Temas em Saúde
Volume 21, Número 5
ISSN 2447-2131
João Pessoa, 2021

Artigo

BIOFILMS FORMED BY *MYCOBACTERIUM ABSCESSUS* SUBSP. *BOLLETII* IN THE PRESENCE OF SUBINHIBITORY CONCENTRATIONS OF GLUTARALDEHYDE - A PUBLIC HEALTH ISSUE

BIOFILMES FORMADOS POR *MYCOBACTERIUM ABSCESSUS* SUBSP. *BOLLETII* NA PRESENÇA DE CONCENTRAÇÕES SUBINIBITÓRIAS DE GLUTARALDEÍDO - UM PROBLEMA DE SAÚDE PÚBLICA

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ABSTRACT - Introduction: In 2008, the National Health Surveillance Agency (Anvisa) released a technical note, stating that from 2003 to 2008, more than 2,000 cases of hospital infections by rapidly growing mycobacteria had been reported in private hospitals in the country, mainly related to procedures videolaparoscopic procedures. A common factor was the predominance of a particular *Mycobacterium abscessus* subsp. *bolletii*, which mainly affected the patients' skin and subcutaneous cellular tissue. After these outbreaks, several measures were taken by Anvisa, including the suspension of chemical sterilization

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DOI: 10.29327/213319.21.5-7
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by immersing surgical instruments in any liquid sterilizing agent. The investigations concluded that the outbreaks were due to failures in the reprocessing of critical medical equipment, which were mainly sterilized using 2% glutaraldehyde. These decisions were based on empirical observations, based exclusively on the observation of what was happening in hospital practice. To date, the relationship of *M. abscessus* subsp. *bolletii* and its high tolerance to glutaraldehyde. This study aimed to evaluate biofilm formation of *M. abscessus* subsp. *bolletii*, from an epidemic outbreak in Brazil, and *M. abscessus* subsp. *abscessus*, a reference strain, at different concentrations of glutaraldehyde. **Methodology**: The biofilms were developed in polycarbonate and stainless-steel discs for colony-forming units analysis and cell culture plates for analysis of confocal laser scanning microscopy (CLSM). **Results**: The results of the comparative analysis of biofilm formation by *M. abscessus* subsp. *bolletii* demonstrated that the microorganism was able to form biofilm on both disc types, even at high concentrations of glutaraldehyde. There was a reduction in the formation of biofilm when exposed to concentrations of 1.0% and 1.5% of glutaraldehyde, without being destroyed. *M. abscessus* subsp. *abscessus* was also able to form biofilm on both disc types, but biofilm was destroyed at glutaraldehyde concentrations of 1.0% and 1.5%. The two strains analyzed by CLSM developed biofilm after 7 and 14 days of incubation. It was also observed the presence of viable cells in the biofilm, even after 14 days of growth, in the presence of high concentrations of glutaraldehyde. **Conclusions**: These results confirmed the ability of the *M. abscessus* species to survive and develop in glutaraldehyde and may be related to the occurrence of the outbreaks. We hope that the data obtained from this work can effectively contribute to the control and prevention of these infections, as it is a serious matter of national public health.

Keywords: *Mycobacterium abscessus* subsp. *bolletii*; *Mycobacterium abscessus* subsp. *abscessus*; Glutaraldehyde; Biofilm

RESUMO - Introdução: Em 2008, a Agência Nacional de Vigilância Sanitária (Anvisa) divulgou uma nota técnica, informando, que de 2003 até 2008, haviam sido notificados mais de 2.000 casos de infecções hospitalares por Micobactérias de Crescimento Rápido em hospitais particulares do país, relacionados principalmente a procedimentos videolaparoscópicos. Um fator comum foi a predominância de um determinado clone de

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Mycobacterium abscessus subsp. *bolletii*, que acometeu principalmente a pele e o tecido celular subcutâneo dos pacientes. Após esses surtos, várias medidas foram tomadas pela Anvisa, entre elas, a suspensão da esterilização química por imersão de instrumental cirúrgico em qualquer agente esterilizante líquido. As investigações concluíram que os surtos se deviam a falhas no reprocessamento de equipamentos médicos críticos, que eram esterilizados principalmente pelo uso de glutaraldeído a 2%. Essas decisões se basearam em observações empíricas, pautadas exclusivamente na observação do que estava ocorrendo na prática hospitalar. Até hoje não foi elucidada a relação de *M. abscessus* subsp. *bolletii* e a sua alta tolerância ao glutaraldeído. Este estudo teve como objetivo avaliar a formação de biofilme de *M. abscessus* subsp. *bolletii*, de um surto epidêmico no Brasil, e *M. abscessus* subsp. *abscessus*, uma cepa de referência, em diferentes concentrações de glutaraldeído. **Metodologia**: Os biofilmes foram desenvolvidos em discos de policarbonato e aço inoxidável para análise de unidades formadoras de colônias e placas de cultura de células para análise em microscopia confocal de varredura a laser (CLSM). **Resultados**: Os resultados da análise comparativa da formação de biofilme por *M. abscessus* subsp. *bolletii* demonstrou que o microrganismo foi capaz de formar biofilme em ambos os tipos de disco, mesmo em altas concentrações de glutaraldeído. Houve redução na formação de biofilme quando exposto às concentrações de 1,0% e 1,5% de glutaraldeído, sem ser destruído. *M. abscessus* subsp. *abscessus* também foi capaz de formar biofilme em ambos os tipos de disco, mas o biofilme foi destruído nas concentrações de glutaraldeído de 1,0% e 1,5%. As duas cepas analisadas por CLSM desenvolveram biofilme após 7 e 14 dias de incubação. Também foi observada a presença de células viáveis no biofilme, mesmo após 14 dias de crescimento, na presença de altas concentrações de glutaraldeído. **Conclusões**: Esses resultados confirmaram a capacidade da espécie *M. abscessus* de sobreviver e se desenvolver em glutaraldeído e podem estar relacionados à ocorrência dos surtos. Esperamos que os dados obtidos com a realização deste trabalho possam contribuir de maneira efetiva no controle e prevenção dessas infecções, por ser uma questão séria de saúde pública nacional.

Palavras-chave: *Mycobacterium abscessus* subsp. *bolletii*; *Mycobacterium abscessus* subsp. *abscessus*; Glutaraldeído; Biofilme

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INTRODUCTION

Infections caused by rapidly growing mycobacteria (RGM) are being recognized as real and growing threats worldwide (AKSAMIT; PHILLEY; GRIFFITH, 2014). *Mycobacterium abscessus* is the RGM most often associated with human diseases and is recognized as a major cause of skin and soft tissue infections, inoculation skin injury, minor trauma, and nosocomial infections in abscesses and surgeries (MEDJAHED; GAILLARD; REYRAT, 2010; MOUGARI et al., 2016).

The *M. abscessus* taxonomy has undergone many changes in recent years (LEÃO et al., 2011; LEE et al., 2015). Genome analyzes have shown a division of the *M. abscessus* species into three subspecies - *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp. *massiliense* (CHO et al., 2013) - altering a previously described classification in which *M. abscessus* was divided into only two subspecies (LEÃO et al., 2011).

M. abscessus subsp. *bolletii* appeared as an emerging pathogen, first described by Adékambi et al. (2004), being isolated from the sputum and bronchoalveolar fluid of a patient with hemoptysis pneumonia. This mycobacterium has been related to reports of postoperative infections such as mammoplasty, liposuction, aesthetic treatments, intramuscular injections, keratitis after laser surgery, and laparoscopic bacteremia (KIM et al., 2007; DUARTE et al., 2009; CHO et al., 2010), and has also been associated with outbreaks that occurred in several states in Brazil from 2003 to 2014, mainly related to video laparoscopic procedures (NUNES et al., 2014; VILLAR et al., 2015).

These microorganisms are ubiquitous and can be found in soils, water, and even in disinfectant or biocidal solutions (BROWN-ELLIOTT; WALLACE, 2002). They survive in adverse conditions (low nutrients, low pH, and extreme temperatures) and can be isolated from biofilms, dust, and aerosols (FALKINHAM III, 2002; DE GROOTE; HUITT, 2006). RGM are resistant to broad-spectrum antibiotics and disinfectants due to their hydrophobicity, impermeability, and slow growth, which is mainly attributed to mycolic acids, which help provide a permeability barrier (ZAMBRANO; KOLTER, 2005; FALKINHAM III, 2009).

The presence of biofilms is a key factor in the resistance of microorganisms to antibiotics and disinfectants (FLORES et al., 2016). This property has been documented related to RGM and appears to be widely distributed among these organisms and has been

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observed for decades in clinical and environmental samples (MARTIN-DE-HIJAS, 2009).

The mechanisms responsible for biofilm formation by these microorganisms are unknown (WILLIAMS et al., 2009). Analysis of mycobacterial genomes suggests that they do not have the property to produce exopolysaccharides. Nevertheless, mycobacteria form biofilms, binding to hydrophobic solid surfaces or floating like films on the surface of liquid culture media (FERNÁNDEZ-ROBLAS et al., 2008). A rapid aggregate formation has also been observed during cell growth in submerged culture in a liquid medium (ZAMBRANO; KOLTER, 2005). The morphology and growth strategy of mycobacterial biofilms depend on the type of attachment surface as well as the nutrient levels resulting in the formation of microcolonies or cord structures (SOCHOROVÁ et al., 2014).

The organization of biofilm makes it difficult to eradicate these mycobacteria with common decontamination practices and is relatively resistant to standard disinfectants such as chlorine, organomercurials, and alkaline glutaraldehydes (WILLIAMS et al., 2009, RÜEGG et al., 2015).

Several authors have described RGM resistance to disinfectants, pointing out problems regarding the use of glutaraldehyde in disinfecting equipment such as the endoscope washer and disinfector (VAN KLINGEREN; PULLEN, 1993; GRIFFITHS et al., 1997). Van Klingeren and Pullen (1993) showed that constant disinfection of glutaraldehyde equipment led to the selection of *M. chelonae* strains with reduced susceptibility to this agent. The same occurred in another study, in which strains of the same bacterium were isolated from two endoscopes and behaved resistantly to the 2% glutaraldehyde solution (GRIFFITHS et al., 1997). Recently, outbreaks of *M. abscessus* subsp. *bolletii* have also been associated with the development of resistance and tolerance to disinfectant (DUARTE et al., 2009; 26].

These outbreaks of post-surgical infections by *M abscessus* subsp *bolletii* in different regions of Brazil were attributed to the selection and dissemination of a virulent epidemic isolate, characterized by a high level of resistance to glutaraldehyde, the disinfectant used in the hospitals involved in the cases (MONEGO et al., 2011; SHANG et al., 2011).

This study aimed to evaluate the biofilm formation of *M. abscessus* subsp. *bolletii* CBRVS (Coleções de Bactérias de Referência em Vigilância Sanitária) 00594 from an epidemic outbreak in Brazil and *M. abscessus* subsp. *abscessus* ATCC 19977 a reference

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strain of the species, at different concentrations of glutaraldehyde. Thus, with these data, Anvisa and local surveillance will be able to take more effective measures to control these infections that occur in Brazil until today.

METHODOLOGY

Microorganisms

M.abscessus subsp. *bolletii* CBRVS 00594 from an outbreak and belonging to clone BRA 100 and the reference strain *M. abscessus* subsp. *abscessus* ATCC 19977 were used in this study (DUARTE et al., 2009). To prepare the inoculums, all strains were cultivated on Middlebrook 7H10 agar (Becton Dickinson, Germany) for 7 days at 37°C.

Glutaraldehyde

To prepare 0.5%, 1.0%, and 1.5% glutaraldehyde solutions the 25% glutaraldehyde solution (1.5 pentanodial, $C_5H_8O_2$) (Merck, Germany) was used.

Biofilm preparation and quantification

Biofilms were developed as described by Williams et al. (2009) with some modifications. Autoclaved stainless steel (SS) (Corning Incorporated, Corning, NY) and polycarbonate (PC) (Corning Incorporated, Corning, NY) discs were incubated in a 24-well tissue culture plate. The discs were covered with 1.0 mL of modified Proskauer Beck (PB) broth containing glutaraldehyde at 0.5%, 1.0%, and 1.5% (v/v) final concentrations per well. The bacterial suspensions, collected from Middlebrook 7H10 plates, were made in a PB medium; the concentration was determined by measuring the absorbance in a spectrophotometer (Shimadzu 1601 Uv-Vis, Japan) at 620 nm. Cells were diluted in PB and 100 µl of approximately 10^9 colony forming units (CFU) were inoculated per well. Inoculation concentration was confirmed by enumerating CFU, these bacterial suspensions were serially diluted, plated on Mueller-Hinton (MH) agar plates, and incubated at 37°C for 7 days. At the end of the incubation, each disk per condition was removed, dipped three times with gentle up and down movements in a beaker containing

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phosphate-buffered saline (PBS) to promote the complete removal of all non-attached bacteria. These discs were processed for CFU and microscopic analysis. For quantification, the discs were placed in 50 mL polypropylene centrifuge tubes containing 10 mL PBS with 0.1% Tween 80. Bacteria were removed from the disks' surfaces by applying three sonication cycles (1 min), followed by stirring for 10 minutes. From this suspension, 0.5 mL aliquots were taken from each tube, and bacterial suspensions were serially diluted from 10^{-1} to 10^{-5} with 4.5 mL of 0.00425% monopotassium phosphate buffer and plated on MH agar. The plates were incubated for 5 to 7 days at 36°C. In the same 24-well plate, sterility controls of discs, culture media used with and without glutaraldehyde, and viability of the microorganism were performed. The experiment was performed in triplicate and was repeated three times.

Confocal laser scanning microscopy

This step was developed as described by Fiallos et al. (2019) with some modifications. The biofilms were developed in glass-bottom 4-compartment cell culture plates (Greiner Bio-One, Brazil). Each compartment was covered with 125 µL of modified PB broth containing glutaraldehyde at 0.5%, 1.0%, and 1.5% (v/v) concentrations and without glutaraldehyde. The suspensions of each mycobacterium were prepared as described previously. Cells were diluted in PB and 125 µl of approximately 10^9 CFU were inoculated per compartment. The plates were incubated for 7 and 14 days at 37°C. After 7 and 14 days, the non-adhering cells were removed by washing three times with PBS buffer. On the 8^{th} day of the incubation, the new medium was replaced. The plates were stained with 50 µL Live /Dead BacLight Feasibility Dye (Invitrogen, Eugene, OR, USA) according to the manufacturer's instructions and kept in the dark until analysis. The SYTO9 reagent allowed visualization of green fluorescence for viable sessile bacterial forms and propidium iodide red fluorescence for dead bacteria. The architecture analysis of the biofilm formed by mycobacteria was performed using the Leica TCS-SPE confocal laser scanning microscope (Leica, Germany) and the images generated were analyzed by the LAS X software. The experiment was conducted in triplicate.

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Statistical analysis

Grubbs's test was used to analyze aberrant values within the same experiment and Mann Whitney's nonparametric test was used to compare the results between experiments, and p-values less than 0.05 were considered statistically significant.

RESULTS

Enumeration of mycobacterial biofilm

Comparative analysis of the adherence level of cells present on SS and PC discs by *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *abscessus* at different concentrations of glutaraldehyde concentrations was performed. There was a significant difference between the numbers of CFU of *M. abscessus* subsp. *bolletii* recovered from 1.0% and 1.5% glutaraldehyde concentrations and the control, on SS (p-value 0.000532 and 0.001453, respectively) and PC (p-value 0.000349 and 0.000349, respectively) disks. No statistically significant difference was evident from 0.5% glutaraldehyde concentration on SS and PC (p-value 0.1557704 and 0.060602, respectively).

Regarding the comparative analysis of the adherence level of cells present on SS and PC discs for *M. abscessus* subsp. *abscessus*, a significant difference was observed only at 0.5% glutaraldehyde concentration (p-value 0.000464 and 0.012355 respectively). There was no observed growth of *M. abscessus* subsp. *abscessus* at 1.0% and 1.5% glutaraldehyde concentrations, in both discs (Table 1 and 2).

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Table 1 - Biofilm formation on stainless steel discs by *M. abscessus* subsp. *bolletii* CBRVS 00594 and *M. abscessus* subsp. *abscessus* ATCC 19977 in the presence of different concentrations of glutaraldehyde. The experiments were performed with incubation for 7 days at 36 ± 1ºC.

		M. abscessus subsp *bolletii*	*M. abscessus* subsp *abscessus*
		Mean (CFU)	Mean (CFU)
STAINLESS STEEL	Control	$7,7 \times 10^7$	$2,3 \times 10^7$
	0,5% Glutaraldehyde	$1,0 \times 10^8$	$4,0 \times 10^6$
	1,0% Glutaraldehyde	$2,2 \times 10^6$	*
	1,5% Glutaraldehyde	$6,4 \times 10^4$	*

* No growth was observed.
Values are expressed as the mean of triplicate in three independent experiments. Grubbs's test was applied to check aberrant values.
CFU = colony forming units.

Table 2 - Biofilm formation on policarbonate discs by *M. abscessus* subsp. *bolletii* CBRVS 00594 and *M. abscessus* subsp. *abscessus* ATCC 19977 in the presence of different concentrations of glutaraldehyde. The experiments were performed with incubation for 7 days at 36 ± 1ºC.

		M. abscessus subsp *bolletii*	*M. abscessus* subsp *abscessus*
		Mean (CFU)	Mean (CFU)
POLICARBONATE	Control	$7,1 \times 10^8$	$9,6 \times 10^7$
	0,5% Glutaraldehyde	$1,2 \times 10^8$	$8,6 \times 10^6$
	1,0% Glutaraldehyde	$7,7 \times 10^6$	*
	1,5% Glutaraldehyde	$2,3 \times 10^5$	*

* No growth was observed.
Values are expressed as the mean of triplicate in three independent experiments. Grubbs's test was applied to check aberrant values.
CFU = colony forming units.

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Image analysis

CLSM images showed mycobacteria biofilms on the surface of cell culture plates with viable and non-viable colonies (green and yellow/red, respectively). Figure 1 shows representative confocal microscopy images of *M. abscessus* subsp. *bolletii* CBRVS 00594 in the biofilm after 7 and 14 days of incubation in the absence and presence of different glutaraldehyde concentrations. *M. abscessus* subsp. *bolletii* CBRVS 00594 in the presence of all glutaraldehyde concentrations showed many viable and non-viable dispersed cells. This growth persists until the 14th day, and the formation of cellular aggregates and a dense layer of dead cells can be observed, demonstrating the persistence of these mycobacteria in high concentrations of glutaraldehyde.

Representative confocal microscopy images of *M. abscessus* subsp. *abscessus* ATCC 1977 in the biofilm after 7 and 14 days of incubation in the presence of different glutaraldehyde concentrations are shown in Figure 2. In *M. abscessus* subsp. *abscessus* ATCC 19977's biofilm, we observed the presence of scattered isolated cells and viable and non-viable aggregated cells.

In these glass surfaces, it was possible to observe the presence of cell growth in all concentrations of glutaraldehyde. These mycobacteria can efficiently form biofilms onto this surface until to 14th day of incubation, despite the growth of *M. abscessus* subsp. *abscessus* ATCC 1977 is smaller than the one presented by *M. abscessus* subsp. *bolletii* CBRVS 00594. Based on the biofilm structures, two types of biofilms could be distinguished. The *M. abscessus* subsp. *bolletii* CBRVS 00594 biofilms were very heterogeneous and contained large cell aggregates whereas the *M. abscessus* subsp. *abscessus* ATCC 1977 biofilms contained more evenly distributed cells.

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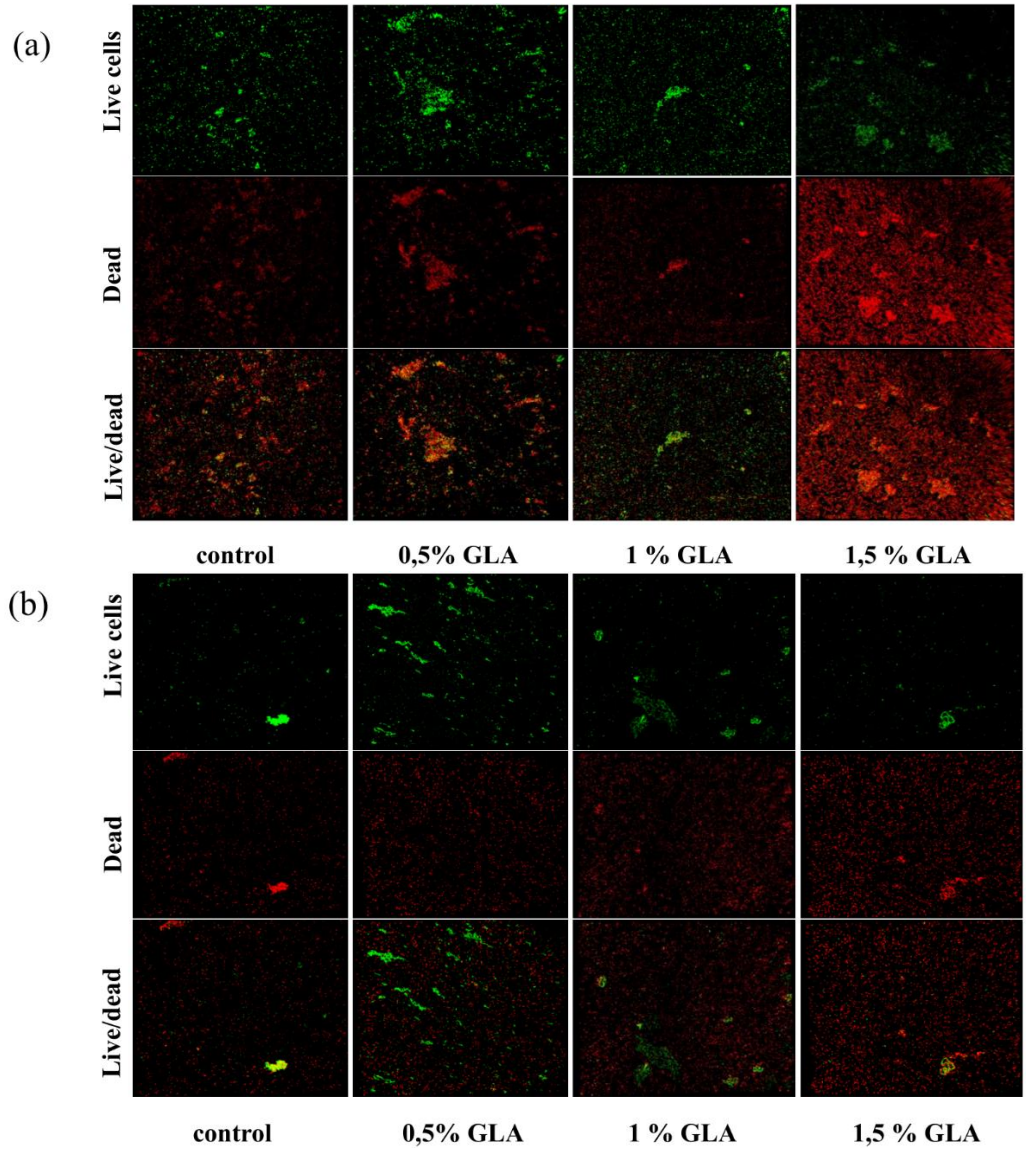

Figure 1. *M. abscessus* subsp *bolletii* CBRVS 00594's biofilm. Images of confocal microscopy obtained after 7 (A) and 14 (B) days of incubation in different assays by

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using Live/Dead BacLight. Green fluorescence labels live cells, whereas red fluorescence labels dead cells. The figure is representative of four experiments.

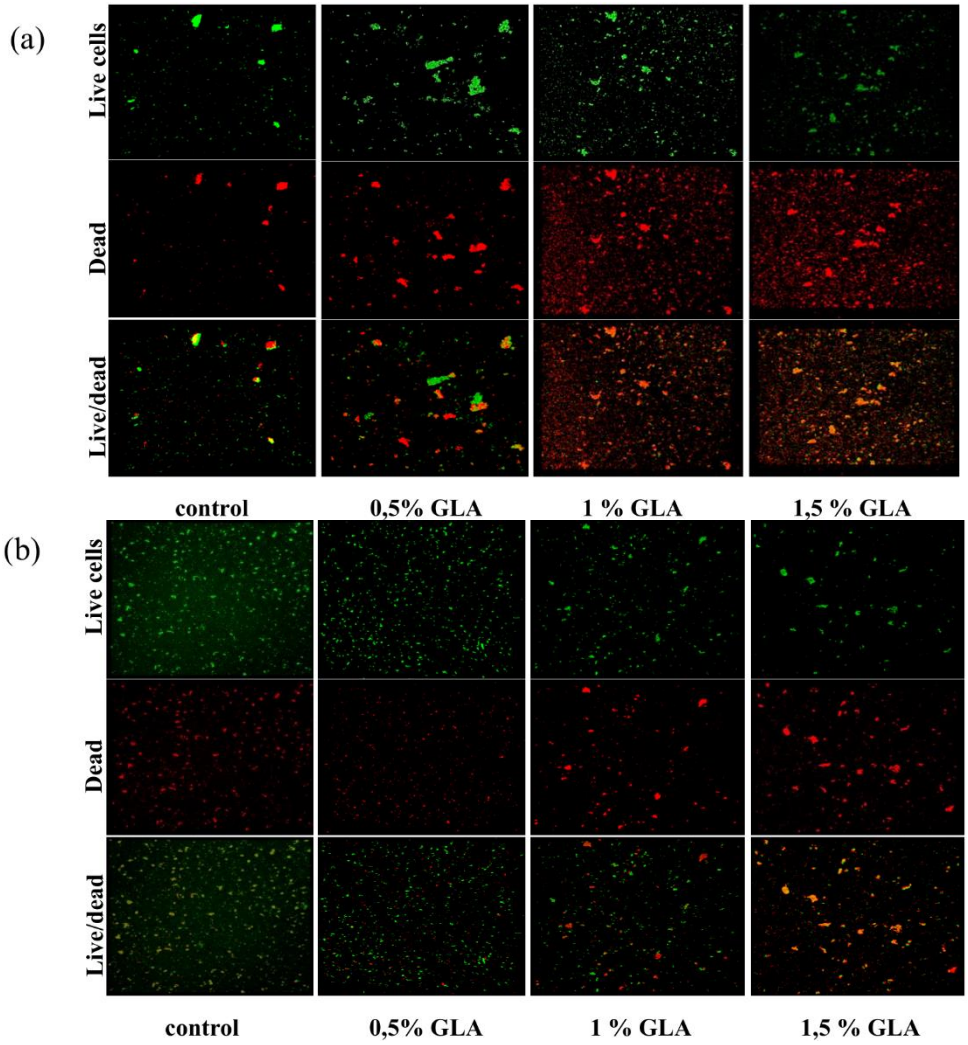

Figure 2. *M. abscessus* subsp *abscessus* ATCC 19977's biofilm. Images of confocal microscopy obtained after 7 (A) and 14 (B) days of incubation in different assays by

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using Live/Dead BacLight. Green fluorescence labels live cells, whereas red fluorescence labels dead cells. The figure is representative of four experiments

DISCUSSION

The first step of biofilm development consists of bacterial adhesion. After mycobacterial adherence, different stages of biofilm formation are observed, such as sessile growth, matrix synthesis, and dispersion (ESTEBAN; GARCÍA-COCA, 2018).

In this study, we found that *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *abscessus* had the property to form biofilm on SS and PC. Similar results were observed by Rodríguez-Sevilla et al. (2018) and Mullis and Falkinham III (2013), in which *M. abscessus* biofilms were cultivated and quickly adhered to polycarbonate membranes and SS surfaces, respectively. Williams et al. (2009) also reported a high affinity of *Mycobacterium fortuitum* to form biofilm on SS and PC.

Although Mycobacteria does not produce the usual exopolysaccharide components of the extracellular matrix, it can bind to different surfaces and form fully developed biofilms (ZAMBRANO; KOLTER, 2005; ZAMORA et al., 2007).

The relationship between RGM and biomaterials has been evaluated for decades and several studies confirm that RGM adheres to these surfaces (RIDGWAY; RIGBY; ARGO, 1984; VESS et al., 1993; MULLIS; FALKINHAM III, 2013; RODRÍGUEZ-SEVILLA et al., 2018). Vess et al. (1993) studied the ability of several species of mycobacteria, including *M. abscessus* and *Mycobacterium chelonae*, to adhere to polyvinyl chloride, and reported that both species were able to colonize the inner surface of polyvinyl chloride tubes and survived after exposure to various disinfectants; Ridgway, Rigby and Argo (1984) analyzed *Mycobacterium* sp. adherence to cellulose diacetate, and Zamora et al. (2007) studied RGM adherence to polypropylene. In addition, several studies have detected mycobacteria in biofilms (SCHULZE-RÖBBECKE; FISCHEDER, 1989; SCHULZE-RÖBBECKE; JANNING; FISCHEDER, 1992; SCHULZE-RÖBBECKE et al., 1995), as well as in vitro biofilm development by standard RGM strains (HALL-STOODLEY; KEEVIL; LAPPIN-SCOTT, 1998; MARTIN-DE-HIJAS et al., 2009). All these works confirm that adhesion to biomaterials is a crucial step for mycobacteria biofilm formation.

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Although no specific adhesion mechanism has been studied for mycobacteria, lipid metabolism and other related bacterial properties, such as sliding motility, have been implicated in the development of biofilm (RECHT; KOLTER, 2001; ZAMBRANO; KOLTER, 2005). The Mycobacterial cell wall comprises a high proportion of lipid molecules, which makes these bacteria more hydrophobic than other genera. Since hydrophobicity is an important mechanism of binding to biomaterials (KATSIKOGIANNI; MISSIRLIS, 2004), it is speculated that variations in cell wall lipids among RGM strains may explain the differences observed in the present study.

CLSM is an optical microscopy technique that has been used to analyze the phenotypic characteristics of biofilms formed by some RGM, such as growth rate, percentage of surface coverage, percentage of live/dead bacteria, and self fluorescence (ESTEBAN; GARCÍA-COCA, 2018), being useful to analyze differences among species and antimicrobial activity of disinfectants (FARIA; JOÃO; JORDÃO, 2015). Studies have been performed using this technique to evaluate the behavior of bacterial biofilms when exposed to microbicide products. Davison, Pitts, and Stewart (2010) demonstrated through CLSM that *Staphylococcus epidermidis* biofilms are not removed using glutaraldehyde. Muñoz-Egea et al. (2016) analyzed the effect of three antimicrobials on RGM biofilms. The analysis of the images from CLSM was used to evaluate the development and behavior of intrinsic fluorescence, covered area, thickness, and cell viability in RGM biofilms after adding antibiotics. Neves et al. (2016) observed the presence of viable cells within biofilms of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *M. abscessus* subsp. *bolletii* by confocal microscopy, after the action of various disinfectants, such as glutaraldehyde, peracetic acid, and orthophataldehyde.

In the present study, CLSM revealed the presence of viable cells in *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *abscessus* biofilms even after 14 days in the presence of glutaraldehyde. Muñoz-Egea et al. (2015) also observed the presence of viable cells in *M. chelonae* and *Mycobacterium smegmatis* biofilms in the presence of the ciprofloxacin antimicrobial. They also observed a significant reduction in mycobacteria viability after 72h of antimicrobial exposure.

Flores et al. (2016) reported that *M. abscessus* and *Mycobacterium massiliense* biofilms displayed resistance to clarithromycin and sulfamethoxazole, respectively. Although biofilms were reduced when other antimicrobials were used, none of them was able to completely eradicate mycobacterial biofilms.

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Aung et al. (2016) investigated the resistance of *M. chelonae* and *M. fortuitum* biofilms to antimicrobial compounds and found that mycobacterial biofilms were resistant to gatifloxacin. *M. chelonae* and *M. fortuitum* formed thick, irregularly shaped biofilms that were relatively resistant to conventional antibiotics even at high minimal inhibitory concentrations.

In our study, the presence of viable cells in *M. abscessus* subsp. *bolletii* CBRVS 00594 and *M. abscessus* subsp. *abscessus* ATCC 19977 biofilms in the presence of high concentrations of glutaraldehyde after 14 days of incubation led us to suppose that there may be a tolerance of these microorganisms to glutaraldehyde. Neves et al. (2016) also observed the presence of viable cells within *M. abscessus* subsp. *bolletii* CBRS 00549 biofilm in endoscope channels, even after glutaraldehyde disinfection. These results are very important because these trials demonstrated and mimicked what happens in hospital practice, indicating that outbreaks of *M. abscessus* subsp. *bolletii* infections could be associated with the development of glutaraldehyde resistance, as described by several authors (DUARTE et al., 2009; SOUTO et al., 2012).

Many human diseases are caused or exacerbated by biofilms, and it is expected that it provides an important reservoir for cells that can become recolonized sites after the drug treatment is finished. However, the mechanism involved in biofilm assembly and persistence needs to be clarified (SOUSA et al., 2015).

The presence of these cells at high glutaraldehyde concentrations reinforces the possibility of the presence of persisters. After 14 days of incubation, nutrient availability decreases, and, at the same time, bacterial stress occurs due to the presence of glutaraldehyde, which could be explained by the development of persistent cells.

Despite more than 70 years after the first report of persisters, the molecular mechanisms underlying bacterial persistence remain largely elusive (DUAN et al., 2016). Persistent bacterial cells play a crucial role, contributing to the recalcitrance of chronic infections and treatment failures. Understanding the molecular mechanism of persistent cell formation and maintenance will inspire the discovery of new antimicrobials (DUAN et al., 2016). Some authors have reported the presence of persister cells in *Mycobacterium tuberculosis*, such as Duan et al. (2016), who demonstrated that the lat gene MSMEG_1764, the *M. tuberculosis* lat Rv3290c homolog, is involved in the formation of persisters (specifical tolerance to norfloxacin) through the mediation of intracellular amino acid content and altering ppGpp synthase expression in *M. smegmatis*. Keren et al.

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(2011) identified 15 genes specifically induced in persisters. Some of these genes have been shown to play a role in persistent infection in mice.

CONCLUSION

The results of this study confirmed the ability of the *M. abscessus* species to survive and develop biofilms in the presence of glutaraldehyde and may be related to outbreaks of mycobacteria infections. Therefore, these results have a great impact, as they alert the global clinical and scientific community, Anvisa and local surveillance agencies in Brazil. We also hope that the data obtained from this work can effectively contribute to the control and prevention of these infections, as it is a serious matter of national public health.

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