

***Mycobacterium massiliense* BRA100 strain recovered from postsurgical infections: resistance to high concentrations of glutaraldehyde and alternative solutions for high level disinfection¹**

Mycobacterium massiliense clone BRA100 associado a infecções pós-cirúrgicas: resistência a altas concentrações de glutaraldeído e produtos alternativos para desinfecção de alto nível

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ABSTRACT

Purpose: To evaluate the minimum inhibitory concentration (MIC) of GTA against these microorganisms and alternative disinfectants for high-level disinfection (HLD). **Methods:** Reference mycobacteria and clinical *M. massiliense* strains were included in this study. Active cultures were submitted to susceptibility qualitative tests with GTA dilutions (ranging from 1.5% to 8%), and commercial orthophthaldehyde (OPA) and peracetic acid (PA) – based solutions, during the period of exposure as recommended by National Agency of Sanitary Surveillance for HLD. **Results:** All reference and *M. massiliense* non-BRA100 strains, recovered from sputum, were susceptible to any GTA concentration, OPA and PA solutions. *M. massiliense* BRA100 strains presented MIC of 8% GTA and were susceptible to OPA and PA. **Conclusion:** *M. massiliense* BRA100 strain is resistant to high GTA concentrations (up to 7%), which proves that this product is non-effective against specific rapidly growing mycobacteria and should be substituted by OPA or PA – based solutions for HLD.

Key words: Mycobacterium Infections. Videolaparoscopy. Disinfection. Glutaraldehyde.

RESUMO

Objetivo: Avaliar a concentração mínima inibitória (CMI) de GTA frente a *M. massiliense* e a susceptibilidade a produtos alternativos para desinfecção de alto nível (DAN). **Métodos:** Cepas de *M. massiliense* de origem clínica e de referência foram incluídas no estudo. As culturas ativas foram submetidas a testes qualitativos com diluições de GTA (de 1,5% a 8%) e com soluções comerciais de orto-ftalaldeído (OPA) ou ácido peracético (PA), utilizando os tempos de exposição recomendados pela Agência Nacional de Vigilância Sanitária para DAN. **Resultados:** Todas as cepas de referência e *M. massiliense* não-BRA100, obtida de escarro, foram susceptíveis às concentrações de GTA, e soluções de OPA e PA. As cepas de *M. massiliense* BRA100 apresentaram CMI de 8% para GTA e foram susceptíveis a OPA e PA. **Conclusão:** *M. massiliense* BRA100 é resistente a altas concentrações de GTA (até 7%), o que demonstra que esse composto não é eficaz, e deve ser substituído por OPA ou PA nos processos de DAN.

Descritores: Mycobacterium Infections. Videolaparoscopy. Disinfection. Glutaraldehyde.

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Introduction

Rapidly-growing mycobacteria (RGM) are usually considered saprophytes and have been recovered from the environment, particularly in dust, watery soil and water distribution systems¹. Moreover, RGM are described as rare opportunistic pathogens involved in nosocomial infections and pseudo-outbreaks, and should hence be considered as an important group of bacteria showing increasing pathological importance. Diseases caused by RGM vary among health-care associated infections, wound infections, disseminated cutaneous disease, bone and joint infections, keratitis, pulmonary disease and other affections¹.

Among the most preeminent RGM species, *Mycobacterium abscessus*, *Mycobacterium chelonae* and *Mycobacterium fortuitum* have been frequently associated to diseases, especially posttraumatic wound infections, in a variety of clinical settings¹. The affected patients are generally healthy and the majority of infections evolves with significant clinical signs after 4 weeks following traumatic procedures or fractures. Some cases and short outbreaks have also been related to medical or surgical procedures including needle injections, videolaparoscopy, cardiac surgery and esthetics procedures^{1,2}.

The species *Mycobacterium massiliense* was proposed in 2004 and the name validated in 2006³. Subsequently to these publications, *M. massiliense* was detected as a cause invasive infections of two patients (pacemaker pocket infection and blood infection, respectively), and cited as an emerging pathogen. The description of this species as a common human pathogen and its epidemiological importance have possibly been hindered by misidentification of *M. massiliense* strains through years, especially due to the high phenotypic and genotypic (PRA-hsp65 and 16S sequencing) similarities between *M. massiliense* isolates and *M. chelonae*-*M. abscessus* group^{4,5}.

In Brazil, infections related to RGM species have been documented and usually described as sporadic cases of skin or lung infections in routine clinical laboratories, or short outbreaks related to breast implant surgery, laser in situ keratomileusis (LASIK) or mesotherapy sessions⁵⁻⁷.

The first significant *M. massiliense* outbreak was recently described in the city of Belém, state of Pará, northern region of Brazil, from 2004 to 2005. Fifty-eight *M. massiliense* isolates were recovered from confirmed patients submitted to laparoscopic surgeries. These individuals presented local hyperemia, abscess formation with inflammatory aspects, serous or purulent secretions and other clinical signs, and no response to common antimicrobial therapy⁵.

Since then, a series of nosocomial outbreaks of wound infections by *M. massiliense* related to video laparoscopic have been notified in several Brazilian states. These include at least the states of Distrito Federal, Espírito Santo, Goiás, Mato Grosso, Minas Gerais, Pará, Rio Grande do Sul, Rio de Janeiro e São Paulo states accounting for over 1000 cases^{8,9}.

From 2006 to 2007, an epidemic (1051 reported cases) of postsurgical infection related to RGM in Rio de Janeiro state, Brazil, was announced to the National Ministry of Health. Out of the confirmed patient-cases submitted to video laparoscopic surgery during this period of time, 148 RGM isolates were recovered and characterized, including one isolate obtained from

surgical instruments¹⁰. Representative isolates were identified as *M. massiliense* and belonged to a single clone named BRA100, which was genotypically identical to isolates obtained from previous outbreaks. Our results showed that *M. massiliense* BRA100 strain was also resistant to 2% glutaraldehyde (GTA) commercial solutions, commonly used for preoperative high-level disinfection in Brazil^{10,11}.

In order to better characterize disinfectant resistance of *M. massiliense* BRA100 strain and other mycobacterial species, the aim of this study was to evaluate the minimal inhibitory concentration (MIC) of GTA against these microorganisms and the susceptibility to alternative high-level disinfection products such as orthophthaldehyde (OPA) and peracetic acid (PA) – based commercial solutions.

Methods

Mycobacterial isolates

Representative isolates (CRM – 0018 and CRM – 0019), recovered from biopsies during the epidemic of postsurgical infections after videolaparoscopic procedures in the state of Rio de Janeiro (2006-2007), and previously identified as belonging to *M. massiliense* BRA100 strain were used for this investigation¹⁰. Additionally, *M. massiliense* isolate (CRM – 270) obtained from sputum of non-epidemiologically related patient and not belonging to BRA100 clone was also evaluated.

For comparative study, reference strains of taxonomically related species (*M. abscessus* ATCC 19977, *M. chelonae* ATCC 35752 and *M. fortuitum* ATCC 6841) and *Mycobacterium smegmatis* INCQS 00061 and *Mycobacterium bovis* INCQS 00062, strains mandatorily used for mycobactericidal efficacy test based on National Agency Sanitary Surveillance (ANVISA) publications (ANVISA, Portaria 15/88), were included.

Glutaraldehyde tolerance assay

The MIC of GTA against reference and clinical mycobacteria were evaluated concerning their ability to survive after 30 min of exposure to 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0%, 4.5%, 5.0%, 5.5%, 6.0%, 7.0% and 8.0% by using qualitative test protocols^{10,12,13}. The above described solutions were obtained by diluting two concentrated commercial solutions prepared as recommended by the manufacturers. The MIC was defined as the lowest GTA concentration able to inhibit the visible mycobacterial growth.

Mycobacterial suspensions with a turbidity equivalent to a McFarland 1 standard were prepared from cultures no older than 7 days grown on Lowenstein-Jensen (LJ) medium at 35°C, except for *M. bovis* INCQS 00062 which was cultivated for 28 days at the same temperature. Five hundred microliters of mycobacterial solutions were added to 4.5 ml of each activated glutaraldehyde solution and incubated at 25°C. After 30 min, an aliquot of 0.5 ml of each mixture containing bacteria and glutaraldehyde was transferred to a new vial containing the same volume of 1% sodium sulfite (1:1, v/v). They were mixed, seeded on LJ slants and incubated at 35°C in ambient air for up to 60 days. Each assay was repeated three times and pH was determined for quality control.

OPA and PA susceptibility assays

The susceptibility of representative *M. massiliense* strains to 0.55% OPA and 3 different PA – based commercial solutions was also determined by using qualitative test assays previously described^{10,12,13}. The strains were evaluated concerning their ability to survive after 15 and 30 min of exposure to disinfectant solutions using the suspension method, as recommended by the manufacturers and ANVISA for high-level disinfection. Each assay was repeated three times and also included *Mycobacterium smegmatis* INCQS 00061, *Mycobacterium bovis* INCQS 00062, *M. abscessus* ATCC 19977, *M. chelonae* ATCC 35752 and *M. fortuitum* ATCC 6841 as quality control strains.

Results

Glutaraldehyde tolerance

Both *M. massiliense* isolates tested belonging to the clonal group BRA100 (CRM-0018 and CRM-0019) survived after

30 min of exposure to usual GTA concentrations used in commercial products such as 1.5%, 2.0% and 2.5% solutions. Assays including higher concentrations of GTA were performed in order to determine the MIC. For these microorganisms, the MIC consisted of 8.0% GTA (Table 1).

All the other strains, including *M. smegmatis* INCQS 00061, *M. bovis* INCQS 00062, *M. abscessus* ATCC 19977, *M. chelonae* ATCC 35752, *M. fortuitum* ATCC 6841 and the unrelated non-BRA100 *M. massiliense* isolate (CRM-0270) did not survive after 30 min of exposure to any GTA concentration. These strains presented MIC < 1.5% GTA solutions as showed in Table 1. The results were reproducible and identical considering the two different commercial concentrated solutions.

OPA and PA mycobacterial susceptibility

All the reference and *M. massiliense* strains presented susceptibility to OPA and PA – based commercial solutions after 15 and 30 min of exposure. The results were also reproducible.

TABLE 1 - Disinfectant susceptibility of *M. massiliense* clinical isolates and reference strains

Strain	1.5% ^a	2.0%	2.5%	3.0%	3.5%	4.0%	4.5%	5.0%	5.5%	6.0%	7.0%	8.0%	OPA ^b	PA ^c
CRM-0018	+	+	+	+	+	+	+	+	+	+	+	-	-	-
CRM-0019	+	+	+	+	+	+	+	+	+	+	+	-	-	-
CRM-0270	-	-	-	-	-	-	-	-	-	-	-	-	-	-
INCQS 00061	-	-	-	-	-	-	-	-	-	-	-	-	-	-
INCQS 00062	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ATCC 19977	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ATCC 35752	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ATCC 6841	-	-	-	-	-	-	-	-	-	-	-	-	-	-

^a GTA concentration; ^b OPA, orthophthaldehyde-based commercial solution after 15 and 30 min of exposure; ^c PA, peracetic acid-based commercial solution after 15 and 30min of exposure.

Discussion

Infections after invasive procedures by RGM had rarely been reported in Brazil before 2004, usually as limited outbreaks or isolated cases. In the state of Rio de Janeiro and São Paulo (southeast region of Brazil), from 1998 to 2003, non-related keratitis outbreaks affecting patients submitted to surgical correction of myopia, related to laser *in situ* keratomileusis (LASIK) surgery caused by *M. abscessus* and *M. chelonae*, were the first detailed description of RGM infections associated to invasive procedures in this country^{6,14}. One specific ocular infections outbreak in 2003 was caused by *M. immunogenum* species, a recently described pathogen, and the clonal relationship among the isolates was detected by PFGE and ERIC-PCR typing techniques⁶. All these outbreaks were restricted to determined ophthalmologic clinics and no specific source of transmission was detected. Other outbreaks of RGM infections were also related to esthetics procedures such as mesotherapy in 2000 and 2002, in the state of São Paulo, and were associated to *M. chelonae* and *M. abscessus* species, respectively⁶. Between 2002 and 2004, another outbreak of post-mammoplasty infections caused by *M. fortuitum* in the city of

Campinas, state of São Paulo, affected 33 patients and the clonal relationship among different clusters of 12 isolates recovered from culture-positive cases was established by different typing methods^{6,7}.

From 2004 to 2005, 311 cases of RGM wound infections after mesotherapy or video laparoscopic surgery caused by *M. bolletii* and *M. massiliense* species, respectively, were notified in the city of Belém, state of Pará⁵. The post-video laparoscopic surgery infections caused by *M. massiliense* species, involving at least 58 patients (positive cultures), were determined by a unique clone and spread around 16 private hospitals and related to different surgeons. The source of the surgical infections was not identified. By the other hand, some aspects were common to all the reported cases such as the fact that all the laparoscopic equipments were high-level disinfected by immersion in 2% glutaraldehyde and inconsistencies in equipment cleaning procedures and exposure times were detected. After this first significant *M. massiliense* postsurgical infections outbreak, several other were described in at least 15 Brazilian states and associated to a specific *M. massiliense* clone (BRA100) probably presenting particular biological properties for environmental survival^{5,8,9,10}.

These apparently uncontrolled outbreaks, or Brazilian epidemic of *M. massiliense* postsurgical infections as named by some authors, exhibit common and specific details which may explain some epidemiological questions. All these described outbreaks were related to videolaparoscopic surgeries and / or other invasive procedures, preceded by high-level disinfection technique through the use of 2% commercial GTA for 15 to 30 min of different brands, and no similar epidemic-like events had been described before.

Previous post-laparoscopic infections by mycobacteria have been described as isolated cases or limited outbreaks^{2,15,16}. Surgical site infections related to *M. chelonae* strains consist of the most frequent published reports, but one of the outbreaks included *Mycobacterium tuberculosis* wound infections affecting 8 patients submitted to videolaparoscopic surgery in a hospital in India¹⁶. In Brazil, the first video laparoscopic surgery (colecistectomy) was practiced in the state of São Paulo in 1990, and almost 70% of the abdominal invasive procedures have been done by using this surgical technique in this country. Most of these surgeries have been preceded by the chemical method for high-level disinfection with 2% GTA. In 2007, this chemical disinfection was prohibited by the ANVISA due to the high suspicion of a possible low activity of the biocide solution. Inadequate procedures for dismantling, cleaning, or remove of organic material from the laparoscopic equipments, the reuse of disposable medical instruments, and the absence of effective control were also suggested as possible additional factors promoting the epidemic in different Brazilian states. No validation schemes for disinfection or specific surveillance procedures had been applied in some hospitals with confirmed cases. Furthermore, many surgeons worked at many different hospitals and had their particular laparoscopic instruments been washed and disinfected together with other surgical teams equipment. The substitution of 2% GTA for PA or physical sterilization were the main procedures used for enough control of the dissemination of infections and blocking the outbreak in all the hospitals.

Although 2% GTA resistance is considered a rare event in mycobacteria, RGM strains with low susceptibility to GTA have already been described, most of them related to *M. chelonae* species¹⁷⁻¹⁹. Nomura *et al.*¹⁸ submitted 4 randomly selected *M. chelonae* isolates recovered from bronchoscope washing machines to GTA suspension tests. The results indicated viable and reproducible mycobacterial cells even after 60-min exposure to this biocide. Svetlíkova *et al.*¹⁹ have proposed that defects of mycobacterial porins may be possibly represent the main mechanisms involved in resistance to aldehyde-based disinfectants.

Our laboratory studies have indicated that *M. massiliense* BRA100 isolates recovered during the epidemic in the state of Rio de Janeiro survive abundantly after 30min up to 10h of exposure to commercial GTA solutions, which may have contributed to the possible dissemination of biocide-tolerant microorganism around the surgical centers in different hospitals^{10,11}. The present study indicated that resistance to high GTA concentrations (MIC = 8%) is a particular characteristic of *M. massiliense* belonging to BRA100 clonal group. This specific clone may harbor potential biological mechanisms of resistance not shared by other *M. massiliense* strains or by other species. It is supposed that the main mode of action of GTA consists of cross-linking with proteins at the mycobacterial

cell wall compounds and cytoplasm causing the inhibition of the DNA, RNA and other macromolecules' synthesis. The inclusion of *M. massiliense* BRA100 in molecular studies for disinfectant's mode of action and mechanisms of resistance will be important to improve the knowledge about mycobacterial survival in stressing environmental conditions. Further studies will be essential for developing new strategies for high-level disinfection and preventing the emergence of new resistant strains.

Conclusion

M. massiliense BRA100 clinical isolates presented significant resistance to high concentrations of GTA, which has been used for hospital high-level disinfection worldwide. Susceptibility to high-level disinfectants such as OPA and PA was detected, which may represent immediate alternatives for preventing other possible outbreaks of postsurgical infections.

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