Resistance to Antiseptics and Disinfectants of Planktonic and Biofilm-Associated Forms of *Corynebacterium striatum*

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Disinfection and antiseptics are of primary importance in controlling nosocomial infections and outbreaks by pathogens expressing multiple resistance to antimicrobial agents (multidrug-resistant [MDR]) used in therapy. Nowadays, infections related to health services (HAIs) due to MDR and multidrug-susceptible (MDS) *Corynebacterium striatum* should not be underestimated, including patients using invasive medical devices. The virulence potential of *C. striatum* needs further investigation. Currently, susceptibility profiles of planktonic and/or sessile forms of four *C. striatum* strains of different pulsed-field gel electrophoresis types were examined as biocides based on the manufacturer's recommendations: 2% glutaraldehyde (GA), 2% peracetic acid (PA), 1% potassium monopersulfate (Virkon®; VK), 1% sodium hypochlorite (SH), and 70% ethyl alcohol (ET). Time-kill assays using 2% bovine serum albumin (BSA) were performed for evaluation of influence of organic matter on biocides effects. Planktonic forms expressed GA resistance at different levels. *C. striatum* viability was observed until 2, 4, 20, and 30 min for MDR 2369/II, MDS 1954/IV, MDR 1987/I, and MDS 1961/III strains, respectively. In contrast to GA, the biocides PA, VK24h, SH, and ET had higher effective bacterial mortality. However, storage of VK (48 hr) reduced their biocide activities. Moreover, mature biofilms were produced on abiotic substrates, including steel surfaces. Post-treatment with GA (30 min), survival of sessile forms was $\geq 100\%$ than planktonic forms of all *C. striatum* tested strains. Independent of biocides tested, BSA increased the survival of planktonic and sessile forms ($p \leq 0.005$). Present data indicated that hospital staff should be aware of dissemination and eradication of HAIs by *C. striatum* presenting resistance to biocides, including high-level disinfectants, such as GA.

Keywords: *Corynebacterium striatum*, biocides, biofilm, glutaraldehyde, nosocomial outbreak, resistance

Introduction

CORYNEBACTERIUM SPP. ARE widely disseminated in the environment and may colonize the skin and mucous membranes of humans as part of the normal microbiota.¹ In addition to these biological properties, most of the corynebacterial species identified during laboratorial procedures remain frequently considered as contaminants by health professionals in different countries of all continents.¹ Multidrug-resistant (MDR) and multidrug-susceptible (MDS) *Corynebacterium striatum* strains have been reported with increased frequency as pathogens of severe nosocomial infections and outbreaks in both industrialized and developing countries, such as Spain,^{2,3} Italy,⁴ Brazil,⁵ Belgium,⁶ China,⁷ Japan,¹ Tunisia,⁸ and Switzerland.⁹

In a Brazilian tertiary care hospital located at the Rio de Janeiro metropolitan area, a nosocomial outbreak caused mostly by MDR *C. striatum* predominantly isolated from tracheal aspirates samples was initially verified in 2009.⁵ Subsequently, cases of bloodstream and catheter-related infections caused by *C. striatum* isolates were noticed in the same hospital. Different studies have increasingly recognized *C. striatum* as pathogen related to varied nosocomial infections, including respiratory tract and urinary infections, surgical wounds, prosthetic joint infections, bloodstream and/or catheter-related infection,^{5,10–12} bacteremia, endocarditis^{13,14} osteomyelitis,¹⁵ meningitis, and infections of patients making use of medical devices, in both immunocompetent and immunocompromised hosts.¹⁶

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Nosocomial spread of MDR *C. striatum* strains, especially in long-term hospitalized patients with prolonged exposure to broad-spectrum antibiotics and admitted in intensive care units and surgical wards using continuous and prolonged medical devices and/or respiratory recuperation, was also reported.^{5,17-21} Accordingly, the pathogenicity of *C. striatum* should not be underestimated and the virulence factors that support *C. striatum* infections related to the health service (HAIs) need further investigation. The ability of *C. striatum* survival and biofilm formation on abiotic surfaces of varied types, including indwelling medical devices, were recently verified.^{22,23}

The use of indwelling medical devices may facilitate colonization and infection by *C. striatum* due to biofilm formation and subsequent invasive dissemination of this pathogen.^{5,12,13,15,22} Previous studies already suggested the relationship between biofilm formation ability, changes in genes, and, consequently, increased resistance to drugs.^{5,6,15,17,24-27} Accordingly, enhanced surveillance and control strategies remain necessary to decrease MDR and MDS *C. striatum* nosocomial infections and outbreaks.

Biocides have been extensively used in hospitals and other health care settings for the disinfection and/or sterilization of medical devices and environmental surfaces. In particular, disinfectants play an essential role in infection control and prevention of transmission of human pathogens. However, resistance to biocides has been previously described for several MDR nosocomial pathogens, including *Mycobacterium* spp. and *Staphylococcus aureus*.²⁸⁻³¹ For these pathogens, studies have also verified correlation between susceptibility to biocides and resistance to antimicrobial agents among hospital-acquired and community-acquired strains. Investigations have been also directed toward the responses of microorganisms to biocides and the effects of antimicrobial agents on surface-attached communities.^{4,17,32-34}

Biofilm formation may facilitate colonization and infections and lesions of human epithelial surfaces that, independent of antibiotic pressure, *C. striatum* strains may get more aggressive by the presence of invasive medical devices, including catheters and endotracheal tubes. Since some foreign materials are previously submitted to high-level disinfection, inappropriate procedures may favor bacterial contamination of foreign materials and, consequently, disseminate bacterial pathogens through human tissues, thus leading to different sites or types of infections.^{3,5,6,7,22,35} In this study, the susceptibility of planktonic and biofilm-associated forms of *C. striatum* strains to different biocidal products currently used in health care facilities was investigated.

Materials and Methods

Bacterial strains

Table 1 shows the epidemiological and microbiological features of the partially studied *C. striatum* strains used in this investigation. *C. striatum* strains in physiological saline solution (0.85% NaCl) without biocides were used as negative controls of experiments.⁵

Susceptibility testing of MDR *C. striatum*

Antimicrobial susceptibility profiles were determined by the disk diffusion method in cation-adjusted Mueller-Hinton

TABLE 1. EPIDEMIOLOGICAL, CLINICAL, AND MICROBIOLOGICAL FEATURES OF *CORYNEBACTERIUM STRIATUM* STRAINS PREVIOUSLY ISOLATED FROM PATIENTS OF A UNIVERSITY HOSPITAL AT THE METROPOLITAN AREA OF RIO DE JANEIRO, BRAZIL

Strains/year	Clinical nosocomial characteristics					Antimicrobial Susceptibility profiles	PFGE- types	Bacterial spontaneous autoaggregating properties (SA)	Biofilm formation on abiotic surfaces Polyurethane catheter (CFU/mL)
	Isolation site	Gender/Age (years-old)	Hospital location	Outcome	Culture				
1987 BR- RJ/09	BAL	F/50	Nursery 18	Death	Pure (2.0×10^5 CFU)	MDR	I	SA-positive	1.5×10^{7a}
2369 BR- RJ/09	TA	M/NI	General ICU	Cure	Pure	MDR	II	SA-positive	1.2×10^7
1961 BR- RJ/09	Urine	F/37	Infectious diseases ward	NI	Pure (2.5×10^5 CFU)	MDS	III	SA-positive	1.2×10^7
1954 BR- RJ/09	Surgical wound	M/NI	Thoracic surgical ward	NI	Pure	MDS	IV	SA-positive	1.3×10^7

Baio et al.⁵ and Souza et al.²²

^aHighest ability of biofilm formation ($p < 0.05$).

BAL, bronchoalveolar lavage; CFU, colony-forming units; F, female; ICU, intensive care unit; M, male; MDR, multidrug resistance presenting resistance to more than three classes of different antibiotics³⁸; MDS, multidrug-susceptible; NI, not informed; PFGE, pulsed-field gel electrophoresis; TA, tracheal aspirate.

agar supplemented with 5% sheep blood. Breakpoints for the susceptible strains were used as suggested by the Clinical Laboratory Standards Institute. As there is not yet a defined standard for interpreting these results, the standard proposed in CLSI document M45-A (ISBN 1-56238-607-7). Breakpoints for *S. aureus* were considered in the cases of penicillin, oxacillin, and ampicillin. For the other antimicrobial agents, there were breakpoints for other microorganisms, but not *Haemophilus* spp. or *Neisseria gonorrhoeae*, whereas intermediate results were considered resistant.^{36,37}

The antibiotics (Oxoid SA, Spain) tested included: penicillin (10 U), ampicillin (30 µg), methicillin (5 µg), cefotaxime (30 µg), cefepime (30 µg), ceftriaxone (30 µg), imipenem (10 µg), erythromycin (15 µg), clindamycin (2 µg), linezolid (30 µg), ciprofloxacin (5 µg), moxifloxacin (5 µg), tetracycline (30 µg), gentamicin (10 µg), rifampin (5 µg), fosfomicin (200 µg), vancomycin (30 µg), mupirocin (200 µg), tobramycin (10 µg), nitrofurantoin (300 µg), and ticarcillin/clavulanate (75 µg/10 µg). *C. striatum* strains presenting resistance to more than three different classes of antibiotics were classified as MDR, as previously defined by Magiorakos *et al.*³⁸

Time-kill assays against *C. striatum* planktonic cells and effects of organic matter on biocides activities

Bactericidal effects of biocides on planktonic forms of *C. striatum* strains were evaluated by the standard time-kill method on 24-well flat-bottomed polystyrene microtiter plates (NUNC™).³⁹ The list of biocides and neutralizers is shown in Table 2. Briefly, microorganisms were previously cultured in TSB medium at 37°C for 48 hr. Aliquots of 1 mL of bacterial suspensions (OD=0.2 at $\lambda=570$ nm; 1.5×10^8 CFU/mL) were centrifuged, and the pellets were resuspended in saline (100 µL) and then in 900 µL of biocides or sterile distilled water (control). High-level biocides 2% glutaraldehyde (2% GA; Vetec Química Fina Ltda) and 2% peracetic acid (2% PA; Sekusept Aktiv Ecolab Deutschland GmbH); intermediate-level biocides 1% sodium hypochlorite (1% SH; Cinord Sudeste Quimica) and 1% potassium peroxymonosulfate (Virkon®—VK24h or VK48h; EPA Label); and low-level biocides, 70% ethyl alcohol (70% ET; Vetec Química Fina Ltda) were tested. Biocide concentrations were chosen based on the manufacturer's recommendations for disinfection procedures.

At the specified contact time (0.25–30 min) with biocides at 22°C, 100 µL of bacterial suspensions was placed into 900 µL of correspondent sterile neutralizing solutions. After serial dilution in phosphate-buffered saline (PBS) 0.1 M pH 7.2, microbial suspensions were plated on to Trypticase Soy Agar medium and incubated at 37°C for 48 hr. The number of viable bacteria was verified by colony-forming units (CFU/mL). The efficacy of each biocide on planktonic forms was evaluated based on the minimum incubation time to completely eliminate bacterial growth and the lower number of CFU/mL counts.³⁹

To evaluate the influence of organic matter on biocides effects, time-kill assays using 2% bovine serum albumin (BSA; Sigma Chemical Co.) were performed based on the Standard Guide for Assessment of Antimicrobial Activity. Moreover, *C. striatum* strains in physiological saline

TABLE 2. CHARACTERISTICS OF BIOCIDES AND NEUTRALIZERS USED IN THIS STUDY

Biocides/concentration	Recommended contact time ^a (min)	Neutralizer	Antimicrobial action	Spectrum action	References
2% Glutaraldehyde	3–30	0.1% sodium sulfite Souza <i>et al.</i> ⁴⁶	Disinfection Preservation	High level ^b	Rutala and Weber ⁴⁰ ; ANVISA 04/2007; Mello ⁵³
2% Peracetic acid	15	6% sodium thiosulfate Souza <i>et al.</i> ⁴⁶	Esterelization Disinfection	High level ^b	Moraes <i>et al.</i> ⁴³ ; Sekusept®
1% Sodium hypochlorite	5–20	0.6% sodium Thiosulfate Souza <i>et al.</i> ⁴⁶	Esterelization Disinfection	Intermediate level ^c	McDonnell and Russell ³⁴ ; ANVISA 2016
1% Potassium monopersulfate (Virkon®)	20	1.0% sodium thiosulfate +10% fetal bovine serum Souza <i>et al.</i> ⁴⁶	Cleaning Disinfection	High level ^b	Dunowska <i>et al.</i> ⁵⁴ ; Martin <i>et al.</i> ⁵⁵ ; Matsuoka <i>et al.</i> ⁵⁶
70% Ethyl alcohol	≥1	2% Tween 80 Souza <i>et al.</i> ⁴⁶	Antiseptics Disinfection Preservation	Low level ^a	McDonnell and Russell ³⁴

Contact time based on manufacturer's recommendations; Virkon, a multipurpose disinfectant containing oxone (potassium monopersulfate); Sekusept, CS-Portugal—January 26, 2009—Aktiv.

^aLow-level biocide is recommended for intact skin or surfaces.

^bHigh-level biocides are recommended for sterilization and high-level disinfection.

^cRecommended for disinfection of semi-critical material.

solution (0.85% NaCl with or without BSA) without biocides were used as negative controls of experiments.³⁹

Effect of 2% GA and 2% BSA on bacterial sessile forms associated to biofilm

Investigation was performed by using the following abiotic substrates lodged into 24-well flat-bottomed polystyrene microtiter plates: (i) 13 mm glass coverslips (Sigma-Aldrich), (ii) sterile 0.5 cm segments of polyurethane catheters (16-gauge percutaneous nephrostomy catheters Intracath; Deseret Pharmaceutical Co.), (iii) 13 mm thermax coverslips (NUNC), and (iv) sterile 0.5 cm segments of metal parts of catheters (16-gauge percutaneous nephrostomy catheters Intracath; Deseret Pharmaceutical Co.). The quantification of bacteria associated to biofilm was determined by biofilm sand abrasion and culture, as previously described by Souza *et al.* Briefly, each abiotic substrate was incubated with 10^8 CFU/mL of bacterial suspension that was mixed with 500 μ L TSB and incubated for 48 hr at 37°C, to allow biofilm formation.

Subsequently, supernatants were removed and biofilms were washed three times with PBS. After that, into each well 2% GA solution with or without 2% BSA was added and incubated at room temperature for 30 min. Wells with PBS were used as negative controls. After that, substrates were rinsed three times with 1% sodium sulfite neutralization solution and three times with PBS pH 7.2; then, they were removed from the 24-well plate and placed into individual glass tubes (18 $\times$ 100 cm) containing 1,000 μ L PBS 0.1 M pH 7.2 plus 1 g of sterile sand (150–212 μ m; Sigma Aldrich Co.). The tubes were then agitated with a vortex mixer of rotation 3,300 RPM speed, and 100 μ L of the suspension was diluted and plated for CFU quantification. Experiments were performed in triplicate, and results were statistically calculated.

Scanning electron microscopy assays

Effects of 2% GA on biofilm formation were evaluated by scanning electron microscopy (SEM). Briefly, sections of polyurethane catheters were fixed in 2.5% GA, postfixed in 1% osmium tetroxide, and dehydrated in a graded series of ethanol. Subsequently, catheter segments were subjected to critical point drying with carbon dioxide, covered with a 10 nm layer of gold palladium, and examined with a JEOL JSM 5310 scanning electron microscope. Sterile polyurethane catheters (negative control) were also processed by SEM directly on removal from commercial packaging.²²

Statistical analysis

Each experiment was carried out in triplicate and repeated three times. Student's *t*-test was used to compare means of experiments; $p < 0.05$ and/or $p < 0.001$ were considered statistically significant.

Results

Phenotypic and genotypic properties and antimicrobial susceptibility profiles of C. striatum clinical isolates

Epidemiological and microbiological features of *C. striatum* strains previously isolated from patients of a University hospital at the metropolitan area of Rio de Janeiro, Brazil

used in this study (Table 1) showed MDR profiles for strains 1987/I—BAL (bronchoalveolar lavage) and 2369/II—tracheal aspirate that were susceptible only to vancomycin, linezolid, and tetracycline. Two other strains (1954/IV surgical wound isolate and 1961/III urine isolate) were susceptible to most of the tested drugs (MDS) except mupirocin, fosfomycin, and ticarcillin/clavulanate.

Bactericidal effects of biocides and influence of organic matter on planktonic forms

Data displayed in Fig. 1 showed the effects of 2% GA on planktonic forms of MDR and MDS *C. striatum* strains. All four *C. striatum* strains of different pulsed-field gel electrophoresis (PFGE) types were found to be resistant to 2% GA, but at different levels. All strains showed resistance up to 2 min of treatment with GA. Bacterial viability was observed until 2, 4, 20, and 30 min of treatment with GA for the strains MDR 2369/II (tracheal aspirate), MDS 1954/IV (surgical wound), MDR 1987/I (BAL), and MDS 1961/III (urine), respectively. For the MDR 1987/I strain, a gradual reduction of viable cells was observed over time; however, the MDS 1954/IV strain presented similar CFU counts from 0.25 to 4 min, whereas for the MDR 2369/II strain an increase at CFU counts at 1 min was observed. Interestingly, for the MDS 1961/III strain, the number of CFU reduced after 0.25 and 0.5 min of incubation with 2% GA, but it increased gradually from 1 to 20 min and decreased at 30 min although it remained viable. Data showed that the presence of 2% BSA favored the ability of MDR and MDS *C. striatum* strains to survive for further periods in the presence of 2% GA (Fig. 1).

Conversely, 2% PA had a far-reaching effect on bacterial mortality (Fig. 1). MDR 1987/I (BAL) and MDS 1954/IV strains were able to survive for 0.25 min, whereas MDR 2369/II and MDS 1961/III strains survived for 0.5 and 1 min, respectively. However, the presence of 2% BSA was able to increase the CFU count of the MDR 1987/I (BAL) strain at 0.25 ($p = 0.005$) and 0.5 min ($p = 0.05$). Interestingly, the presence of BSA increased the resistance of the MDS 1961/III (urine) strain from 1 to 4 min. No significant difference was observed for MDS 1961/III, MDR 2369/II, and MDS 1954/IV strains (Fig. 1).

Data displayed in Fig. 1 showed bactericidal effects of the disinfectants and/or antiseptics 1% SH, Virkon (VK) and 70% ethanol (70% ET) and the influence of organic matter on planktonic forms of *C. striatum* strains. Resistance to 1% SH was observed for MDR and MDS strains: MDR 1987/I, MDR 2369/II, and MDS 1954/IV strains until 0.25 min and for MDS 1961/III strain until 1 min. The presence of 2% BSA significantly increased ($p \leq 0.005$) the resistance to 1% SH of MDS 1961/III strain quantitatively (0.25, 0.5, and 1 min) and survival time (until 2 min).

Results also showed the effects of a commercial formula of potassium monopersulfate—1% VK freshly prepared and used on the same day and after stock for 48 hr (VK48h) on survival/resistance of planktonic forms of all *C. striatum* tested strains. Higher resistance to freshly prepared 1% VK was shown for MDR 1987/I and 1961/III strains survived until 0.5 and 2 min, respectively. The presence of 2% BSA was able to increase the resistance of MDR 1961/III strain from 2 to 4 min and for MDS 1954/IV strain from 0.25 to 0.5 min. However, the storage of VK solution for 48 hr

FIG. 1. (A) Bactericidal effects of 2% GA and 2% PA on planktonic forms of *Corynebacterium striatum* strains in the presence or not of organic matter (2% BSA); (B) bactericidal effects of Virkon® (VK) on planktonic forms of *C. striatum* strains in the presence or not of organic matter (2% BSA); (C) bactericidal effects of 1% sodium hypochloride (1% NaClO) and 70% EtOH on planktonic forms of *C. striatum* strains in the presence or not of organic matter (2% BSA). BSA, bovine serum albumin; GA, glutaraldehyde; PA, peracetic acid.

(VK48) reduced its biocide activity in all opportunities: Bacterial survival was observed for the MDR 2369/II and MDR 1961/III strains until 4 min of incubation, whereas for the MDR 1987/I and MDS 1954/IV strains CFU were detected until 2 and 0.25 min, respectively. The presence of 2% BSA significantly increased survival in VK48 of the planktonic forms of all strains tested, especially MDR 2369/II (from 4 to 10 min) and MDS 1961/III strains (from 4 to 20 min) (Fig. 1).

For 70% ET, the MDS 1961/III strain survived until 1 min and MDR 1987/I, MDR 2369/II, and MDS 1954/IV strains were able to survive until 0.5 min. Interestingly, the presence of 2% BSA increased time of survival and viability of

all *C. striatum* strains, especially the MDS 1961/III (urine) strain until 30 min. The presence of BSA decreased bacterial survival at 0.25 min for the MDS 1961/III strain, whereas this organic matter significantly increased bacterial viability of planktonic forms at 0.25 min for MDR 1987/I and MDS 1954/IV strains ($p=0.005$) (Fig. 1).

Effects of 2% GA and 2% BSA on mature biofilm produced by MDR and MDS *C. striatum* strains

Data displayed in Fig. 2 indicated that all four *C. striatum* strains of different PFGE types were found capable of producing mature biofilm (48 hr) at different levels on

FIG. 2. *Corynebacterium striatum* cellular viability counting (CFU/mL) recovered from biofilm formation on different hydrophilic and hydrophobic abiotic surfaces post 2% GA exposure (30 min) and influence of organic matter (2% BSA); % survival compared with untreated sessile forms. Experiments were performed in triplicate, and data were statistically calculated ($p < 0.05$). CFU, colony-forming units.

abiotic surfaces, such as: hydrophilic and positively charged (glass, metal from nephrostomy catheter), polyurethane (thermanox), and negatively charged (polystyrene). Biofilms produced on these abiotic and hydrophobic surfaces were as follows: metal, glass, and polyurethane.

Although data showed the effects of 2% GA and 2% BSA on mature biofilm produced by *C. striatum*, sessile forms of MDR and MDS strains were capable of surviving in the presence of 2% GA, but at different levels.

Experiments with MDS 1961/III (urine) followed by MDR 1987/I (BAL) strains showed that a higher number of viable sessile bacterial cells recovered from biofilm formation on metal surfaces whereas the MDR 1987/I strain showed that a higher number of viable sessile bacterial cells recovered from biofilm formation on glass, polyurethane, and, especially, polystyrene surfaces.

Bacterial pathogenic potential due to biofilm formation in the presence of organic matter (2% BSA) was able to increase the survival of *C. striatum*, independent of PFGE type, multidrug resistance, clinical site, and biofilm formation, especially on hydrophilic and positively charged glass and polyurethane abiotic surfaces. The ability of dissemination of this pathogen in biotic and abiotic environments may be emphasized by the observation of higher numbers of CFU/mL of planktonic forms of *C. striatum* strains tested, especially in the presence of GA, VK, and alcohol (Figs. 1 and 2).

All four MDR and MDS *C. striatum* strains of different PFGE types were found to be capable of surviving post-treatment for 30 min with 2% GA, considered as a high level disinfectant, although in these treatment conditions GA was found to be able to kill all planktonic forms and to eliminate most of *C. striatum* strains, except MDS 1961/III.

The lowest ability formation of all abiotic surfaces tested was observed for both MDR 2369/II (tracheal aspirate) and MDS 1954/IV (surgical wound), independent of antimicrobial susceptibility profiles.

Biofilm formation recovery from glass surface without 2% GA was positive and was at least 1.0×10^7 CFU. After 2% GA treatment, the number of CFU reduced significantly, varying from 0.008% to 0.21%. Interestingly, 2% GA treatment was incapable of completely eliminating sessile bacterial forms of any *C. striatum* strains tested: The CFU number recovered after 2% GA treatment were MDS 1954/IV (2.33×10^3) < MDR 2369/II (8.0×10^3 CFU/mL) < MDS 1961/III (8.33×10^3 CFU/mL) < MDR 1987/I (8.67×10^3 CFU/mL). The presence of BSA was able to increase bacterial survival in all strains tested by at least 150%.

Bacterial recovery from metal formed biofilm without 2% GA varies but bacterial recovery was at least of 1.0×10^7 CFU/mL except for the MDR 2369/II strain (3.0×10^5 CFU/mL). After 2% GA treatment, the number of CFU/mL reduced significantly, varying from 0.04% to 6.0%. The CFU/mL number recovered after 2% GA treatment was MDS 1961/III (7.5×10^4 CFU/mL) > MDR 1987/I (2.5×10^4 CFU/mL) > MDR 2369/II (1.8×10^4 CFU/mL) > MDS 1954/IV (7.0×10^3 CFU/mL). Moreover, the presence of BSA was able to increase bacterial survival in all strains tested by at least 106%.

Moreover, biofilm formation was observed on thermanox and polyurethane without 2% GA (Fig. 2). *C. striatum* strains showed high ability (at least of 1.2×10^7 CFU/mL) of biofilm formation on thermanox (hydrophobic and negative

charged), independent multidrug resistance, PFGE type, and clinical site. Treatment with 2% GA significantly reduced the number of CFU/mL, varying from 0.085% to 0.542%: MDS 1954/IV (8.0×10^4 CFU/mL) > MDS 1961/III (6.5×10^4 CFU/mL) > MDR 2369/II (2.0×10^4 CFU/mL) > MDR 1987/I (1.1×10^4 CFU/mL). The presence of BSA was able to increase bacterial survival in all strains tested by at least 118% (Fig. 2).

Similar to the thermanox surface, biofilm formation tested on polyurethane catheter fragments treated with 2% GA was also significantly reduced for all *C. striatum* strains tested: MDS 1954/IV (8.0×10^4 CFU/mL) > MDS 1961/III (6.5×10^4 CFU/mL) > MDR 2369/II (2.0×10^4 CFU/mL) > MDR 1987/I (1.1×10^4 CFU/mL). Similarly, BSA was able to increase bacterial survival of all strains tested by at least 125% (Fig. 2).

Scanning electron microscopy

Scanning electron micrographs displayed in Fig. 3 illustrate the effects of biocides on morphological features of biofilm arrangements of MDR and MDS *C. striatum* strains of different PFGE types formed on abiotic surfaces. Biofilm was present in all conditions tested. Before and after treatment with 2% GA solution (30 min), a large amount of biofilm material on polyurethane catheter surfaces (48 hr) was observed for all four strains tested. The presence of autoaggregative microcolonies, extracellular slime, and hollow voids on the polyurethane surface were indicative of mature biofilm structure. It was also observed that in *C. striatum* biofilm the presence of GA, a high number of elongated sessile bacterial cells were present, which may indicate an adaptation response of the pathogen to stress conditions with important implications for persistence and dissemination of *C. striatum* in a nosocomial environment.

Discussion

Antiseptics and disinfectants are used extensively in hospitals and other health care settings for a variety of topical and hard-surface applications.^{34,35} However, less is known about the mode of action of biocides than about antibiotics. In general, biocides have a broader spectrum of activity than antibiotics, and antibiotics tend to have specific intracellular targets, whereas biocides may have multiple targets. The widespread use of antiseptic and disinfectant products has prompted some speculation on the development of microbial resistance, in particular cross-resistance to antibiotics.^{34,35,40–45} Disinfection and sterilization are essential for ensuring that medical and surgical instruments do not transmit infectious pathogens to patients, medical professionals, and/or nosocomial environment. Failure to properly disinfect or sterilize equipment carries not only risk associated with breach of host barriers but also risk for person-to-person transmission and transmission of environmental pathogens.^{40–45}

In this study, resistance to antiseptics and disinfectants of planktonic and biofilm-associated forms of MDR and MDS *C. striatum* nosocomial strains were demonstrated.

Sessile forms of bacteria associated to biofilm are usually more resistant to killing than bacteria growing under planktonic conditions, since biofilms play an important role in the nosocomial environment by protecting microorganisms

FIG. 3. Scanning electron micrographs illustrating mature biofilm on polyurethane catheter surfaces (48 hr) treated (A, C, E, G) and untreated (B, D, F, H) with 2% GA solution (30 min) by MDR and MDS *Corynebacterium striatum* strains of different pulsed-field gel electrophoresis: (A, B) MDR 1987/I (bronchoalveolar lavage); (C, D) MDR 2369/II (tracheal secretion); (E, F) MDS 1961/III (urine); (G, H) MDS 1954/IV (wound lesion); (A, C) amorphous material on catheter surface was kept evident post-treatment with biocide; elongated cells of *C. striatum* were observed and may indicate an adaptation mechanism to stress conditions. MDR, multidrug-resistant; MDS, multidrug-susceptible.

against killing by antibiotics and biocides.^{40–43,45} Biocides have been currently used to control and inactivate biofilms, although resistance to some biocides may decrease efficacy of disinfection.^{40–46} Previous studies highlighted the importance of biofilm formation for infectious potential and resistance to biocides of *Mycobacterium* spp. and/or capability to adapt to environmental stresses that favored persistence in biofilms of other bacterial pathogens.^{22,28–30,47}

Bacterial infections acquired in a nosocomial environment may be associated with medical devices, including catheters and endotracheal tubes, as previously shown with *C. striatum*, among other pathogens.^{4,17,22,48} Interaction and persistence of MDR and MDS *C. striatum* strains with surfaces of semi-critical and critical medical devices, such as catheters and endotracheal tubes, may be aided by affinity of this pathogen to abiotic hydrophilic and positively charged and hydrophobic and negatively charged abiotic surfaces and subsequent ability of biofilm formation. In a previous study, 1987/I strain was found to be the one that

presented the highest ability to make biofilm on glass, polyurethane, and polystyrene surfaces.²² Currently, all *C. striatum* strains tested were able to form biofilm, independently of the substrate tested, including metal.

The 1987/I strain was the most adherent since it presented the highest CFU numbers recovered from biofilm associated to most of the substrates tested. Although all PFGE types were able to form biofilm in all substrates tested, 2396/III strain was poorly adherent to metal and 1961/III strain was highly adherent to metal. The mechanisms that influence *C. striatum* ability to adhere to different abiotic surfaces at varied levels remain unknown. Since the widespread use of antiseptic and disinfectant products has prompted some investigation on the development of microbial resistance, in particular cross-resistance to antibiotics, resistance to biocides of planktonic and biofilm-associated forms of *C. striatum* was currently verified.

The presence of medical devices and the exposure to antimicrobials may possibly enable infection of the human

lower respiratory tract by *C. striatum*. Recently, we documented the first Brazilian nosocomial outbreak caused by MDR *C. striatum* and most of the patients presented lower respiratory tract infection (64%) and were also receiving ventilatory support (89%). PFGE analysis confirmed the isolation of a single clone of MDR *C. striatum* (PFGE I) from the lower respiratory tract.⁵ However, there is little research about disinfectant efficacy on contaminated medical devices and appliances by *C. striatum*.

In Japan, Wong *et al.*⁴⁸ also showed that *C. striatum* may be responsible for serious hospital-acquired respiratory infection (empyema, pneumonia, or acute bronchitis) and community-acquired pneumonia. Only 50% of patients who received active antibiotics against this pathogen survived the infection. Most of the patients received ventilatory support by using noninvasive ventilators and/or invasive mechanical ventilators.

GA is one of the most commonly used disinfectants in endoscopy units. It is effective and does not damage endoscopes, accessories, or automated processing equipment.^{17,28,29} Similar to *C. striatum*, resistance to GA has been previously reported in several bacterial species, including *Pseudomonas aeruginosa*.^{49–51} Although GA has been withdrawn from use in some countries,⁵² GA remains a biocide commonly used to control microbial growth in hospitals, water treatment, food production, and industries, including Brazil.⁵³

Given that GA is a widely used biocide, it is necessary to elucidate microbial biofilm responses to GA to inform proper biocide application. Although widely used, to the best of the authors' knowledge, the response of *C. striatum* biofilm to GA has not been previously reported. The biofilms are often known to demonstrate increased antimicrobial resistance compared with planktonic cells, however, *C. striatum* planktonic forms and sessile forms showed phenotypic adaptation when exposed to treatment of GA. High-level disinfection procedure experiments showed that exposure to 2% GA during 30 min significantly reduced the number of viable bacteria associated to biofilm in all substrates tested. However, the survival of sessile forms was at least 100% higher than planktonic forms of *C. striatum*. GA resistance was observed for planktonic and sessile forms of all four *C. striatum*, PFGE types, and antimicrobial susceptibility profiles (MDR and MDS). Interestingly, planktonic forms of the MDS 1961/III strain remained alive until 2% GA treatment (30 min).

Antimicrobial activity can be influenced by many factors such as formulation effects, synergy, temperature, dilution, test method, and/or presence of an organic load.³⁴ Organic matter in the form of serum, blood, pus, or fecal or lubricant material can interfere with the antimicrobial activity of biocides in at least two ways. Most commonly, interference occurs by a chemical reaction between the germicide and the organic matter, resulting in a complex that is less germicidal or nongermicidal, leaving less of the active germicide available for attacking microorganisms. Alternatively, organic material can protect microorganisms from attack by acting as a physical barrier, and also by the duration of exposure; microorganisms may be protected from disinfectants by the production of thick masses of cells and extracellular materials or biofilms. Guidelines for disinfection and sterilization in health care facilities describe that the use

of GA-based solutions in health care facilities is widespread because of their advantages, including excellent biocidal properties; activity in the presence of organic matter (20% bovine serum); and noncorrosive action to endoscopic equipment, thermometers, rubber, or plastic equipment.⁴⁰

Conversely, current data suggested that the presence of organic matter (BSA 2%) was capable of enhancing resistance to GA treatment (30 min) of *C. striatum* strains, independent of clinical sites, PFGE types, and multidrug-susceptibility (MDS and MRD) profile. Moreover, the survival of sessile forms was significantly higher than planktonic forms during GA treatment (30 min) in the presence of 2% BSA. However, biofilms of MDR clinical isolates of PFGE-type I mostly responsible for a Brazilian nosocomial outbreak and predominantly isolated from patients submitted to endotracheal intubation²² showed resistance to 2% GA at higher levels also in the presence of organic matter (2% BSA) on both abiotic–glass (hydrophilic) and polystyrene (hydrophobic) surfaces. The mechanisms that influence *C. striatum* ability to adhere to different hydrophilic and hydrophobic materials with different intensities remain unknown.

Sterilization and high-level disinfection of critical medical devices are mandatory in nosocomial environments. Therefore, PA, a highly effective disinfectant, may be an alternative to GA.⁵⁴ However, the use of PA may lead to higher costs, including costs for processing, purchasing and training, installation, and endoscope repairs. Although this product is rapidly effective against a broad range of microorganisms, it tarnishes the metal of endoscopes and is unstable, resulting in only a 24 hr use life.

PA is characterized by rapid action against several microorganisms. Special advantages of PA are that it lacks harmful decomposition products (*i.e.*, acetic acid, water, oxygen, hydrogen peroxide), enhances removal of organic material, and leaves no residue. It remains effective in the presence of organic matter and is sporicidal even at low temperatures. However, PA can corrode copper, brass, bronze, plain steel, and galvanized iron but these effects can be reduced by additives and pH modifications. It is considered unstable, particularly when diluted; for example, a 1% solution loses half its strength through hydrolysis in 6 days, whereas 40% PA loses 1–2% of its active ingredients per month. For a high-level disinfection procedure, PA may be used at variable concentrations during 12–30 min exposure time at $\geq 20^{\circ}\text{C}$. An alternative high-level disinfectant available in the United Kingdom contains 0.35% PA.⁵⁵

In this study, all MDR and MDS *C. striatum* tested strains of different PFGE types showed resistance until 1 min of exposure to immediately prepared PA solution at a high concentration (2%). Conversely to data previously described by Rutala and Weber,⁴⁰ the presence of 2% BSA increased the resistance of all MDR and MDS *C. striatum* tested at different levels, including the MDS 1961/III strain from 1 to 4 min. Although PA has shown good results on *C. striatum*, the use of PA may lead to higher costs, including costs for processing, purchasing and training installation, and endoscope repairs, stains metal of endoscopes in addition to being unstable, resulting in only a 24 h use life.⁵⁴

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VIRKON (VK) is a multipurpose disinfectant of intermediate level that is typically used for cleaning up hazardous spills, disinfecting surfaces, and soaking equipment in hospitals, laboratories, and dental and veterinary facilities. Previous studies have confirmed bactericidal activity of VK products for pathogens, including *Streptococcus pyogenes* and *S. aureus*.^{55,56}

SH, a disinfectant with a broad antimicrobial spectrum, is widely used on hard-surfaces and human blood spillages.⁴⁰ However, its use becomes problematic due to corrosion of metal and other equipment surfaces, generation of irritating chlorine gas, and complicated concentrations.

On the other hand, disinfectants and cleaning agents formulated with VK are improved over chlorine-based disinfectants and cleaning. In addition, it has been reported that with hypochlorous acid as the active substance, there is little metal corrosion and no chlorine odor, and this agent is highly efficacious against many infectious organisms, including methicillin-resistant *S. aureus*,⁴⁰ cleaning, and disinfection of environmental surfaces.⁵⁶

In this study, 1% VK and 1% SH were tested against planktonic forms of *C. striatum*. Overall, both biocides were 100% effective against *C. striatum*. No viable bacterial cells were detected after 1 or 2 min of incubation. However, it is important to highlight that VK was more effective than VK48h since viable bacterial cells detected after VK48h exposure were at least two times higher than that observed VK post-treatment.

Ethyl alcohol at 70% (EtOH) is largely used for skin and instrument disinfection. Most of the critical medical devices are coated with organic matter, which may contribute to the reduction of the effect of biocides and favor bacterial survival.⁴⁰

Alcohol exhibits rapid broad-spectrum antimicrobial activity against vegetative bacteria, including mycobacteria,⁴⁰

and is widely used for both hard-surface disinfection and skin antiseptics.

Efficacy is dependent on the concentration of both the active agent and the microorganism. Generally, the antimicrobial activity of alcohol is significantly optimal in the 60–90% range. Little is known about the specific mode of action of alcohol, but based on the increased efficacy in the presence of water; it is generally believed that it causes membrane damage and rapid denaturation of proteins, with subsequent interference.⁴⁰

Currently, planktonic forms of all *C. striatum* strains tested showed resistance of 70% (EtOH) during only 1 min of exposure. Interestingly, the presence of 2% BSA increased bacterial survival for all biocides tested, especially VK (from 4 to 20 min) and alcohol (from 1 to 30 min).

For *C. striatum*, overall observations demonstrated that the MDS 1961/III strain presented the highest resistance level to most of the biocides tested, whereas the MDS 1954/IV strain is the most sensitive strain except to 2% GA and 70% ET treatments.

In addition to results currently demonstrated with GA, the presence of organic matter was able to influence the effects of four other biocides tested on planktonic forms of different *C. striatum* strains.

For the effective prevention of hospital-acquired infections, it is necessary that clinicians have access to the most current knowledge regarding efficacy and reliability of the most widely used disinfectants. It is also important to have available an extensive knowledge about the behavior and possible resistances of emerging infectious agents such as *C. striatum* under disinfection conditions. Therefore, the data presented here could be of value to the clinical community.

In conclusion, the resistance of sessile and planktonic forms to products used in disinfection and antiseptic procedures, also in the presence of organic matter, contributes to the establishment and dissemination of infections (HAIs) caused by MDR and MDS *C. striatum*. Moreover, the survival of sessile forms was at least 100% higher than planktonic forms for all tested *C. striatum* strains of different PFGE types. Biocides may reduce or eliminate bacteria associated to biofilms but the biofilm matrix may remain undisrupted for some pathogens.^{8,22,45,57} Therefore, hospital staff should be aware of the circulation of MDR and MDS *C. striatum* strains presenting resistance to high-level disinfectants, such as GA and other biocides. This study also indicated that difficulties for bacterial eradication in chronic and device-related infections may occur for MDR corneobacterial species, including *C. striatum*.

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In memoriam: Doctor Raphael Hirata Júnior.

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