associated with pulmonary disease (1, 4, 7, 15). Other diseases, such as osteomyelitis, also have been reported in connection with this organism (6). In 1996, there was one case of lung infection caused by *M. szulgai* at the VAMC. The patient had a clinical presentation similar to tuberculosis, had six positive AFB smears and seven positive cultures from sputum, and was successfully treated with a four-drug tuberculosis treatment regimen (isoniazid, ethambutol, rifampin, and pyrazinamide). In contrast, all patients harboring the nonpigmented pseudoepidemic *M. szulgai* strain had negative AFB smears and only a single positive specimen, isolated from a single medium (BACTEC vials). Most of the patients were immunocompromised, and there was no primary disease associated with the pseudoepidemic strain of *M. szulgai*. Similarly, there was no disease associated with the pigmented strains of *M. szulgai* isolated during the 1997 to 2000 study period.

Neither CFA nor 16S rRNA gene sequencing analysis could uniquely differentiate pseudoepidemic strains from other strains. However, in this study, the RAPD-PCR patterns of the nonpigmented *M. szulgai* isolates were identical and were distinct from those of the pigmented strains, suggesting that a single unique clone was responsible. Furthermore, the RAPD pattern indicated that the pigmented strains were varied; they were found in three different genotypic clusters with at least four different RAPD patterns and thus did not represent a single source. The RAPD pattern was particularly valuable in distinguishing the six pigmented strains that were in the same 16S rRNA genogroup (cluster I) as the pseudoepidemic strains. Although 16S rRNA gene sequence analysis is excellent for bacterial identification (3, 12), it was not definitive in recognizing these pseudoepidemic strains. However, both the buff phenotype and a common RAPD-PCR pattern were uniquely associated with the pseudoepidemic strains.

Several characteristics made these pseudoepidemic strains difficult to investigate and evaluate. First, the strains of *M. szulgai* were nonpigmented, and pigment production has been considered a necessary characteristic before an identification of *M. szulgai* can be made. Because of this unusual phenotype,

the strains could have been misidentified as *Mycobacterium terrae* or *Mycobacterium triviale* by conventional methods. Second, the strains were isolated over a period exceeding 2 years and were not associated with laboratory procedures, methodological changes, or reagents. Third, the pseudoepidemic strains originally could not be connected to an environmental source. They appeared abruptly in December 1998 but were unconnected to any known geographic location or water supply. The initial search for an environmental source within the hospital was negative, a finding that could have been due to the low concentration of organisms. In addition, the water tests were not performed at the peak period in August and September, when more organisms might be expected. At the time of the investigation, we found that the hospital water tanks were 2 years past their scheduled maintenance time for descaling and cleaning. Subsequently, we have made sure that the tanks get regular preventive maintenance cleaning, since *M. szulgai* could be just an indicator organism, signaling the presence of other pathogens of a more serious nature, such as *Legionella* spp. There have been no further isolations of *M. szulgai* at the VAMC.

We found an unusually high rate of *M. szulgai* isolations at the VAMC in recent years. The identical RAPD-PCR patterns and phenotypes of the 31 pseudoepidemic strains indicated that they were from a single source. Isolation of the identical strain from one of the hospital water supply tanks suggested that the pseudoepidemic of *M. szulgai* at the VAMC between 1998 and 2000 most likely originated from hospital water, which transiently inoculated or colonized our patients. The precursor source has not been determined, but nonpigmented *M. szulgai* strains occasionally have been isolated elsewhere in Texas (K. C. Jost, Texas State Health Laboratory, personal communication). We do not have a clear explanation as to why the strains first appeared in December 1998 or why there were increased isolations in September 2000 and from patients in a single nursing unit (unit 4A). However, our extensive investigation has proved valuable in that it focused attention on the probability of our stored water supply being a source of waterborne contaminants. In addition, the description of this unique strain increases the known characteristics associated with this species and makes correct identification in other laboratories more likely. Although no disease was associated with this cluster of isolates, the event was costly because, since most of the patients were immunocompromised and candidates for a mycobacterial disease, we could not easily discount the isolations. The identification of *M. szulgai* in a respiratory specimen as a nonpathogen and the analysis of the pseudoepidemic strains subsequently prevented unnecessary diagnostic workups and treatments.

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