ORIGINAL ARTICLE

# Molecular identification and typing of *Mycobacterium massiliense* isolated from postsurgical infections in Brazil

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Submitted on: 01/18/2011
Approved on: 06/29/2011

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We declare no conflict of interest.

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## *ABSTRACT*

**Objective:** One hundred thirty-one cases of postsurgical infections were reported in Southern Region of Brazil between August 2007 and January 2008. Thirty-nine (29.8%) cases were studied; this report describes epidemiological findings, species identification, antimicrobial susceptibility and clonal diversity of rapidly growing mycobacteria isolated in this outbreak. **Methods:** All 39 isolates were analyzed by Ziehl-Nielsen stained smear, bacterial culture and submitted to *rpoB* partial gene sequencing for identification. The isolates were also evaluated for their susceptibility to amikacin, cefoxitin, clarithromycin, ciprofloxacin, doxycycline, tobramycin and sulfamethoxazole. **Results:** Thirty-six isolates out of the confirmed cases were identified as *Mycobacterium massiliense* and the remaining three were identified as *Mycobacterium abscessus*, *Mycobacterium chelonae* and *Mycobacterium fortuitum*. All *M. massiliense* isolates were susceptible to amikacin ($MIC_{90}$ = 8 µg/mL) and clarithromycin ($MIC_{90}$ = 0.25 µg/mL) but resistant to cefoxitin, ciprofloxacin, doxycycline, tobramycin and sulfamethoxazole. Molecular analysis by pulsed-field gel electrophoresis clustered all 36 *M. massiliense* isolates and showed the same pattern (BRA 100) observed in three other outbreaks previously reported in Brazil. **Conclusions:** These findings suggest a common source of infection for all patients and reinforce the hypotheses of spread of *M. massiliense* BRA100 in Brazilian hospital surgical environment in recent years.

**Keywords:** mycobacteria, atypical; mycobacterium infections; microbiological analysis.

## *INTRODUCTION*

Human infections after cosmetic procedures, surgery, postinjection and nipple piercing[1,2] by rapidly growing mycobacteria (RGM) have been described worldwide as mainly associated with *Mycobacterium chelonae, Mycobacterium abscessus*, *Mycobacterium fortuitum* and *Mycobacterium smegmatis* groups.[3] These microorganisms have already been isolated from soil, water treatment plants, hospital tap water and distilled water, and considered environmentally adapted species.[4] Some RGM strains have been described as being able to develop biofilm and infections related to biofilm represent more than two-thirds of all infections caused by these organisms.[5]

Hospital outbreaks as well as isolated cases of RGM infections have been reported in different scenarios involving chronic lung disease, disseminated cutaneous infections and postsurgical wound infections.[6,7] Outbreaks and pseudo-outbreaks associated with these bacteria have been generally related to contaminated medical equipments, solutions and laboratory reagents.[8,9] Strains resistant to disinfectants have also been isolated from endoscope washer disinfector after decontamination with 2% glutaraldehyde solution.[10] Since 2% glutaraldehyde is one of the basic compounds most widely used as a chemical disinfectant for surgical equipment in several countries, particularly for non-autoclavable devices, resistance to biocides have become a great concern in hospital practice.

In Brazil, the first reported outbreaks caused by *M. chelonae*-abscessus group species were related to laser *in situ* keratomileusis, mesotherapy sessions and breast implant surgeries.[11,12] *Mycobacterium massiliense* has been described as the main agent isolated during these recent RGM outbreaks in several Brazilian states, mainly those that occurred after 2004. Furthermore, in all described postsurgical outbreaks, a specific *M. massiliense* clone, named BRA100, has emerged as an opportun-

istic pathogen, usually causing postsurgical wound infections, including superficial abscesses and granulomas.[13] The Brazilian Public Health Surveillance System has registered an overall of 1,937 confirmed cases of RGM postsurgical infections since 2001. Up to now, only three *M. massiliense* outbreaks have been reported in Brazil, all related to surgical site infections following video-assisted surgeries. Even though each of the three Brazilian *M. massiliense* outbreaks occurred in distant geographical regions (Northern, Central and Southeastern regions of Brazil), strains isolated from each outbreak were identified as clonal by molecular techniques.[2,13,14] We report a new postsurgical infection outbreak of *M. massiliense* in Curitiba, Brazil, which includes clinical findings, microbiological investigation, and molecular typing by pulsed-field gel electrophoresis (PFGE) and *rpoB* partial gene sequencing.

## MATERIAL AND METHODS

### General aspects and microbiological procedures

From August 2007 to January 2008, 131 patients were submitted to surgical procedures at one of the seven major private hospitals located in the city of Curitiba, in the South region of Brazil. All patients showed signs of postsurgical infections clinically suggestive of RGM, such as wound with local inflammation, presence of abscess, delayed wound healing and no response to the treatment commonly used in cutaneous infections. Out of the 131 patients, a sample of 39 patients were collected by either biopsy or aspiration of abscesses fluids and were cultivated on Lowenstein-Jensen solid medium for up to four weeks at 37°C.[15] A detailed history of patients was obtained by the Public Health Surveillance System. We were not able to inspect environmental conditions such as sterilization measures or antiseptic method applied. This study was approved by the Internal Review Board, Hospital do Trabalhador Ethics Committee.

### Species identification

Observation of the growth rate was taken as an evidence of RGM. Definitive confirmation and species identification was based on partial sequencing of the *rpoB* gene. Extraction of DNA from clinical isolates was carried out using the Kit Nuclisens Basic Nasba Diagnostics (bioMérieux) based on methods previously published.[16] DNA amplification and sequencing of the PCR products was performed with primers MycoF (5'–GCA AGG TCA CCC CGA AGG G–3') and MycoR (5'–AGC GGC TGC TGG GTG ATC ATC–3'), that amplify a 764 bp within the *rpoB* gene.[17] PCR mixtures (50 µL) contained 5 µL of 10 X *Taq* buffer (included with *Taq* polymerase), 200 µM each deoxynucleoside triphosphate, 2 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Invitrogen), 10 mmol of each primer (Invitrogen), 2 µL of the extracted DNA and ultrapure water. PCR mixtures were subjected to 35 cycles of denaturation at 94°C for 30s, primer annealing at 64°C for 30s, and DNA elongation at 72°C for 90s. Every amplification program began with a denaturation step of 95°C for 1 min and ended with a final elongation step of 72°C for 5 min. Amplicons were purified with PureLink™ PCR purification kit (Invitrogen) and cycle-sequenced using the Big dye terminator kit v.3.1 according to the manufacturer's instructions (Applied Biosystems) with the following program: 30 cycles of denaturation at 94°C for 10s, primer annealing at 50°C for 15s, and extension at 60°C for 4 min. The cycling-sequenced products were purified by Big Dye XTerminator™ Purification kit (Applied Biosystems) and detected on an ABI Prism 3110 DNA Sequence Analyzer (Applied Biosystems). The resulting sequences were aligned with BioEdit software (version 7.0.5.3)[18] using *M. tuberculosis* H37Rv (GenBank accession BX842574.1) as the reference sequence. The homology analysis was performed by comparison of the consensus sequence obtained from each isolate with those deposited in the GenBank using the BLAST algorithm (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov/BLAST).

### Antimicrobial susceptibility test

All 39 isolates were evaluated for their susceptibility to amikacin, cefoxitin, clarithromycin, ciprofloxacin, doxycycline, sulfamethoxazole and tobramycin as recommended by the Clinical and Laboratory Standards Institute.[19] *Staphylococcus aureus* ATCC 29213 was used as a control strain.

### Pulsed-field gel electrophoresis analysis

All *M. massiliense* isolates were submitted to genotypic analysis by PFGE. One isolate of *M. massiliense* BRA 100 clone from a previous outbreak occurred in Rio de Janeiro state (CRM 0020) and two epidemiologically unrelated strains recovered from sputum samples in 2007 in Rio de Janeiro city (CRM 0270 and CRM 0273) were also included for comparison. The agarose plugs were first treated as previously described, and then digested with *Dra*I (Promega).[20,21] Agarose gel (1%) was used to separate the restriction fragments in a CHEF-DRIII system (Bio-Rad Laboratories) with pulse times increasing from 1.6 to 21.3s over 22 hr at 14°C, at a voltage gradient of 6 V/cm and with included angle of 120°. The PFGE profiles generated were analyzed by a commercial molecular analysis fingerprinting GelCompar software (Applied Maths). Fragments patterns were interpreted as previously described.[22]

## RESULTS

### Clinical findings and outbreak description

We studied 39 cases of post-surgical infection, which were confirmed by microbiological culture while 92 remained as suspected cases. The median age was 50 years old (range

24-87) and most patients (73.0%) were female. All patients showed abscesses only on the surgical site. One hospital concentrated 77% of the cases. The time from surgery to the manifestation of clinical signs ranged from 5 to 60 days. Most patients (n = 33; 84.6%) were treated with a combined antimicrobial therapy consisting of clarithromycin, amikacin and terizidone. The remaining patients received an initial treatment with clarithromycin, amikacin and minocycline. The majority of cases (n = 37; 94.9%) progressed to cure and two reported deaths were associated with RGM infection.

### Species identification

Growth of the isolates was observed in less than seven days and Ziehl–Nielsen stained smears showed acid-fast bacilli. The organisms were identified as rapidly growing non-pigmented mycobacteria and pure culture isolates were used for molecular species identification. Analysis of the *rpoB* gene sequences of all isolates were identical and presented 100% similarity (695/695) to the sequence from *M. massiliense* strain INCQS 594 (GenBank accession number EU117207). One sequence was identical (695/695) to *M. abscessus* type strain P02 (GenBank accession number FJ590436.1). One isolate was similar (695/695) to the *M. chelonae* ATCC 19237 type strain, retrieved from GenBank under the accession number AY262740.1. The other isolate had the similar sequence (695/695) to *M. fortuitum* type strain CIP 104534T (GenBank accession number AY147165.1).

### Antimicrobial susceptibility test

All *M. massiliense* isolates were susceptible to amikacin ($MIC_{90}$ = 8 μg/mL) and clarithromycin ($MIC_{90}$ = 0.25 μg/mL) but resistant to cefoxitin, doxycycline, sulfamethoxazole and tobramycin. Thirty-five *M. massiliense* isolates were ciprofloxacin-resistant and a single isolate was characterized as susceptible. *M. abscessus* isolate was susceptible to amikacin (MIC = 8 μg/mL) and clarithromycin (MIC = 0.25 μg/mL) and intermediate to cefoxitin ($MIC_{90}$ = 128 μg/mL). However, this isolate was resistant to ciprofloxacin, doxycycline, tobramycin and sulfamethoxazole. *M. chelonae* isolate was susceptible to clarithromycin (MIC = 0.25 μg/mL) and intermediate to amikacin (MIC = 32 μg/mL) and tobramycin (MIC = 16 μg/mL), but resistant to cefoxitin, ciprofloxacin, doxycycline and sulfamethoxazole. *M. fortuitum* isolate was susceptible to amikacin (MIC = 8 μg/mL), clarithromycin (MIC = 0.25 μg/mL) and ciprofloxacin (MIC = 16 μg/mL) but resistant to doxycycline, sulfamethoxazole and tobramycin. This isolate presented intermediate resistance to cefoxitin (MIC = 128 μg/mL).

### Molecular pattern

PFGE analysis revealed that *M. massiliense* isolates presented indistinguishable patterns and according to the criteria proposed by Tenover et al.[22] these isolates were considered highly related and to belong to the same strain. The PFGE patterns were similar to those of the isolates recovered from a recent epidemic in Rio de Janeiro, Southeastern Brazil (strain CRM 0020, Figure 1).[13] *M. massiliense* isolates obtained from sputum samples (CRM 0270 and CRM 0273) showed no identical eletrophoretic patterns when compared to those isolates from Curitiba and Rio de Janeiro (Figure 2).

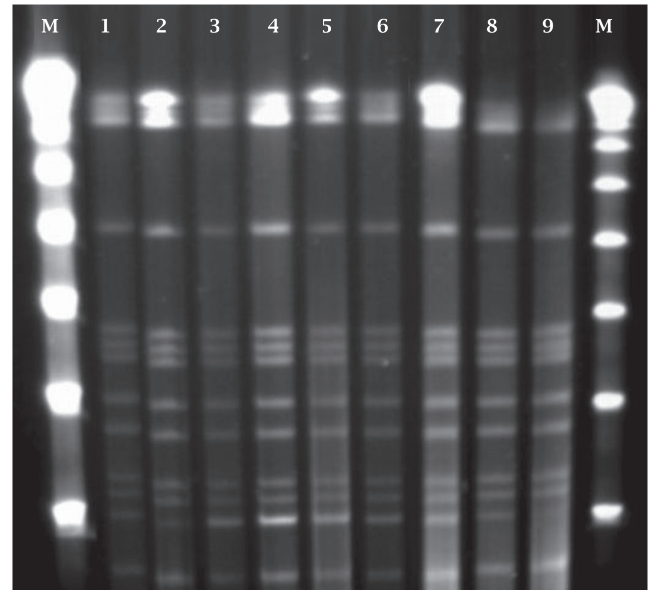



**Figure 1:** PFGE pattern of *M. massiliense* isolates from Curitiba outbreak. M, molecular size markers (Lambda DNA concatemers ranging from 48.5 to 1,018.5 kb); lane 1, CRM-587; lane 2, CRM-591; lane 3, CRM-592; lane 4, CRM-593; lane 5, CRM-595, lane 6, CRM- 596; lane 7, CRM-598; lane 8, CRM-602 and lane 9, CRM-604.

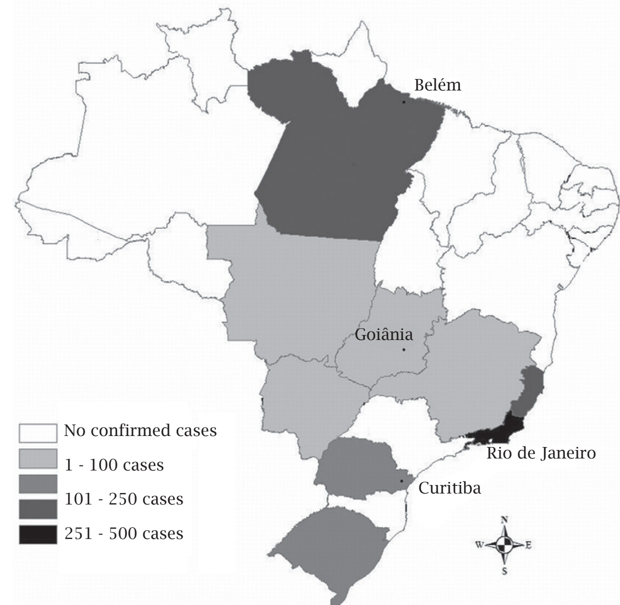



**Figure 2:** Map of rapidly growing mycobacteria confirmed cases by the Brazilian Public Health Surveillance System from 2001 to 2008. The map indicates the cities of Goiânia, Belém, Rio de Janeiro and Curitiba where outbreaks have been reported.

## *DISCUSSION*

The Brazilian Public Health Surveillance System has presented cases of RGM infections from 2001 to date. Since 2001, a total of 1,937 confirmed cases were registered, with an evident increase of outbreaks in the period (Figure 3). However, there are only three reports of *M. massiliense* outbreaks in Brazil, all related to surgical infection following video-assisted surgeries.

This study reports the identification and molecular epidemiological features of a single clone of *M. massiliense* isolated from a new outbreak of surgical site infections caused by RGM in Curitiba, Southern region of Brazil. Partial *rpoB* sequence analysis was considered discriminatory for identification of *M. massiliense* clinical isolates described here, since we obtained the highest similarity index (100%) when comparing our sequences to that of the *M. massiliense* type strain. The *rpoB* partial sequencing and PFGE analysis confirmed the similarity of our isolates with those of previously reported outbreaks in Brazil.[2,13,14] The molecular patterns obtained suggest a common source of infection and spread of a single clone of *M. massiliense* in different regions of the country.

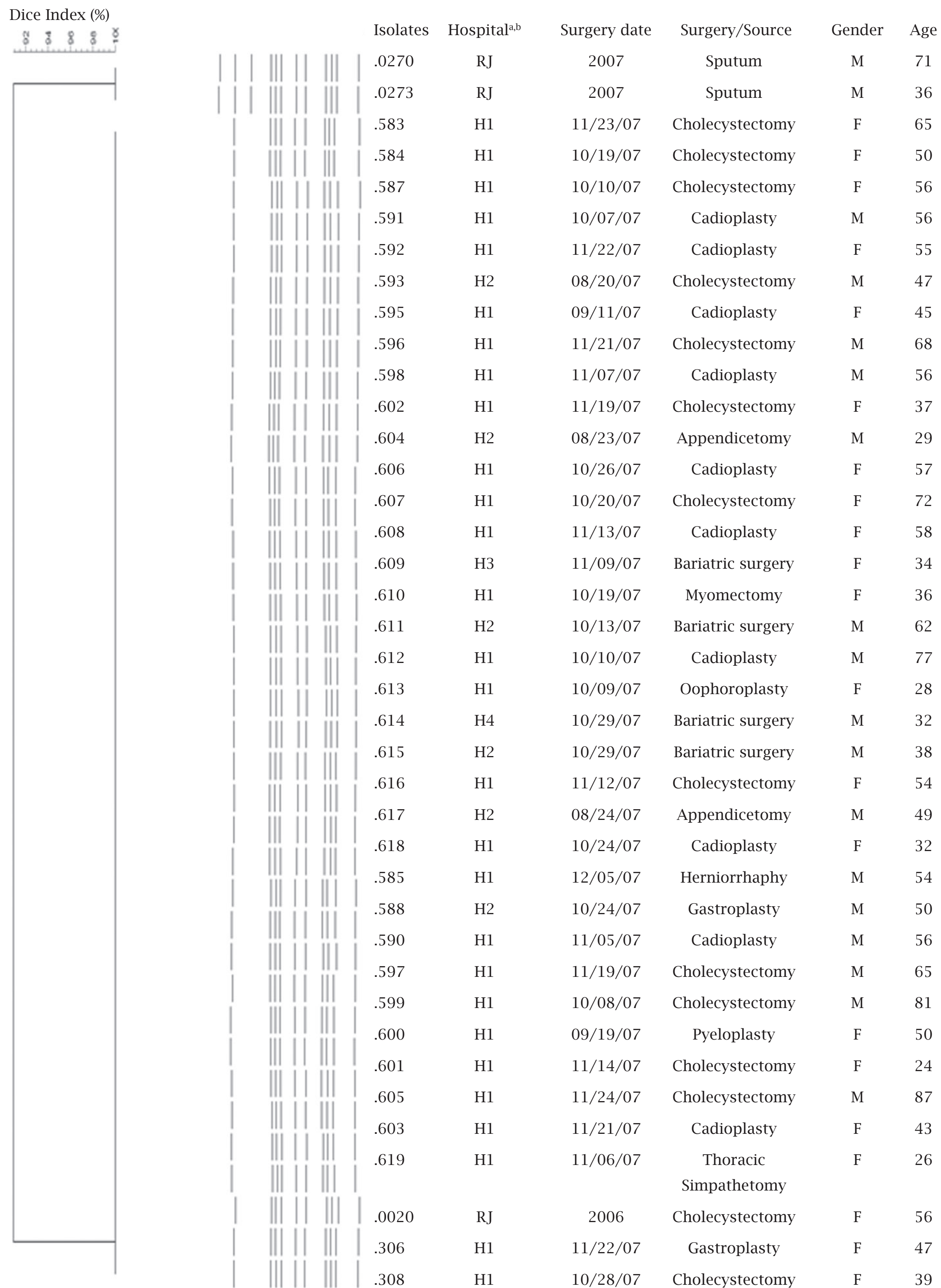
There were some limitations in this study. First a recall bias might have occurred since only 39 cases were included causing the reduced availability of other strains for analysis. Additionally, the lack of standardized procedures or protocols for isolation of RGM from surgical supplies made it difficult to determine the infection sources and its relation with surgical devices and equipments. Since no environmental isolates were obtained, we were not able to definitely identify the source of the infection. In this outbreak surgical equipments were disinfected by immersion in 2% glutaraldehyde and were used in different hospitals by different surgical teams that brought along their own instruments and performed surgeries in other cities and states. Inspections made by public health authorities evidenced that the disinfection protocol was unsettled by some of the hospitals in different ways. Thus, strictly monitoring concerning disinfection in 2% glutaraldehyde solution may explain why some hospitals in Curitiba have not had cases of surgical infections caused by RGM.

The sources of the infections for the surgical cases have not been identified in the first outbreak, in Northern Brazil. Patient procedures were performed by different surgeons, who used their own laparoscopic equipment, referred to be disinfected by immersion in 2% glutaraldehyde between surgeries and which were used in different hospitals.[2] Inconsistencies in equipment cleaning, glutaraldehyde concentrations, or contact times were suggested as the cause of the *M. massiliense* strain selection. The disinfection procedure used in arthroscopic and laparoscopic surgeries in Central Brazil outbreak was also performed by immersion in 2% glutaraldehyde. According to inspections made by public health authorities, the disinfection protocol was incorrectly implemented in some hospitals. So, inadequate aseptic techniques during surgeries could have been the possible cause.[13] In the Southeastern outbreak, all hospitals that presented cases of *M. massiliense* infection used 2% alkaline glutaraldehyde solution to sterilize surgical instruments. Interestingly, all *M. massiliense* isolates consistently presented tolerance to 2% glutaraldehyde.[14] The authors suggested that 2% glutaraldehyde tolerance may partially explain the occurrence of outbreaks in three different regions in Brazil.

Based on reported RGM outbreaks, potential source of infection was either the lack of adequate disinfection procedures (that could result in biofilm formation) or resistance to common disinfectants, or even both. Biofilms have been described in a high number of human infections, especially those related to biomaterials.[23] Although biofilm formation may contribute to tolerance to biocide solutions,[24] biofilm itself could not explain the single clone found on different time periods in distinct Brazilian regions.[13] However, since a significant relationship between biofilm development ability and clinical infection has been experimentally demonstrated, biofilm development may be an important pathogenic risk factor for RGM, contributing to development of human infections.[25] Moreover, biofilms are a well-known form of bacterial resistance against antibiotics,[26] and therefore the facility to develop these structures can explain treatment failures.[27]

Non-tuberculosis mycobacteria are often resistant to standard antituberculosis drugs and can be very difficult to treat. If RGM infection is identified and treated early, adequate recovery is possible, otherwise death can ensue. In this report, 33 patients (n = 39, 84.6%) were treated with a combination of amikacin, clarithromycin and terizidone. Treatment with multiple agents is preferable because of a high rate of relapse and emergence of drug resistance.[28] Amikacin and clarithromycin exhibited the greatest activity against all RGM isolates in this study and demonstrated to be effective when used in a multidrug regimen. All *M. massiliense* isolates from Rio de Janeiro outbreak tested *in vitro* were susceptible to amikacin and clarithromycin and resistant to cefoxitin, ciprofloxacin and doxycycline;[13] the same susceptibility pattern observed in our isolates. Although no treatment data was presented in the Rio de Janeiro outbreak, patients from the Central Brazil outbreak were successfully treated with a combination of clarithromycin and amikacin.

After the occurrence of RGM outbreaks, the Brazilian Public Health Surveillance System recommended preventive measures to reduce the infections in Brazilian hospitals. These measures include the discontinuation of chemical sterilization by immersion for invasive items used in abdominal surgeries, conventional laparoscopic and plastic surgery. For disinfection of non-autoclavable equipments used in surgical procedures such as endoscopes, sterilization should be performed by ethylene oxide gas, hydrogen per-




| Isolates | Hospital[a,b] | Surgery date | Surgery/Source | Gender | Age |
|---|---|---|---|---|---|
| .0270 | RJ | 2007 | Sputum | M | 71 |
| .0273 | RJ | 2007 | Sputum | M | 36 |
| .583 | H1 | 11/23/07 | Cholecystectomy | F | 65 |
| .584 | H1 | 10/19/07 | Cholecystectomy | F | 50 |
| .587 | H1 | 10/10/07 | Cholecystectomy | F | 56 |
| .591 | H1 | 10/07/07 | Cadioplasty | M | 56 |
| .592 | H1 | 11/22/07 | Cadioplasty | F | 55 |
| .593 | H2 | 08/20/07 | Cholecystectomy | M | 47 |
| .595 | H1 | 09/11/07 | Cadioplasty | F | 45 |
| .596 | H1 | 11/21/07 | Cholecystectomy | M | 68 |
| .598 | H1 | 11/07/07 | Cadioplasty | M | 56 |
| .602 | H1 | 11/19/07 | Cholecystectomy | F | 37 |
| .604 | H2 | 08/23/07 | Appendicetomy | M | 29 |
| .606 | H1 | 10/26/07 | Cadioplasty | F | 57 |
| .607 | H1 | 10/20/07 | Cholecystectomy | F | 72 |
| .608 | H1 | 11/13/07 | Cadioplasty | F | 58 |
| .609 | H3 | 11/09/07 | Bariatric surgery | F | 34 |
| .610 | H1 | 10/19/07 | Myomectomy | F | 36 |
| .611 | H2 | 10/13/07 | Bariatric surgery | M | 62 |
| .612 | H1 | 10/10/07 | Cadioplasty | M | 77 |
| .613 | H1 | 10/09/07 | Oophoroplasty | F | 28 |
| .614 | H4 | 10/29/07 | Bariatric surgery | M | 32 |
| .615 | H2 | 10/29/07 | Bariatric surgery | M | 38 |
| .616 | H1 | 11/12/07 | Cholecystectomy | F | 54 |
| .617 | H2 | 08/24/07 | Appendicetomy | M | 49 |
| .618 | H1 | 10/24/07 | Cadioplasty | F | 32 |
| .585 | H1 | 12/05/07 | Herniorrhaphy | M | 54 |
| .588 | H2 | 10/24/07 | Gastroplasty | M | 50 |
| .590 | H1 | 11/05/07 | Cadioplasty | M | 56 |
| .597 | H1 | 11/19/07 | Cholecystectomy | M | 65 |
| .599 | H1 | 10/08/07 | Cholecystectomy | M | 81 |
| .600 | H1 | 09/19/07 | Pyeloplasty | F | 50 |
| .601 | H1 | 11/14/07 | Cholecystectomy | F | 24 |
| .605 | H1 | 11/24/07 | Cholecystectomy | M | 87 |
| .603 | H1 | 11/21/07 | Cadioplasty | F | 43 |
| .619 | H1 | 11/06/07 | Thoracic Simpathetomy | F | 26 |
| .0020 | RJ | 2006 | Cholecystectomy | F | 56 |
| .306 | H1 | 11/22/07 | Gastroplasty | F | 47 |
| .308 | H1 | 10/28/07 | Cholecystectomy | F | 39 |

**Figure 3:** PFGE patterns of 36 *M. massiliense* isolates from Curitiba outbreak. Identical patterns clustered the isolates from surgical patients. Comparison was performed with a commercial software (Applied Maths). The percentages of similarity among the profiles were calculated using Dice coefficient. The PFGE patterns of *M. massiliense* isolated from Rio de Janeiro outbreak (CRM 0020) and two epidemiologically unrelated strains from Rio de Janeiro patients (CRM 0270 and CRM 0273) were included in the analysis. [a] Hn, mycobacterial isolate from patient who undergone surgery at hospital number n; [b] RJ, mycobacterial isolate collected from Rio de Janeiro city.

oxide plasma or low-temperature steam with formaldehyde gas. Since these measures have been adopted by the hospitals no additional case of RGM infection was registered by the Brazilian Public Health Surveillance System.

The obtained results suggest that a single *M. massiliense* clone might be responsible for the infections that have occurred in Northern, Central and Southeastern regions of Brazil and reinforce the concept of *M. massiliense* BRA100 as an emergent pathogen in Brazilian hospital surgical environment.

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