Molecular Characterization of *Mycobacterium massiliense* and *Mycobacterium bolletii* in Isolates Collected from Outbreaks of Infections after Laparoscopic Surgeries and Cosmetic Procedures[▽]

Cristina Viana-Niero,[1] Karla Valéria Batista Lima,[2] Maria Luiza Lopes,[2]
Michelle Christiane da Silva Rabello,[1] Lourival Rodrigues Marsola,[3]
Vânia Cristina Ribeiro Brilhante,[3] Alan Mitchel Durham,[4]
and Sylvia Cardoso Leão[1]*

*Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo, São Paulo, Brazil[1];
Instituto Evandro Chagas, Belém, Brazil[2]; Hospital Universitário João de Barros Barreto, Belém, Brazil[3]; and
Instituto de Matemática e Estatística da Universidade de São Paulo, São Paulo, Brazil[4]*

An outbreak of infections affecting 311 patients who had undergone different invasive procedures occurred in 2004 and 2005 in the city of Belém, in the northern region of Brazil. Sixty-seven isolates were studied; 58 were from patients who had undergone laparoscopic surgeries, 1 was from a patient with a postinjection abscess, and 8 were from patients who had undergone mesotherapy. All isolates were rapidly growing nonpigmented mycobacteria and presented a pattern by PCR-restriction enzyme analysis of the *hsp65* gene with BstEII of bands of 235 and 210 bp and with HaeIII of bands of 200, 70, 60, and 50 bp, which is common to *Mycobacterium abscessus* type 2, *Mycobacterium bolletii*, and *Mycobacterium massiliense*. *hsp65* and *rpoB* gene sequencing of a subset of 20 isolates was used to discriminate between these three species. *hsp65* and *rpoB* sequences chosen at random from 11 of the 58 isolates from surgical patients and the postinjection abscess isolate presented the highest degrees of similarity with the corresponding sequences of *M. massiliense*. In the same way, the eight mesotherapy isolates were identified as *M. bolletii*. Molecular typing by pulsed-field gel electrophoresis (PFGE) grouped all 58 surgical isolates, while the mesotherapy isolates presented three different PFGE patterns and the postinjection abscess isolate showed a unique PFGE pattern. In conclusion, molecular techniques for identification and typing were essential for the discrimination of two concomitant outbreaks and one case, the postinjection abscess, not related to either outbreak, all of which were originally attributed to a single strain of *M. abscessus*.

Rapidly growing mycobacteria (RGM) are widely distributed in the environment, especially in water (rivers, lakes, potable water), and can contaminate reagents and medical equipment. Most RGM infections in humans are caused by species belonging to the *Mycobacterium fortuitum*, *Mycobacterium chelonae-Mycobacterium abscessus*, and *Mycobacterium smegmatis* groups (6).

The *M. chelonae-M. abscessus* group comprises two genomospecies, *M. chelonae* and *M. abscessus*, which have been differentiated on the basis of <70% genomic homology by DNA-DNA hybridization (16, 18). These species have been isolated from sporadic cases of chronic lung disease associated with bronchiectasis and cystic fibrosis, disseminated cutaneous infections, and postsurgical wound infections. They have also been implicated in outbreaks in cardiac, ophthalmologic, and plastic surgeries and pseudo-outbreaks related to contaminated bronchoscopes and contaminated laboratory reagents (6, 12, 20, 25, 26). *Mycobacterium immunogenum* was included in

this group in 2001. It was isolated from metalworking fluids and was associated with cases of hypersensitivity pneumonitis in factory workers. This species has also been detected in cutaneous, catheter-related, articular, and lung infections; in an outbreak related to ophthalmologic surgeries; and in a pseudo-outbreak related to bronchoalveolar lavage procedures (19, 27). *Mycobacterium massiliense* was validated as a species separate from the *M. chelonae-M. abscessus* group in 2006 (11). It has been isolated from pulmonary specimens, from a pacemaker pocket infection, and during an outbreak associated with intramuscular injections (4, 15, 22). *Mycobacterium bolletii* has been isolated from 4 of 59 specimens from a collection of clinical RGM isolates and was recognized as a species separate from the *M. chelonae-M. abscessus* group in 2006 (1).

The discrimination of species belonging to this group is important for epidemiological studies, the prediction of antibiotic susceptibility, and the recognition of medically important taxa. Few phenotypic tests can distinguish RGM species, and the discriminatory power of lipid analysis by high-performance liquid chromatography is limited due to profile similarities (5). Individual species of the *M. chelonae-M. abscessus* group present differences in their antimicrobial susceptibilities. The most pronounced differences between *M. abscessus* and *M. chelonae* are in their susceptibilities to cefoxitin (with *M. abscessus* being generally susceptible and *M. chelonae* being

* Corresponding author. Mailing address: Universidade Federal de São Paulo, Escola Paulista de Medicina, Departamento de Microbiologia, Imunologia e Parasitologia, Rua Botucatu, 862, 3° andar, São Paulo 04023-062, Brazil. Phone: 55 11 55764537. Fax: 55 11 55724711. E-mail: sylvia.leao@unifesp.br.

resistant) and tobramycin (with *M. abscessus* being resistant and *M. chelonae* being susceptible) (6). *M. immunogenum* is resistant to both drugs (27). It was reported that *M. massiliense* could be distinguished by its susceptibility to doxycycline, while other members of this group are resistant to this drug (4). *M. massiliense* isolates with intermediate susceptibility to doxycycline were also identified (15). *M. bolletii* was described as a species highly resistant to antimicrobial drugs, including clarithromycin, to which the other members of the group are highly susceptible (1). Five different patterns have been observed by PCR-restriction enzyme analysis of the *hsp65* gene (PRA-*hsp65*) among species belonging to the *M. chelonae-M. abscessus* group: *M. chelonae* type 1, *M. abscessus* type 1 and type 2, and *M. immunogenum* type 1 and type 2, respectively (8, 19, 27). *M. massiliense* and *M. bolletii* present an indistinguishable PRA-*hsp65* pattern, described as *M. abscessus* type 2, precluding the separation of these species by this method. By 16S rRNA sequencing, *M. chelonae* and *M. abscessus* differ by only 4 bp and have identical 16S rRNA hypervariable region A sequences; *M. immunogenum* differs by 8 bp from *M. abscessus* and by 10 bp from *M. chelonae*; *M. massiliense* and *M. bolletii* show 100% sequence similarity with *M. abscessus*. Therefore, 16S rRNA sequencing is not adequate for the discrimination of these species. Differentiation is best achieved by analysis of polymorphisms either in the *rpoB*, *hsp65*, *sodA*, and *recA* genes or in the 16S-23S rRNA internal transcribed sequence (2, 3, 9).

This report describes the molecular identification and typing of isolates obtained during an outbreak of infections after invasive procedures in Belém, Brazil. To our knowledge, this is the first report of infections caused by the recently described species *M. massiliense* and *M. bolletii* in Brazil.

MATERIALS AND METHODS

Mycobacterial isolates. From February 2004 to June 2005, 311 patients who had undergone different invasive procedures, performed in 16 private hospitals and clinics in the city of Belém, state of Pará, in the northern region of Brazil, presented with signs and symptoms of localized infection. One-hundred seventy specimens were collected at the Instituto Evandro Chagas by needle aspiration of secretions, by surgical biopsy, or from the open drainages of abscesses and were cultivated at 37°C on Löwenstein-Jensen solid medium for up to 4 weeks. Sixty-seven isolates were included in this study. Fifty-eight isolates were obtained from patients who had undergone surgery; eight were from patients who had undergone mesotherapy, a procedure comprising multiple subcutaneous injections of pharmaceutical and/or homeopathic medications for cosmetic purposes; and one was from an abscess after the intramuscular injection of a contraceptive.

Species identification. All isolates were identified by analysis of their characteristics on culture (the time for growth and pigment production) and by PRA-*hsp65*. In brief, a 441-bp fragment of the *hsp65* gene, Telenti's fragment, was amplified with primers Tb11 (ACCAACGATGGTGTGTCCAT) and Tb12 (CTTGTCGAACCGCATACCCT), as described before (17, 23). The PCR mixture was used as a negative control. The amplicons were digested with BstEII (Promega) and HaeIII (Invitrogen), and the restriction fragments were separated by electrophoresis in 3% Seakem LE (BioWhittaker Molecular Applications) agarose gels stained with ethidium bromide. Fifty- and 25-bp DNA ladders (Invitrogen) were used as molecular standards.

As the PRA-*hsp65* pattern obtained was common to the three different species, a subset of isolates was submitted to DNA sequencing to discriminate between these species. Two DNA targets were sequenced: a 667-bp fragment of the *hsp65* gene containing Telenti's fragment was amplified with primers hsp667forward (GGCCAAGACAATTGCGTACG) and hsp667-reverse (GGAGCTGACCAGCAGGATG) (21), and a 752-bp fragment of *rpoB* region V was amplified with primers MycoF (GGCAAGGTCACCCCGAAGGG) and MycoR (AGCGGCTGCTGGGTGATCATC) (2). The PCR mixture was used as negative control. To obtain consensus *hsp65* and *rpoB* sequences, the amplicons from isolate B5, chosen at random from among the 58 isolates from surgical patients,

were cloned before sequencing by using the TOPO TA cloning kit for sequencing (Invitrogen), according to the manufacturer's recommendations. Recombinant plasmids were purified from the isolated colonies with the Wizard Plus SV Minipreps DNA purification system (Promega) and were sequenced with BigDye Terminator cycle sequencing reagents and primers M13F and M13R (Invitrogen). Cycle sequencing was performed with a Perkin-Elmer 9600 GeneAmp PCR system programmed for 25 cycles at 96°C for 20 s, 50°C for 10 s, and 60°C for 4 min. The sequencing products were cleaned with CentriSep spin columns, according to the manufacturer's instructions (Princeton Separations, Applied Biosystems), and were then analyzed on a ABI Prism 3100 sequencer (Perkin-Elmer, Applied Biosystems).

The sequences were assembled from the ABI chromatograms by using the PHRED, PHRAP, and CONSED programs (13). The quality of the assembled sequences (the phred scores) ranged from 0 to 1.45 per 10 kb, as estimated with the CONSED program. Consensus sequences corresponding to 401 bp of the *hsp65* gene (the 441-bp Telenti's fragment without the sequences of the primers at both ends) and to 711 bp of the *rpoB* gene (752 bp from *rpoB* region V without the sequences of the primers at both ends) were identified by similarity analysis with the sequences in the GenBank database by use of the BLAST program (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST).

The *hsp65* and *rpoB* amplicons of 19 additional isolates were submitted to direct sequencing: 10 isolates (isolates B51, B49, B37, B31, B25, B58, B52, B43, B50, and B30) randomly chosen from among the 58 available isolates from surgical patients operated on in different hospitals, 8 isolates from mesotherapy patients (isolates B59 to B66), and 1 isolate (isolate B67) from the postinjection abscess. As these sequences were obtained from noncloned PCR products, the data output from the sequencer had to be processed, and a pipeline was developed for this purpose by using the Egene platform (10). The trace files were initially submitted to analysis with the PHRED program for base calling and quality assessment. Then, the primers sequences were masked by use of the cross_match program (P. Green, 1997, http://www.phrap.org/phredphrapconsed.html). After masking of the primer sequences, a trimming process was performed to eliminate masked and low-quality (phred value, 15) regions from the 3′ and the 5′ ends of the sequence. The surviving sequences were subjected to a size filter (minimum sizes, 350 bp for *hsp65* and 670 bp for *rpoB*). Next, the resulting sequences with less than 90% of the bases with phred values above 15 across the minimum size were filtered. These sequences were aligned by using the BioEdit program (version 7.0.5.3) (14) and the CLUSTALW algorithm.

Molecular typing. Pulsed-field gel electrophoresis (PFGE) was performed as described by Coleman and Spain (7), with modifications. Colonies were cultivated in 30 ml of Mueller-Hinton broth supplemented with 0.1% Tween 80 at 37°C on a rotatory shaker until an optical density of 0.64 at 650 nm was achieved. The cells were pelleted and frozen at −80°C for 1 h. After they were thawed, the cell pellets were resuspended in a solution of 100 mM NaCl, 10 mM Tris, pH 8.0, 50 mM EDTA, and 0.1% Tween 80. The suspension was mixed with an equal volume of 1% low-melting-point preparative-grade agarose (Bio-Rad Laboratories) in 125 mM EDTA prewarmed to 55°C, and the mixture was cast into plug molds. The plugs were treated with 2 ml of 10 mg/ml lysozyme in a solution of 100 mM NaCl, 10 mM Tris, pH 8.0, and 50 mM EDTA at 37°C overnight and were then incubated at 4°C for 1 h in 0.5 M EDTA plus 1% Sarkosyl. Proteinase K (BioAmerica, Brazil) was added at a final concentration of 2 mg/ml, and the plugs were incubated at 55°C for 24 h and then at 4°C for 1 h. The plugs were washed with 1× Tris-EDTA (TE) and incubated in 1× TE containing 0.12 mg/ml phenylmethylsulfonyl fluoride (Sigma) for 1 h at 55°C. The plugs were washed once in 1× TE and once in 0.1% Triton for 90 min and digested with 30 U of DraI (Promega) at 37°C overnight or were stored in 0.5 M EDTA at 4°C until they were used. After digestion, the plugs were washed in 0.05 M EDTA, loaded into 1% pulsed-field-certified agarose gels (Bio-Rad) prepared in 0.5× TBE buffer (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA), and subjected to electrophoresis in 0.5× TBE buffer. PFGE was carried out in a CHEF-DR III system (Bio-Rad) at 14°C for 24 h at 6 V/cm, with a switch time of 1.6 to 21.3 s. A bacteriophage lambda ladder PFG marker (New England BioLabs) was used as a molecular standard.

The PFGE gel images were analyzed with the BioNumerics program (version 4.5; AppliedMaths, Sint-Martens-Latem, Belgium). The PFGE patterns of type strains *M. abscessus* ATCC 19977 and *M. chelonae* ATCC 35752 were included in the analysis. The band-based Dice unweighted pair group method with arithmetic mean method was used to prepare dendrograms of the PFGE profiles, based on 2% optimization and position tolerance. The PFGE interpretative criteria were those described by Tenover et al. (24).

Nucleotide sequence accession numbers. The consensus sequences obtained from isolate B5 were deposited in GenBank under accession numbers EU117205 (*hsp65*) and EU117207 (*rpoB*). The *hsp65* and *rpoB* gene sequences from isolates

TABLE 1. Type of surgery or invasive procedure performed for patients from this study

Procedure	No. of patients	% of all patients
Cholecystectomy	182	58.52
Hiatal hernia	22	7.07
Diagnostic laparoscopy	15	4.82
Bariatric surgery	11	3.54
Other surgical procedures[a]	42	13.5
Mesotherapy	14	4.5
NR[b]	25	8.04
Total	311	100

[a] Other surgical procedures included mammaplasty, liposuction, ooforectomy, miomectomy, appendectomy, salpingectomy, cystectomy, and kidney stone extraction.

[b] NR, not reported.

B67, B60, B62, and B66, which represented all sequevars detected in this work, were deposited in GenBank under accession numbers EU220417 to EU220424.

RESULTS

Outbreak description. From February 2004 to June 2005, 311 patients who underwent different types of surgeries involving the use of laparoscopes or cannulas or other invasive procedures, such as mesotherapy and intramuscular injection (Table 1), presented with signs and symptoms of localized infection. The clinical manifestations included local hyperemia; vesicles; abscess formation with either an acute inflammatory reaction and festering or a chronic evolution; nodules; ulceration at the portal of entry of laparoscopes; fistulization with serous, bloody, or purulent secretions; difficulty in cicatrization; low fever; and a lack of a response to conventional treatment for common skin bacterial pathogens. Generalized infections or deaths were not reported.

All surgeries were performed in 16 private hospitals in the city of Belém and mesotherapy was performed in one private clinic in Belém. Sixty-seven percent of the cases were concentrated in three hospitals, and in 17 cases, the institutions where the procedures were performed were not reported. One patient received a contraceptive injection in a different city and developed a local abscess, which was initially attributed to a *Proteus* sp. Treatment for this abscess, including antibiotics and

surgical debridement, was performed in Belém, and an additional specimen yielded a positive culture for RGM.

A total of 225 (72.35%) patients were female and 86 (27.65%) were male, and for 1 (0.31%) patient this information was unknown. The patients' ages ranged from 10 to 89 years (mean, 45 years; standard deviation, 13.87 years).

Identification results. Sixty-seven isolates, which were obtained at the Instituto Evandro Chagas, were identified as rapidly growing nonpigmented mycobacteria and presented a pattern by PRA-*hsp65* with BstEII with bands of 235 of 210 bp and with HaeIII with bands of 200, 70, 60, and 50 bp, which is common to *M. abscessus* type 2, *M. bolletii*, and *M. massiliense*.

To discriminate between these three species, a subset of 20 isolates, including 11 isolates chosen at random from among the 58 isolates from surgical patients, all 8 isolates from mesotherapy patients, and the single isolate from the postinjection abscess, was submitted to DNA sequencing of the *hsp65* and *rpoB* genes. Consensus *hsp65* and *rpoB* sequences were initially obtained by cloning the amplicons from isolate B5, which was chosen at random for this purpose. BLASTn analysis of the *hsp65* and *rpoB* consensus sequences showed the highest indexes of similarity with the corresponding sequences from *M. massiliense* (GenBank accession numbers AY596465 and AY593981) and *M. bolletii* (GenBank accession numbers AY859675 and AY859692) (Table 2).

The other sequences were obtained by direct sequencing of the PCR products. CLUSTALW analysis of the *hsp65* sequences revealed that the sequences from the surgical isolates and the postinjection abscess isolate presented 100% similarity to the sequence of *M. massiliense* CIP 108297 deposited in the GenBank database and differed from the sequence of *M. bolletii* CIP 108541 at three positions (99.25% similarity). Six of the eight sequences from the mesotherapy isolates were 100% similar to the sequence from the *M. bolletii* type strain (GenBank accession number AY859675); two isolates (isolates B59 and B60) presented a single substitution at position 253 (99.75% similarity).

The *rpoB* sequences from the surgical isolates analyzed were identical. Their sequences presented five mismatches compared to the sequence of *M. massiliense* type strain CIP 108297 deposited in the GenBank database (99.29% similarity) and 11 mismatches compared to the sequence of *M. bolletii* CIP

TABLE 2. BLASTn analysis of the consensus *hsp65* (401 bp) and *rpoB* (711 bp) sequences from isolate B5

Gene region	GenBank accession no.	Strain	No. of identical nucleotides/total no.	Index of similarity (%) to isolate B5
hsp65	AY596465	*M. massiliense* CIP 108297	401/401	100
	AY859675	*M. bolletii* CIP 108541	398/401	99.25
	AY458075	*M. abscessus* CIP 104536	396/401	98.75
	AY458074	*M. chelonae* CIP 104535	371/401	92.5
	DQ288262	*M. immunogenum* F1112	370/401	92.26
	AY458081	*M. immunogenum* CIP 106684	367/401	91.52
rpoB	AY593981	*M. massiliense* CIP 108297	706/711	99.29
	AY859692	*M. bolletii* CIP 108541	700/711	98.45
	DQ288264	*M. immunogenum* F1112	633/651	97.23
	AY262739	*M. immunogenum* CIP 106684	691/711	97.18
	AY147164	*M. abscessus* CIP 104536	686/711	96.48
	AY147163	*M. chelonae* CIP 104535	679/711	95.49

108541 (98.45% similarity). The *rpoB* sequence of the postinjection abscess isolate presented 99.1% similarity with the sequence of *M. massiliense* CIP 108297 and 98% similarity with that of *M. bolletii* CIP 108541. It also differed from the *rpoB* sequence of the surgical isolates at five positions (99.25% similarity).

The mesotherapy isolates comprised three *rpoB* sequvars. The *rpoB* sequences from two isolates (isolates B59 and B60) presented 100% similarity to the corresponding sequence of *M. bolletii* CIP 108541 and 98% similarity to the sequence of *M. massiliense* CIP 108297. Compared to the *rpoB* sequence of *M. bolletii* CIP 108541, the *rpoB* sequences of three isolates (isolates B61, B62, and B65) had two substitutions (99.56% similarity) and those of the remaining isolates (isolates B63, B64, and B66) had one substitution (99.85% similarity). Compared to the *rpoB* sequence of *M. massiliense* CIP 108297, these two sequvars presented 97.66% and 97.93% similarities, respectively.

In conclusion, the isolates from the surgical patients and from the postinjection abscess were identified by the highest similarity of sequences as *M. massiliense*, while the mesotherapy isolates were classified as *M. bolletii*.

Molecular typing results. All isolates generated interpretable patterns by PFGE. Except for eight isolates that lacked one band, the isolates from surgical patients presented indistinguishable patterns (Fig. 1). According to the criteria proposed by Tenover et al. (24), these isolates were considered highly related and to belong to the same strain. The *M. massiliense* isolate from the postinjection abscess presented a clearly distinct PFGE pattern, showing 46.7% similarity with the patterns from the surgical isolates.

The mesotherapy isolates were distributed in three clusters, each of which showed indistinguishable PFGE patterns. The clusters comprised four isolates (isolates B63 to B66), two isolates (isolates B59 and B60), and two isolates (isolates B61 and B62), respectively. These PFGE patterns differed from the patterns of the surgical isolates and the postinjection abscess isolate (Fig. 1). The overall similarity of the PFGE patterns of the mesotherapy isolates was 68.7%.

DISCUSSION

From 1999 to 2003, between 4 and 12 nontuberculous mycobacterial infections were diagnosed each year at the Mycobacteria Laboratory of the Instituto Evandro Chagas in Belém, Brazil, and no more than one isolate per year presented the PRA-*hsp65* pattern reported here. Most isolates were obtained from respiratory specimens. Unexpectedly, from October 2004 on, several isolates that presented this PRA-*hsp65* pattern were isolated from specimens from patients who had undergone invasive procedures. An epidemiological investigation was initiated, and in parallel, the isolates were identified and typed by the use of molecular methods.

This PRA-*hsp65* pattern was initially described as characteristic of that of *M. abscessus* (8) and was later observed in two recently described species from the *M. chelonae-M. abscessus* group, *M. massiliense* and *M. bolletii* (1, 4). Discrimination of these closely related species is best achieved by DNA sequencing. By consideration of the 401-bp fragment from the *hsp65* gene, the sequences of *M. massiliense* (GenBank accession

number AY596465) and *M. bolletii* (GenBank accession number AY859675) differ at three nucleotides (99.25% similarity); however, this polymorphism does not affect the PRA-*hsp65* pattern. Region V of the *rpoB* gene was shown to be more polymorphic (1, 2). In general, the RGM interspecies sequence divergence is >3%, and isolates presenting less than 1.7% sequence divergence are considered to belong to the same species (2). *M. massiliense* CIP 108297 and *M. bolletii* CIP 108541 have 98% similarity in the *rpoB* region V sequences and are presently considered to be two separate species.

As the differentiation of these species by *hsp65* and *rpoB* sequencing relies on few polymorphisms, the quality of the sequences obtained is of utmost importance. For this reason, a strategy was devised in which one isolate (isolate B5), chosen from among the 58 isolates from surgical patients which presented indistinguishable PFGE patterns, had *hsp65* and *rpoB* amplicons cloned to obtain reliable consensus sequences. The highest similarities of the consensus sequences of isolate B5 were observed with the respective sequences of *M. massiliense* (Table 2). The *hsp65* and *rpoB* sequences of 10 additional isolates from surgical patients, obtained by direct sequencing of the PCR products, were indistinguishable from the *hsp65* and *rpoB* sequences of isolate B5.

It is important to point out that even though the highest sequence similarity for *rpoB* (99.29%) was observed with the corresponding sequence from *M. massiliense*, the similarity to the *rpoB* sequence from *M. bolletii* was also high (98.45%), corresponding to divergences of <1.7% in both cases. According to the criteria proposed by Adekambi et al. (2), these isolates could be identified as either *M. massiliense* or *M. bolletii*. The *rpoB* sequences from surgical isolates showed 100% similarity to two recently deposited *rpoB* sequences (GenBank accession numbers EU090065 and EU090066) from isolates identified as *M. massiliense* in a recent publication (22). Taking all of this into account, *M. massiliense* was considered the conclusive identification for the isolates from the surgical patients described here.

The *hsp65* and *rpoB* sequences of the postinjection abscess isolate also showed the highest similarities to the corresponding sequences of *M. massiliense*. Compared to the sequences of the surgical isolates, the *hsp65* sequence of the postinjection abscess isolate was identical and the *rpoB* sequence of the postinjection abscess isolate presented five mismatches, suggesting that this isolate belonged to a different strain of *M. massiliense* and indicating that this patient was not epidemiologically related to the surgical cases. Molecular typing results confirmed that this isolate presented a distinct PFGE pattern, strongly suggesting that this abscess represented an isolated infection not related to the outbreak.

M. massiliense was reported to be susceptible to doxycycline, which could distinguish it from resistant *M. abscessus* and *M. bolletii* isolates (4). *M. massiliense* isolates with intermediate susceptibility to doxycycline have also been described (15). In the present study, nine *M. massiliense* isolates from surgical patients (isolates B51, B41, B31, B5, B22, B28, B43, B48, and B19) were shown to be resistant to doxycycline (MICs > 64 μg/ml) (data not shown) These results agree with those reported by Simmon et al. (22), indicating that this species does not have a unique pattern of susceptibility to this drug. Moreover, the doxycycline resistance and the identity of the *rpoB*

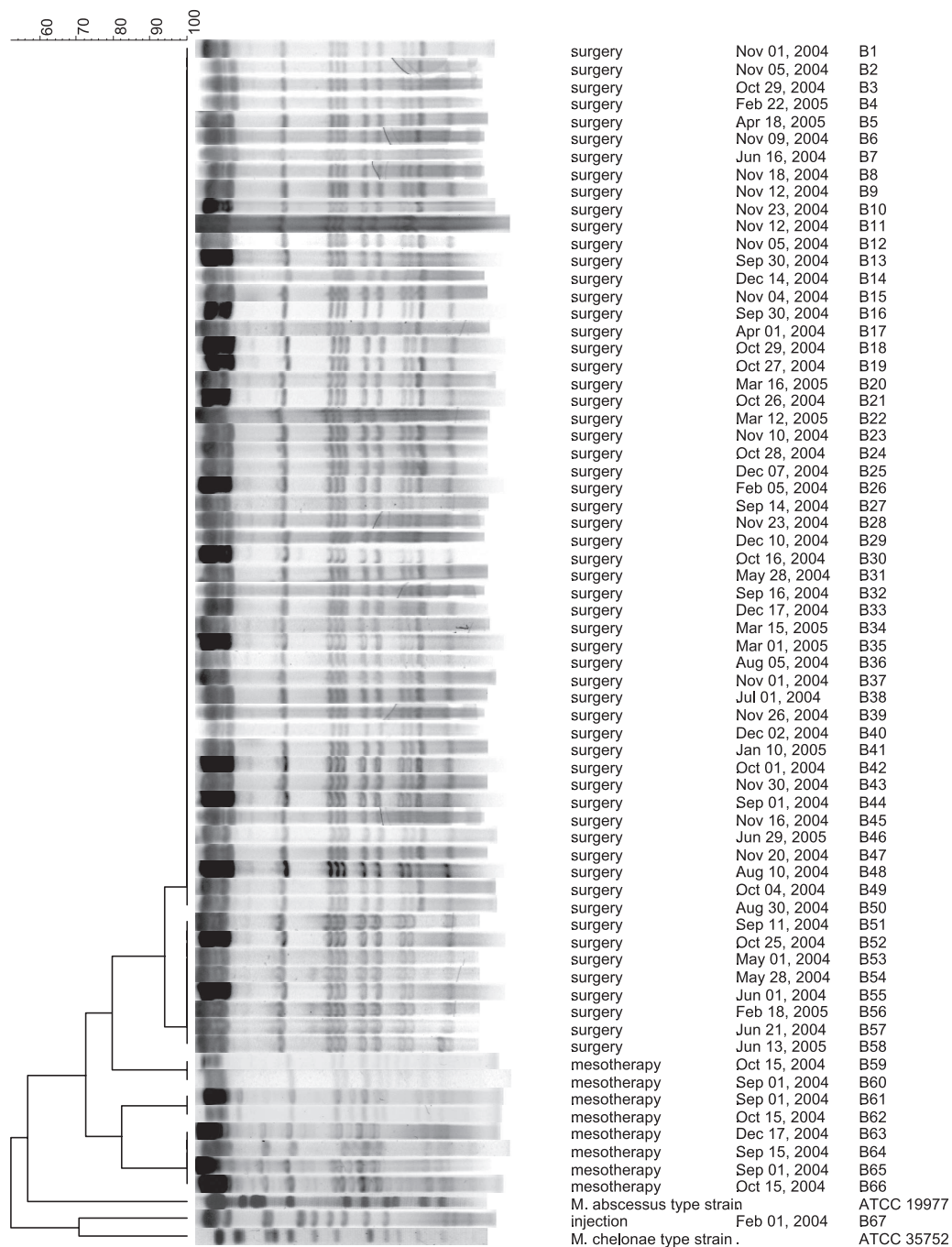| Procedure	Date	Isolate
surgery	Nov 01, 2004	B1
surgery	Nov 05, 2004	B2
surgery	Oct 29, 2004	B3
surgery	Feb 22, 2005	B4
surgery	Apr 18, 2005	B5
surgery	Nov 09, 2004	B6
surgery	Jun 16, 2004	B7
surgery	Nov 18, 2004	B8
surgery	Nov 12, 2004	B9
surgery	Nov 23, 2004	B10
surgery	Nov 12, 2004	B11
surgery	Nov 05, 2004	B12
surgery	Sep 30, 2004	B13
surgery	Dec 14, 2004	B14
surgery	Nov 04, 2004	B15
surgery	Sep 30, 2004	B16
surgery	Apr 01, 2004	B17
surgery	Oct 29, 2004	B18
surgery	Oct 27, 2004	B19
surgery	Mar 16, 2005	B20
surgery	Oct 26, 2004	B21
surgery	Mar 12, 2005	B22
surgery	Nov 10, 2004	B23
surgery	Oct 28, 2004	B24
surgery	Dec 07, 2004	B25
surgery	Feb 05, 2004	B26
surgery	Sep 14, 2004	B27
surgery	Nov 23, 2004	B28
surgery	Dec 10, 2004	B29
surgery	Oct 16, 2004	B30
surgery	May 28, 2004	B31
surgery	Sep 16, 2004	B32
surgery	Dec 17, 2004	B33
surgery	Mar 15, 2005	B34
surgery	Mar 01, 2005	B35
surgery	Aug 05, 2004	B36
surgery	Nov 01, 2004	B37
surgery	Jul 01, 2004	B38
surgery	Nov 26, 2004	B39
surgery	Dec 02, 2004	B40
surgery	Jan 10, 2005	B41
surgery	Oct 01, 2004	B42
surgery	Nov 30, 2004	B43
surgery	Sep 01, 2004	B44
surgery	Nov 16, 2004	B45
surgery	Jun 29, 2005	B46
surgery	Nov 20, 2004	B47
surgery	Aug 10, 2004	B48
surgery	Oct 04, 2004	B49
surgery	Aug 30, 2004	B50
surgery	Sep 11, 2004	B51
surgery	Oct 25, 2004	B52
surgery	May 01, 2004	B53
surgery	May 28, 2004	B54
surgery	Jun 01, 2004	B55
surgery	Feb 18, 2005	B56
surgery	Jun 21, 2004	B57
surgery	Jun 13, 2005	B58
mesotherapy	Oct 15, 2004	B59
mesotherapy	Sep 01, 2004	B60
mesotherapy	Sep 01, 2004	B61
mesotherapy	Oct 15, 2004	B62
mesotherapy	Dec 17, 2004	B63
mesotherapy	Sep 15, 2004	B64
mesotherapy	Sep 01, 2004	B65
mesotherapy	Oct 15, 2004	B66
M. abscessus type strain		ATCC 19977
injection	Feb 01, 2004	B67
M. chelonae type strain		ATCC 35752

FIG. 1. Dendrogram of PFGE patterns of isolates from this study. Indistinguishable patterns grouped the isolates from surgical patients; however, the patterns of eight isolates lacked one restriction band. Three clusters of indistinguishable patterns were detected in the mesotherapy isolates, but none of the patterns matched the patterns of the surgical isolates. The columns on the right indicate the type of procedure, the procedure date, and the isolate number. Comparison was performed with the BioNumerics (version 4.5) program and by analysis by the Dice unweighted pair group method with arithmetic mean method, based on 2% optimization and position tolerance. The PFGE patterns of *M. abscessus* ATCC 19977 and *M. chelonae* ATCC 35752 were included in the analysis.

region V sequences of Brazilian and U.S. isolates (22) could suggest the existence of a variant clone of *M. massiliense* in the Americas.

A different result was obtained with isolates from mesotherapy patients. The *hsp65* and *rpoB* sequences presented the highest similarities with the corresponding sequences from *M.*

bolletii CIP 108541, and this is probably the correct identification for these isolates. Isolates from mesotherapy patients also presented three distinct PFGE patterns that were clearly separated from the patterns observed for the surgical isolates. Therefore, even though the mesotherapy infections were concomitant with the surgical cases, they are most likely unrelated

to the outbreak of laparoscopic surgeries and may have had different sources.

The sources of the infections for the surgical cases have not been identified here. The patients were operated on by different surgeons, who used their own laparoscopic equipment, which was disinfected by immersion in 2% glutaraldehyde between surgeries and which was used in different hospitals. Glutaraldehyde, a frequently employed biocide, can be used for the high-level disinfection of some medical equipment after careful cleaning for the elimination of organic substances. Without adequate cleaning, surgical or medical equipment can accumulate organic material, generating biofilms and preventing the action of disinfectants. Our hypothesis is that inconsistencies in equipment cleaning, glutaraldehyde concentrations, or contact times may have contributed to the selection of the *M. massiliense* strain described here, which contaminated the surgical equipment. The use of laparoscopic equipment in several hospitals disseminated the strain to the different outbreak settings. Cases of infection after laparoscopic surgeries subsided after the cleaning and sterilization procedures were reviewed. No RGM infections were reported in patients who underwent different surgical procedures, in which laparoscopic equipment or cannulas and disinfection with glutaraldehyde were not employed, during the same period and in the same hospitals.

The prevalence of this particular *M. massiliense* strain could have originated from its atypical survival ability in different environments, resistance to biocides, and/or enhanced biofilm formation. It is also possible that this particular strain may be unusually resistant to the disinfectant used, and experiments to test this hypothesis are under way.

In conclusion, this outbreak, which was initially attributed to *M. abscessus*, in fact consisted of two concomitant but different outbreaks. The main outbreak grouped 298 surgical patients who were operated on in several hospitals and was probably caused by a single strain of *M. massiliense*. The other outbreak clustered 14 mesotherapy patients who were treated in a private clinic and involved multiple strains of *M. bolletii*. The postinjection abscess case was not related to either outbreak.

ACKNOWLEDGMENTS

REFERENCES

1. **Adekambi, T., P. Berger, D. Raoult, and M. Drancourt.** 2006. *rpoB* gene sequence-based characterization of emerging non-tuberculous mycobacteria with descriptions of *Mycobacterium bolletii* sp. nov., *Mycobacterium phocaicum* sp. nov. and *Mycobacterium aubagnense* sp. nov. Int. J. Syst. Evol. Microbiol. **56:**133–143.
2. **Adekambi, T., P. Colson, and M. Drancourt.** 2003. *rpoB*-based identification of nonpigmented and late-pigmenting rapidly growing mycobacteria. J. Clin. Microbiol. **41:**5699–5708.
3. **Adekambi, T., and M. Drancourt.** 2004. Dissection of phylogenetic relationships among 19 rapidly growing *Mycobacterium* species by 16S rRNA, *hsp65*, *sodA*, *recA* and *rpoB* gene sequencing. Int. J. Syst. Evol. Microbiol. **54:**2095–2105.
4. **Adekambi, T., M. Reynaud-Gaubert, G. Greub, M. J. Gevaudan, B. La Scola, D. Raoult, and M. Drancourt.** 2004. Amoebal coculture of "*Mycobacterium massiliense*" sp. nov. from the sputum of a patient with hemoptoic pneumonia. J. Clin. Microbiol. **42:**5493–5501.
5. **Brown-Elliott, B. A., D. E. Griffith, and R. J. Wallace, Jr.** 2002. Diagnosis of nontuberculous mycobacterial infections. Clin. Lab. Med. **22:**911–925, vi.
6. **Brown-Elliott, B. A., and R. J. Wallace, Jr.** 2002. Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. Clin. Microbiol. Rev. **15:**716–746.
7. **Coleman, N. V., and J. C. Spain.** 2003. Distribution of the coenzyme M pathway of epoxide metabolism among ethene- and vinyl chloride-degrading *Mycobacterium* strains. Appl. Environ. Microbiol. **69:**6041–6046.
8. **Devallois, A., K. S. Goh, and N. Rastogi.** 1997. Rapid identification of mycobacteria to species level by PCR-restriction fragment length polymorphism analysis of the *hsp65* gene and proposition of an algorithm to differentiate 34 mycobacterial species. J. Clin. Microbiol. **35:**2969–2973.
9. **Devulder, G., M. Perouse de Montclos, and J. P. Flandrois.** 2005. A multigene approach to phylogenetic analysis using the genus *Mycobacterium* as a model. Int. J. Syst. Evol. Microbiol. **55:**293–302.
10. **Durham, A. M., A. Y. Kashiwabara, F. T. Matsunaga, P. H. Ahagon, F. Rainone, L. Varuzza, and A. Gruber.** 2005. EGene: a configurable pipeline generation system for automated sequence analysis. Bioinformatics **21:**2812–2813.
11. **Euzéby, J. P.** 2007, posting date. List of bacterial names with standing in nomenclature. http://www.bacterio.cict.fr/m/mycobacterium.html. Société de Bactériologie Systématique et Vétérinaire, Toulouse, France.
12. **Freitas, D., L. Alvarenga, J. Sampaio, M. Mannis, E. Sato, L. Sousa, L. Vieira, M. C. Yu, M. C. Martins, A. Hofling-Lima, and R. Belfort, Jr.** 2003. An outbreak of *Mycobacterium chelonae* infection after LASIK. Ophthalmology **110:**276–285.
13. **Gordon, D., C. Abajian, and P. Green.** 1998. Consed: a graphical tool for sequence finishing. Genome Res. **8:**195–202.
14. **Hall, T. A.** 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. **41:**95–98.
15. **Kim, H. Y., Y. J. Yun, C. G. Park, D. H. Lee, Y. K. Cho, B. J. Park, S. I. Joo, E. C. Kim, Y. J. Hur, B. J. Kim, and Y. H. Kook.** 2007. Outbreak of *Mycobacterium massiliense* infection associated with intramuscular injections. J. Clin. Microbiol. **45:**3127–3130.
16. **Kusunoki, S., and T. Ezaki.** 1992. Proposal of *Mycobacterium peregrinum* sp. nov., nom. rev., and elevation of *Mycobacterium chelonae* subsp. *abscessus* (Kubica et al.) to species status: *Mycobacterium abscessus* comb. nov. Int. J. Syst. Bacteriol. **42:**240–245.
17. **Leao, S. C., A. Martin, G. I. Mejia, J. C. Palomino, J. Robledo, M. A. S. Telles, and F. Portaels.** 2004. Practical handbook for the phenotypic and genotypic identification of mycobacteria. Vanden Broelle, Bruges, Belgium.
18. **Lévy-Frébault, V., F. Grimont, P. A. D. Grimont, and H. L. David.** 1986. Deoxyribonucleic acid relatedness study of the *Mycobacterium fortuitum-Mycobacterium chelonae* complex. Int. J. Syst. Bacteriol. **36:**458–460.
19. **Sampaio, J. L., D. N. Junior, D. de Freitas, A. L. Hofling-Lima, K. Miyashiro, F. L. Alberto, and S. C. Leao.** 2006. An outbreak of keratitis caused by *Mycobacterium immunogenum*. J. Clin. Microbiol. **44:**3201–3207.
20. **Sampaio, J. L., C. Viana-Niero, D. de Freitas, A. L. Hofling-Lima, and S. C. Leao.** 2006. Enterobacterial repetitive intergenic consensus PCR is a useful tool for typing *Mycobacterium chelonae* and *Mycobacterium abscessus* isolates. Diagn. Microbiol. Infect. Dis. **55:**107–118.
21. **Selvaraju, S. B., I. U. Khan, and J. S. Yadav.** 2005. A new method for species identification and differentiation of *Mycobacterium chelonae* complex based on amplified *hsp65* restriction analysis (AHSPRA). Mol. Cell. Probes **19:**93–99.
22. **Simmon, K. E., J. I. Pounder, J. N. Greene, F. Walsh, C. M. Anderson, S. Cohen, and C. A. Petti.** 2007. Identification of an emerging pathogen, *Mycobacterium massiliense*, by *rpoB* sequencing of clinical isolates collected in the United States. J. Clin. Microbiol. **45:**1978–1980.
23. **Telenti, A., F. Marchesi, M. Balz, F. Bally, E. C. Bottger, and T. Bodmer.** 1993. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J. Clin. Microbiol. **31:**175–178.
24. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33:**2233–2239.
25. **Tiwari, T. S., B. Ray, K. C. Jost, Jr., M. K. Rathod, Y. Zhang, B. A. Brown-Elliott, K. Hendricks, and R. J. Wallace, Jr.** 2003. Forty years of disinfectant failure: outbreak of postinjection *Mycobacterium abscessus* infection caused by contamination of benzalkonium chloride. Clin. Infect. Dis. **36:**954–962.
26. **Wallace, R. J., Jr.** 1994. Recent changes in taxonomy and disease manifestations of the rapidly growing mycobacteria. Eur. J. Clin. Microbiol. Infect. Dis. **13:**953–960.
27. **Wilson, R. W., V. A. Steingrube, E. C. Bottger, B. Springer, B. A. Brown-Elliott, V. Vincent, K. C. Jost, Jr., Y. Zhang, M. J. Garcia, S. H. Chiu, G. O. Onyi, H. Rossmoore, D. R. Nash, and R. J. Wallace, Jr.** 2001. *Mycobacterium immunogenum* sp. nov., a novel species related to *Mycobacterium abscessus* and associated with clinical disease, pseudo-outbreaks and contaminated metalworking fluids: an international cooperative study on mycobacterial taxonomy. Int. J. Syst. Evol. Microbiol. **51:**1751–1764.