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Biofilm-Producing Ability of *Staphylococcus* Spp. Multidrug-Resistance Isolated from Hospitalized Patients with Osteomyelitis

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Paula Marcele Afonso Pereira-Ribeiro*[1], Guilherme Goulart Cabral-Oliveira[1], Julianna Giordano Botelho Olivella[1], Felipe Caldas Ribeiro[1], Barbara Araújo Nogueira[1,3], Bruna Ribeiro Sued-Karam[1,4]; Ana Luíza Mattos-Guaraldi[1,2]

[1]Departament of Microbiology, Imunology and Parasitology, Medical Sciences Faculty, Rio de Janeiro State University (DMIP/FCM/UERJ), Rio de Janeiro, RJ, Brazil.
[2]Departament of Microbiology, Universidade Federal do Rio de Janeiro (CCS/UFRJ), Rio de Janeiro, RJ, Brazil.
[3]Hospital Infection Research Laboratory, Institute Oswaldo Cruz, Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, Brazil;
[4]Laboratory of Bacterial Technology, Bio-Manguinhos, Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, Brazil;

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ABSTRACT

Osteomyelitis is an inflammatory bone disease that is caused by a microorganism and leads to progressive bone destruction and loss. Staphylococcal infections are a global concern, due to the resistance mechanisms developed by these bacteria to evade the host immune system and antibiotic treatment difficult. In addition to the ability of staphylococci, biofilm formation is described as a multifactorial associated with infection. The aim was to evaluate the epidemiological and microbiological aspects from patients hospitalized with osteomyelitis infectious as well as the influence of antibiotics on biofilm formation by different strains of multidrug-resistance *Staphylococcus* spp. The identification was performed by the MALDI-TOF method. Assays for Disc Diffusion and MIC; biofilm formation on polystyrene in the presence of antibiotics and PCR assays to detect *mec*A and *ica*A genes were analyzed. Strains were identified as *S. aureus*, *S. epidermidis, S. warneri,* and S*. capitis*. All strains were oxacillin-resistant and all *S. epidermidis* strains were vancomycin-intermediate. The strains were able to produce biofilm on the polystyrene surface at different levels independent of the presence of the *ica*A and *mec*A genes and antibiotics. The increase in the number of cases of infections and the multifactorial aspects that favor the pathogenicity of *Staphylococcus spp*. should continue to be investigated, since the spread in the hospital environment has become a growing public health challenge.

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INTRODUCTION

Osteomyelitis is an inflammatory bone disease marrow that can progress to bone destruction. It is characterized grossly by purulent secretions, necrotic bone, compromised soft tissue, or fistulas. Diagnosing osteomyelitis is often a difficult challenge, as there are vast variations in clinical presentation. Prescription of treatment for osteomyelitis in the clinical setting largely depends on the classification as either “acute” or “chronic”. Early diagnosis is the key to the successful treatment of osteomyelitis[1-3].

Staphylococcus species are among the most frequently encountered bacteria in hospital settings and have been incriminated for many infections in humans. *Staphylococcus* spp. can be divided into two major groups based on the production of the coagulase enzyme – coagulase-positive Staphylococci (CoPS) and coagulase-negative Staphylococci (CoNS). The CoPS include *Staphylococcus aureus* as the major pathogen. There is now increasing evidence that some of the CoNS species (*Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus saprophyticus, Staphylococcus warneri, Staphylococcus capitis*) could also be pathogenic and cause nosocomial infections[4,5].

Identifying the causative pathogen should be a priority in diagnosing osteomyelitis since it allows the best choice of antibiotic. Gram-positive microorganisms, especially *Staphylococcus aureus* and *Staphylococcus epidemidis* are the most common isolates in osteomyelitis [1,6,7].

Appropriate antibiotic therapy is crucial for a favorable outcome. Treatment should usually be systemic and typically prolonged. Beta-lactam agents are among the most used antibiotics for osteomyelitis. The *mec*A gene is responsible for the expression of methicillin resistance through PBP2a, an altered penicillin-binding protein that is characterized by its low affinity to penicillin and other beta-lactam drugs. For both *S. aureus* and CoNS methicillin-resistant, glycopeptides, such as vancomycin, are used as a drug of the first choice for osteomyelitis treatment. Difficulties in treating osteomyelitis have been aggravated by the rise of antibiotic-resistant bacterial strains[1,3,8,9].

Colonization of bone can occur through direct interaction with the bone cells, plasma proteins, or the organic extracellular matrix. Once colonized, staphylococci can produce a biofilm, a highly

structured heterogeneous community of sessile bacteria surrounded by an extracellular polymer matrix[1,3].

Biofilm development is a multifactorial process in which an altered microenvironment protects microorganisms and blocks the access of antibiotics and the host's immune cells. These biofilms can be difficult to penetrate, and often require surgical intervention [6,8,10]. Biofilm formation is generally described as a three-step process: (a) initial adhesion to the surface (initial attachment); (b) biofilm accumulation due to intercellular aggregation (maturation); and (c) bacterial cell detachment caused by the direct action of bacterial products (dispersion). *Staphylococcus* biofilm can be mediated by polysaccharide intercellular adhesion (PIA) is synthesized by the N-acetylglucosamine transferase icaA enzyme encoded by the *ica*A gene[11,12]. Although there is an ongoing development of therapeutic techniques, a combined surgical and pharmaceutical approach has been the gold standard in clinical practice for the treatment of osteomyelitis[13].

The aim of this study was to evaluate the epidemiological and microbiological aspects from patients hospitalized with osteomyelitis infectious as well as the influence of antibiotics on biofilm formation by different strains of multidrug-resistance *Staphylococcus* spp.

METHODS

Origin and identification of *Staphylococcus* spp. strains: A total of seven *Staphylococcus* spp. strains were previously isolated from blood cultures of patients with osteomyelitis, in a Brazilian hospital, in the year 2019, were identified by phenotypic methods (Gram, catalase, DNAse, coagulase, and growth on mannitol salt agar) and MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) assays. *Staphylococcus haemolyticus* ATCC 29970, *S. epidermidis* ATCC 35984, *S. epidermidis* ATCC 12228, *S. aureus* ATCC 29213, and *S. aureus* ATCC 25923 strains were used as controls for antimicrobial susceptibility tests, PCR, and biofilm assays [14-16].

Antibiotic susceptibility testing of *Staphylococcus*spp. strains: Antimicrobial susceptibility profiles were determined by the disk diffusion method, employing the following drugs: cefoxitin (FOX, 30µg), gentamicin (GEN, 10µg), sulfamethoxazole-trimethoprim (SXT, 25µg), ciprofloxacin (CIP, 5µg), clindamycin (DA, 2µg), erythromycin (E, 15µg), penicillin (P, 10 µg), rifampicin (RIF, 5 µg) and chloramphenicol (CLO, 30 µg) (purchased from CECON, São Paulo,

Brazil and Oxoid, Basingstoke, England) [17]. Minimum Inhibitory Concentration (MIC) of oxacillin (OXA) and vancomycin (VA) (Sigma, St. Louis, MO, USA) were evaluated by the broth microdilution method[16,17].

Influence of antibiotics on biofilm formation by polystyrene method: Biofilm formation on the surface of 96-well polystyrene microtiter plates were performed with and without antibiotics based on previously described methods The biofilm formation assays were performed using their respective media added of sub-MICs of OXA and VA, equivalent to1/4 MIC[15,16].

PCR assay for the presence of *ica*A and *mec*A genes: For detection of the presence *ica*Agene involved in *Staphylococcus* biofilm formation and the presence of *mec*A gene (methicillin resistance) were performed respectively by methods previously described. The primers used in this study were listed in **Table 1**[18,16].

Table 1. Primers used in this study in PCR assays to determine methicillin resistance by the presence of *mec*A gene (MRS) and *ica*A genes that exerts a significant role in biofilm formation.

Primers	Sequence of forward and reverse primers 5'→ 3'	Product size (bp)	References
MRS1	TAGAAATGACTGAACGTCCG	154 bp	[16]
MRS2	TTGCGATCAATGTTACCTAG		
*ica*A-F	CGATGGGCTCAAGGTGG	287 bp	[18]
*ica*A-R	TTCTTTTCGTAGCGACTGTC		

RESULTS

Genotypic identification and epidemiological aspects of *Staphylococcus* strains: Data shown in **Table 2** indicated that seven *Staphylococcus* spp. strains, isolated from patients with osteomyelitis in a Brazilian hospital, were identified as *S. aureus* (n=2; 28.6%), *S. epidermidis* (n=3; 42.8%), *S. warneri* (n=1; 14.3%), and *S. capitis* (n=1; 14.3%) by the MALDI-TOF assays, with scores ≥ 2.0.

Antimicrobial susceptibility patterns and presence of the *mec*A gene: Results of experiments using eleven antimicrobial agents of *Staphylococcus* spp. strains were shown in **Table 2**. Data

demonstrated that *Staphylococcus* spp. strains isolated from osteomyelitis presented heterogenic antimicrobial resistance properties. All *Staphylococcus* spp. strains showed multidrug-resistance (MDR), were phenotypically resistant to oxacillin and *mec*A-positive. Interestingly, a decreased susceptibility to vancomycin was detected for all *S. epidermidis* strains. OXA and VA MIC values were 256 µg/mL to 512 µg/mL and 2.0 µg/mL to 8.0 µg/mL, respectively.

Biofilm formation on polystyrene surfaces and presence of the *ica*A gene: In the present study, all MDR *Staphylococcus* strains isolated from patients with osteomyelitis expressed the ability of biofilm formation on hydrophobic (polystyrene) surfaces, but at varying levels (**Table 2**). MDR *Staphylococcus* strains were characterized to express a heterogenic ability of biofilm formation on polystyrene surface, as one *S. epidermidis* (7A) and two *S. aureus* (2A/9A) strains were classified as strongly (+++) adherent. The presence of *ica*A gene was detected in all *S. aureus* strains and two (66.6%) *S. epidermidis* strains (7A, 8A). Interestingly, one *S. epidermidis* strain (1A), *S. warneri* strain, and *S. capitis* strain were *ica*A-negative. Correlation of the presence of *ica*A gene with biofilm formation on polystyrene surface was not observed for *Staphylococcus* strains isolated from patients with osteomyelitis, indicating that *Staphylococcus* strains can produce biofilm via *ica*-independent pathways.

Influence of antibiotics in biofilm formation on polystyrene surface: Data displayed in **Table 2** showed that all MDR *Staphylococcus* strains isolated from patients with osteomyelitis were able to produce biofilm on polystyrene surface in the presence of both OXA and VA. Curiously, one (50%)*S. aureus* strain (2A) and one (33.3%) *S. epidermidis* strain (7A), showed a decrease in biofilm formation in the presence of OXA and/or VA, and one *S. epidermidis* strain (8A) and *S. capitis* strain (3A), showed an increase on biofilm formation in the presence of OXA and/or VA. Biofilm formation levels polystyrene surface of MDR *Staphylococcus* strains grown in the presence of OXA and VA were not associated with the presence of *mec*A and *ica*A genes.

Table 2. Antimicrobial resistance properties and biofilm formation ability on the abiotic surface of seven *Staphylococcus* spp. strains isolated from patients with osteomyelitis, in a Brazilian hospital.

Strains	Species	*mec*A/ *ica*A genes*	Antimicrobial multi resistance profiles	MIC[µg/ml] (susceptibility)		Polystyrene binding assays#	
				OXA	VA	C	OXA/VA
1A	*Staphylococcus epidermidis*	+ / -	P, FOX, DA, E, CIP, GEN, CLO	256 (R)	8 (I)	++	++ / ++
2A	*Staphylococcus aureus*	+ / +	P, FOX, DA, SXT, E, GEN, CLO	512 (R)	4 (S)	+++	++ / ++
3A	*Staphylococcus capitis*	+ / -	P, FOX, E, CIP	512 (R)	4 (S)	++	+++ / ++
6A	*Staphylococcus warneri*	+ / -	P, FOX, E, CIP, CLO, RIF	32 (R)	2 (S)	++	++ / ++
7A	*Staphylococcus epidermidis*	+ / +	P, DA, SXT, E, CIP, RIF	512 (R)	8 (I)	+++	++ / ++
8A	*Staphylococcus epidermidis*	+ / +	P, FOX, SXT, CIP, GEN, CLO, RIF	512 (R)	8 (I)	+	+++/ ++
9A	*Staphylococcus aureus*	+ / +	P, DA, SXT, E, CIP, GEN, CLO, RIF	512 (R)	4 (S)	+++	+++ / +++

*, +, positive, -, negative; MIC, minimum inhibitory concentration; OXA, oxacillin, VA, vancomycin; C, control; #, weakly (+), moderately (++), strongly (+++) adherent; P, penicillin, FOX, cefoxitin, DA, clindamycin, E, erythromycin, CIP, ciprofloxacin, GEN, gentamicin, CLO, chloramphenicol, SXT, sulfamethoxazole-trimethoprim, RIF, rifampicin; R, resistant; I, intermediary; S, susceptible.

DISCUSSION

Osteomyelitis is a debilitating inflammatory process with a tendency for progression until bone destruction and is caused by an infecting microorganism. Most cases occur after trauma to a bone or bone surgery or secondary to vascular insufficiency. The infection can be limited to a single

portion of the bone or can involve several regions. The development of osteomyelitis is related to microbial and host factors. Antimicrobial therapy and surgical debridement are the primary modalities of osteomyelitis treatment, requiring a large commitment between patient and clinician[19,20].

Even though antibiotic resistance bacterial is a natural phenomenon, the alarming increase in pathogenic bacteria refractory to a wide range of antimicrobials is attracting attention worldwide. Anti-staphylococcal parenteral β-lactam antibiotics agents are suitable options for the initial treatment of chronic staphylococcal osteomyelitis. β-lactam antibiotics and vancomycin penetrate bone, however, these agents are not available in oral formulation, and the bioavailability of oral penicillin and cephalosporins is usually low. In patients with penicillin allergy, clindamycin and vancomycin are good therapeutic alternatives. Antimicrobial therapy should be based on the final culture helping avoid multidrug resistance and ensuring a more favorable outcome[2,20,21,7].

Among pathogenic microorganisms, *S. aureus* is the most commonly involved in osteomyelitis infection. This organism elaborates on a range of extracellular and cell-associated factors contributing to its virulence. The treatment recommended for osteomyelitis caused by *S. aureus* is a long course of the parenterally administered β-lactam antibiotics or vancomycin[19,21,7]. Mboutol-Mandavo et al (2019) [22] describe *S. aureus* remains the most common cause of hematogenous osteomyelitis in children, in the USA.

In the present study, all *S. aureus* strains isolated from osteomyelitis, demonstrated resistance to oxacillin, penicillin, clindamycin, erythromycin, gentamycin, chloramphenicol, and sulfamethoxazole-trimethoprim, however, in Korea, Oh et al (2019)[23], related all methicillin-susceptible *S. aureus* (MSSA) strains were susceptible to sulfamethoxazole-trimethoprim, 97% were susceptible to clindamycin and 93% were susceptible to erythromycin. Mboutol-Mandavo et al (2019) [22], related seven *S. aureus* isolated from osteomyelitis, including one *S. aureus* multi-resistant.

A reduction in the efficacy of vancomycin has been described in studies of methicillin-resistant *S. aureus* infections treated with this antibiotic and the mechanism of reduced vancomycin susceptibility in CoNS is unclear but may be related to the selection of resistant subpopulations under the pressure of antimicrobial exposure[24].

S. epidermidis, an important commensal bacterium, was described as a cause of osteomyelitis in association with foreign bodies and is often drug-resistant. The microbiological diagnosis of *S. epidermidis* osteomyelitis can be difficult. A diagnosis can be made when the organism is grown from multiple blood cultures, however confirmation of the diagnosis should be made by isolation of the organism from a bone biopsy or aspirate[25,6].

In the present study, all *S. epidermidis* strains were oxacillin-resistantsimilarly to data from previous reports[6,26]. Supporting our data, Aragón-Sánches et al (2010) [27], described that 8.6% of *S. epidermidis* isolated by osteomyelitis were vancomycin-resistant. In 2020, a case was reported, in Indonesia, which a patient presented with stage 4 chronic osteomyelitis, caused by *S. epidermidis*[28].

S. capitis is part of the normal flora of the skin of the scalp, face, ears, neck, and has been occasionally implicated in catheter-related bloodstream infections, device-related bone and joint infections(Brooks et al 2019)[29].In contrast to our findings, Fukuda et al (2010)[30]. described in Japan, a case of osteomyelitis involving the acetabulum caused by *S. capitis* sensitive to penicillin, Brooks et al (2019) [29], reports osteomyelitis in a child caused by methicillin-susceptible *S. capitis*.*S. capitis* osteomyelitis is an uncommon occurrence with a literature review demonstrating three total cases, one of the acetabulum, one of the tibia, and one of the jaw (Brooks et al 2019)[29].

S. warneri generally is not considered pathogenic and has rarely been reported to cause disease in healthy people, probably because of a lack of aggressive virulence properties. Nowadays *S. warneri* is already appointed as a newly emerging pathogen, capable of causing serious infections (Diaconu et al 2020) [31]. The first case report of osteomyelitis caused by *S. warneri*, was in 1986, in Australia[32].In 1987 and 1989, in EUA, other two cases of osteomyelitis were reported caused by *S. warneri*[33,34]. In Italy, 2010, was a characterization of 26 oxacillin and vancomycin-susceptible *S. warneri* isolates from orthopedic infections in large part related to implant materials[35].

Dudareva et al (2019)[36], compared two prospectively identified cohorts of patients with osteomyelitis from the same specialist bone infection treated ten years apart in the UK and describe that 9.6% of isolates were identified as *S. aureus* and 16.3% was identified as CoNS

from 2001 to 2004. Already from 2013 to 2017, 4.3% of isolates were identified as *S. aureus*, and 6% of isolates were identified as CoNS.

Pincher et al (2019)[37].carried out a systematic review and thirteen eligible studies were identified with 505 patients and reported that *S. aureus* was the most common single organism isolated (35.2%) and CoNS accounted for 7% of positive microbiology samples.

The biofilm is a microbial community characterized by cells that attach to substratum or interface or each other, embedded in a matrix of extracellular polymeric substance (EPS), including proteins, DNA, RNA, polysaccharides, and peptidoglycans, and showing an altered phenotype in terms of growth, gene expression, and protein production. Bacterial biofilms play a crucial role in the pathogenesis of relevant human infections, such as osteomyelitis, endocarditis, and infections related to the use of biomedical devices. The EPS materials provide physical barriers to the penetration of antibiotics to the inner viable population of bacteria on biofilm[19,38].

The capacity for biofilm formation is determined by many genetic factors. The main biofilm component is the polysaccharide intercellular adhesin (PIA) molecule synthesized by enzymes encoded by the *ica*A gene that forms the *ica*ABCD operon and mediates cell-to-cell adhesion and *slime* production [39,15].In this study, not all strains harbored the *ica*A gene, which suggests a biofilm formation independent of *ica*A gene presence.

The ability of biofilm formation on the abiotic surface may act as a significant virulence factor that also contributes to the response to harmful and environmental conditions, including exposure to antimicrobial agents, and/or host immune response. Several studies have been investigating surface-attached communities and mechanisms of biofilm resistance to antimicrobial compounds. Biofilm formation has been related to increased expression of antimicrobial resistance by some clinical isolates, due to the acquisition of additional resistance to environmental conditions[40,41,16].

Presently, *Staphylococcus* spp. biofilm formation activity on the abiotic surface was found independent of the presence of *mec*A gene coding for oxacillin resistance. Strains with resistance to oxacillin, related to the *mec*A gene, generally present simultaneous resistance to other β-lactams and are generally related to multi-resistance to non-beta-lactam antimicrobials [15].

Staphylococcus spp. strains in osteomyelitis patients were able to produce biofilm on polystyrene surface in the presence of both OXA and VA, but two strains showed a decrease in biofilm formation in the presence of OXA and/or VA, and interestingly *S. capitis* strain showed an increase in biofilm formation in the presence of antimicrobials tested. The strains studied showed no correlation to the presence of *mec*A and *ica*A genes. Pereira-Ribeiro et al (2019)[16], demonstrate that OXA and VA did not exert any inhibitory effects on biofilm formation on *ica*A-positive and *ica*A-negative *Staphylococcus* strains. Less information is available regarding the effects of antibiotics on biofilm formation by *Staphylococcus*.

The ability of biofilm formation has been demonstrated in some studies with*S. aureus* inoculated onto the surface of different implant materials placed subcutaneously in mice. The authors emphasized the ability of biofilm formation on the surfaces of implanted prostheses as one of the major causes of persistent infection following surgery of patients[42].

During the last decades, multiple virulence factors, including antibiotic resistance, biofilm formation, and surface proteins with adhesive properties have been involved in mechanisms of CoNS survival, dissemination, and persistence in both human hosts and nosocomial environment have been investigated, mostly for CoNS[43,24,15].

CONCLUSION

Osteomyelitis is difficult to treat infection of the bone, which requires a combined medical and surgical approach and often persists intermittently for years. A better understanding of how bacteria invade, survive within, and trigger pathological remodeling of bone could therefore lead to new therapies and the emergence of multi-drug resistant organisms poses major therapeutic challenges. The complex biology of *Staphylococcus*, which includes the ability to form biofilms, contributes to the difficulty of developing a comprehensive strategy applicable to the treatment of all infections caused by *Staphylococcus*. The increase in the number of cases of infections and the multifactorial aspects that favor the high pathogenicity and antibiotic resistance of many *Staphylococcus spp*. should continue to be investigated, since the spread of microorganisms in the hospital environment has become a growing public health challenge and has been severely affecting healthcare systems worldwide. Identification of the causative agent and subsequent targeted antibiotic treatment has a major impact on patients' outcomes.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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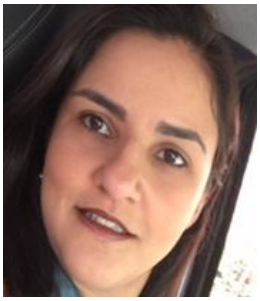
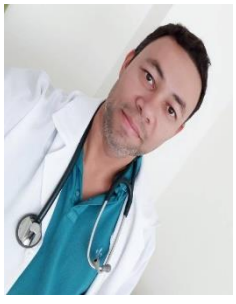
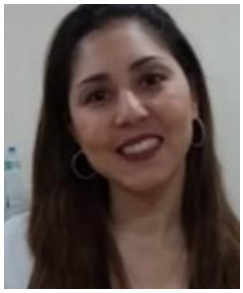
	**Paula Marcele Afonso Pereira Ribeiro – *Corresponding Author*** Departamento de Microbiologia, Imunologia e Parasitologia, Faculdade de Ciências Médicas, Universidade do estado do Rio de Janeiro – UERJ, Rio de Janeiro, RJ, Brasil. Avenida 28 de setembro, 87, fundos, 3ºandar, Vila Isabel, Rio de Janeiro, Brasil. CEP: 20551-030.
	**Guilherme Goulart Cabral-Oliveira** Departamento de Microbiologia, Imunologia e Parasitologia, Faculdade de Ciências Médicas, Universidade do estado do Rio de Janeiro – UERJ, Rio de Janeiro, RJ, Brasil. Avenida 28 de setembro, 87, fundos, 3ºandar, Vila Isabel, Rio de Janeiro, Brasil. CEP: 20551-030.
	**Julianna Giordano Botelho Olivella** Departamento de Microbiologia, Imunologia e Parasitologia, Faculdade de Ciências Médicas, Universidade do estado do Rio de Janeiro – UERJ, Rio de Janeiro, RJ, Brasil. Avenida 28 de setembro, 87, fundos, 3ºandar, Vila Isabel, Rio de Janeiro, Brasil. CEP: 20551-030.
	**Felipe Caldas Ribeiro** Departamento de Microbiologia, Imunologia e Parasitologia, Faculdade de Ciências Médicas, Universidade do estado do Rio de Janeiro – UERJ, Rio de Janeiro, RJ, Brasil. Avenida 28 de setembro, 87, fundos, 3ºandar, Vila Isabel, Rio de Janeiro, Brasil. CEP: 20551-030.

	**Barbara Araújo Nogueira** Departamento de Microbiologia, Imunologia e Parasitologia, Faculdade de Ciências Médicas, Universidade do estado do Rio de Janeiro – UERJ, Rio de Janeiro, RJ, Brasil; e Instituto Oswaldo Cruz, Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, Brazil; Avenida 28 de setembro, 87, fundos, 3ºandar, Vila Isabel, Rio de Janeiro, Brasil. CEP: 20551-030.
	**Bruna Ribeiro Sued Karam** Departamento de Microbiologia, Imunologia e Parasitologia, Faculdade de Ciências Médicas, Universidade do estado do Rio de Janeiro – UERJ, Rio de Janeiro, RJ, Brasil; e Laboratorio de Bacteriologia e Technologia, Bio-Manguinhos, Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, Brazil; Avenida 28 de setembro, 87, fundos, 3ºandar, Vila Isabel, Rio de Janeiro, Brasil. CEP: 20551-030.
	**Ana Luíza Mattos-Guaraldi** Departamento de Microbiologia, Imunologia e Parasitologia, Faculdade de Ciências Médicas, Universidade do estado do Rio de Janeiro – UERJ, Rio de Janeiro, RJ, Brasil; e Departamento de Microbiologia, Universidade Federal do Rio de Janeiro (CCS/UFRJ), Rio de Janeiro, RJ, Brazil. Avenida 28 de setembro, 87, fundos, 3ºandar, Vila Isabel, Rio de Janeiro, Brasil. CEP: 20551-030.