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Detecção e análise genômica de bactérias Gram-negativas multirresistentes de anfíbios

Dissertação apresentada ao Programa de Pós-graduação em Microbiologia do Instituto de Ciências biomédicas da Universidade de São Paulo, para obtenção do título de mestre em Ciências

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Detection and genomic analysis of multidrug-resistant Gram-negative bacteria from amphibians

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Certificamos que o Protocolo CEP-ICB nº **1154/2021** referente ao projeto intitulado: “**Detecção e análise genômica de bactérias Gram-negativas multirresistentes de anfíbios“**, sob a responsabilidade do(a) aluno(a) ***Zuleyma Johana Becerra Tellez,*** e orientação do(a) Prof.(a) Dr.(a) ***Nilton Erbet Lincopan Huenuman***, do Microbiologia, foi analisado pela **CEUA** - Comissão de Ética no Uso de Animais e pelo **CEPSH -** Comitê de Ética em Pesquisa com Seres Humanos, tendo sido deliberado que o referido projeto não utilizará animais que estejam sob a égide da Lei nº 11.794, de 8 de outubro de 2008, nem envolverá procedimentos regulados pela Resolução CONEP nº 466/2012.

São Paulo, 26 de fevereiro de 2021.

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RESUMO

Becerra, J. Detecção e análise genômica de bactérias Gram-negativas multirresistentes de anfíbios. [Dissertação (Departamento de Microbiologia)] Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2022

A resistência aos antibióticos é considerada uma das maiores ameaças à saúde global. A ocorrência de bactérias resistentes em animais silvestres vem aumentando em todo o mundo, paralelamente à situação da medicina humana e veterinária. A presença de bactérias resistentes em animais silvestres pode ser explicada por: (i) a exposição a contaminantes e a bactérias resistentes devido a mudanças antrópicas, e (ii) o surgimento de mecanismos de resistência na microbiota como um processo ancestral de competição e seleção. O principal mecanismo de resistência em bactérias Gram-negativas é a produção de ESBL e β-lactamases AmpC que hidrolisam penicilinas, cefalosporinas, e em alguns casos, carbapenêmicos. Nesse sentido, bactérias ambientais e hospedeiras não humanas podem ser consideradas reservatórios de β-lactamases de interesse clínico. O presente estudo teve como objetivo identificar e caracterizar genomicamente bactérias Gram-negativas resistentes aos antibióticos, isoladas da microbiota de anfíbios [*Phyllomedusa distincta* (*n*= 11), *Rhinella ornata* (*n*= 5), *Scinax fuscovarius* (*n*= 3), e *Physalaemus cuvieri* (*n*= 2)], da Mata Atlântica no Brasil. Os isolados bacterianos foram inicialmente identificados por MALDI-TOF/MS e/ou sistema automatizado Vitek 2. O perfil de susceptibilidade antibacteriana foi determinada pelo método de Kirby-Bauer para 67 isolados bacterianos. O sequenciamento de genoma completo (WGS) foi realizado para caracterizar isolados de *Enterobacter huaxiensis*, *E. bugandensis*, *Pseudomonas putida*, *P. japonica*, e *Stenotrophomonas maltophilia* resistentes a cefalosporinas de amplo espectro, carbapenêmicos, fluoroquinolonas, sulfametoxazol-trimetoprim e/ou tetraciclinas de uso humano e veterinário. Os resultados apresentados são inéditos enquanto a identificação de cepas multirresistentes em anfíbios no Brasil. A identificação de *Enterobacter huaxiensis*, e *E. bugandensis* constituem os primeiros reportes e genomas destas espécies de relevância clínica como parte da microbiota de anfíbios. De grande interesse, novas variantes alélicas de AmpC foram identificada em *E. huaxiensis*. Adicionalmente, uma nova espécie foi identificada (proposta como *Pseudomonas brasiliensis*). Em conclusão, o conjunto de dados compilados neste trabalho contribui para o conhecimento da composição da microbiota de anfíbios, novas variantes alélicas de genes de resistência, e a descoberta de novas espécies que compõem uma biodiversidade a ser ainda descoberta em ecossistemas brasileiros.

Palavras-chave: resistência bacteriana, anfíbios, resistoma, beta-lactamase, WGS.

ABSTRACT

Becerra, J. Detection and genomic analysis of multidrug-resistant Gram-negative bacteria from amphibians. [Master thesis (Departamento de Microbiologia)] Instituto deCiências Biomédicas, Universidade de São Paulo, São Paulo, 2022

Antibiotic resistance is considered one of the greatest threats to global health. The occurrence of resistant bacteria in wild animals has been increasing worldwide in parallel to the situation in human and veterinary medicine. Resistant bacteria in wild animals can be explained by: (i) exposition to contaminants and resistant bacteria due to anthropogenic changes, and (ii) the emergence of resistance mechanisms as an ancient competition process. The primary resistance mechanism in Gram-negative bacteria is the production of ESBL and AmpC β-lactamasesthat hydrolyze penicillins, cephalosporins, and in some cases carbapenems. In this sense, environmental bacteria and non-human hosts can be considered reservoirs of β-lactamases of clinical interest. This study was carried out to characterize antibiotic-resistant Gram-negative bacteria isolated from the microbiota of amphibians (*Phyllomedusa distincta* n=11; *Rhinella ornata* n=5; *Scinax fuscovarius* n=3 and *Physalaemus cuvieri* n=2) in the Brazilian Atlantic Forest. Bacterial isolates were initially identified by MALDI-TOF/MS and/or Vitek2. Kirby-Bauer method was carried out for antimicrobial susceptibility profile in 67 bacterial isolates, and Whole-genome sequencing (WGS) was carried out to characterize isolates such as *Enterobacter huaxiensis*, *E. bugandensis*, *Pseudomonas putida*, *P. japonica*, and *Stenotrophomonas maltophilia* resistant to broad-spectrum cephalosporins, carbapenems, fluoroquinolones, trimethoprim-sulfamethoxazole, and tetracyclines for human and veterinary use. The results presented are unprecedented in terms of identifying multidrug-resistant strains in amphibians in Brazil. The identification of *Enterobacter huaxiensis* and *E. bugandensis* constitute the first reports and genomes of these clinical relevance isolates as part of the amphibian microbiota (Chapter 3). Of great interest is the presence of new allelic variants of AmpC identified in *E. huaxiensis* (Chapter 4). Additionally, a new species wasidentified proposed as *Pseudomonas brasiliensis* (Chapter 5). In conclusion, the dataset compiled in this work contributes to the knowledge of the composition of the amphibian microbiota, new allelic variants of AmpC β-lactamase, and the discovery of new species that contribute to the knowledge of microbial biodiversity.

Keywords: antibiotic resistance, amphibian, resistome, β-lactamase, WGS.

SUMMARY

CHAPTER 1.

GENERAL INTRODUCTION

Antimicrobial resistance (AMR) is considered one of the greatest threats to the global health (WHO, 2019). The origin is complex, driven by environmental, ecological, and socioeconomic factors that facilitate the dissemination resistant-bacteria (WELLINGTON et al. 2013). Since the first description of chloramphenicol-resistant *Escherichia coli* isolates in Japanese wild birds, in 1978, several studies have reported that occurrence of AMR-bacteria in wild animals, which has increased worldwide in parallel to the situation in human and veterinary medicine (SATO et al. 1978; WANG et al. 2017).

Although wild animals are not directly exposed to antibiotics, the water or food contaminated with fertilizer, residues from factories, hospitals, sewage, and the livestock can enhance their contact with selective agents, as well as with AMR-bacteria (ANDERSSON et al. 2014; TORRES, 2020). After ingestion, these bacteria pass to the intestine, transfer AMR-genes to the commensal microbiota and are released into the environment returning to human and animal hosts (Fig. 1) (SINGER et al. 2006; VALDES et al. 2021).

On the other hand, considering that secretion of antimicrobial compounds by microbes is an ancient strategy to improve the competing with other microorganisms, the emergence of antimicrobial resistance mechanisms is also an ancient natural response process (DAVIES et al. 2010; D'COSTA et al. 2011). Additionally, the short generation time and horizontal gene transfer enhance the resistance dissemination and mutation at a relatively high rate (BRANDT et al. 2017).

The main mechanism of resistance in Gram-negative bacteria is the production of β-lactamases (BLs), which hydrolyze the β-lactam ring, inactivating monobactams,

penicillins, cephalosporins, and in some cases carbapenems (LAVIGNE et al. 2004). In this context, the emergence of multidrug-resistance (MDR) Gram-negative bacteria deserves special attention. MDR is defined as acquired nonsusceptibility to at least one agent in three or more antimicrobial categories (MAGIORAKOS et al. 2012). They are been widely reported in non-clinical settings, from pets, farm animals and in wildlife (RADHOUANI et al. 2014; NOWAKIEWIC et al. 2020).

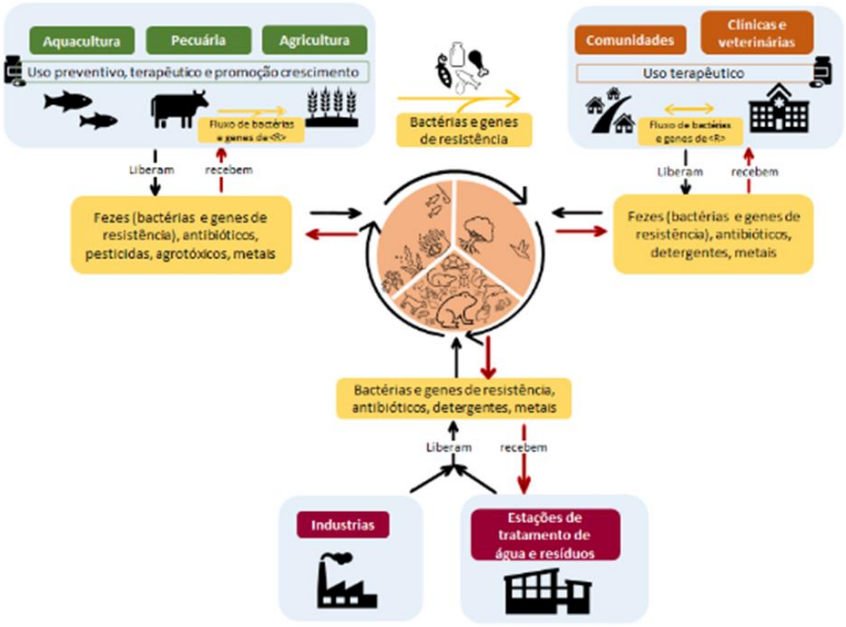

Figure 1. Scheme of transfer of bacteria and resistance genes and their reservoirs. Modified from Andersson et al. 2014. Aquaculture, livestock, and agriculture, highlighted in green; clinical and veterinary communities, highlighted in orange; industries and treatment plants, highlighted in red. The black arrows indicate the output of matter and energy from the systems, and the red arrows return these to the systems.

Environmental bacteria and non-human hosts may be considered as underestimated reservoirs of BLs genes widely associated with pathogens in clinical settings (BUSH et al. 2018; BRANDT et al. 2017; WHITE et al. 2019). Resistance in environmental bacteria typically goes unnoticed until the emerging resistance genes appear in a clinical setting. The most alarming rise has been observed in resistance to β-lactam antibiotics as a result of

global dissemination of extended-spectrum beta-lactamases (ESBL) and AmpC cephalosporinases and carbapenemases (WHO, 2019).

ESBL and AmpC-producing isolates have been documented in commensal microbiota of wild mammals (CEVIDANES et al. 2020; NOWAKIEWICZ et al. 2020), and birds (MAROTTA et al. 2019; PLAZA-RODRÍGUEZ et al. 2021; YAHIA et al. 2018), showing the complexity of wild animals as reservoirs and vectors of clinically relevant genes. Nonetheless, there are few studies focused on MDR Gram-negative bacteria in frogs and toads (HACIOGLU et al. 2014; MORRISON et al. 2020).

In Brazil, amphibian populations represent the highest diversity worldwide, with 1188 species (SEGALLA et al., 2021). In addition, this country is the largest producer of frogs for human consumption in captivity conditions, with São Paulo and Rio de Janeiro being the states with the most significant number of frog farms. In natural settings, despite the challenges in intervened systems, they are present in areas with different degrees of anthropization, being found in little-intervened, agricultural, peri-urban and urban areas (HUTTO et al. 2021; WESTGATE et al. 2015).

Therefore, in this study, is proposed to genomic characterization of multidrug-resistant Gram-negative bacteria isolated from amphibians present in ecosystems with different degrees of anthropization in areas of Brazilian Atlantic Forest in São Paulo State. This study was carried out from individuals of the species *Phyllomedusa distincta*, sampled from conserved areas, and *Rhinella ornate*, *Scinax fuscovarius*, and *Physalaemus* spp., sampled from an urban area with anthropic intervention. These analyses were discussed from a One Health perspective, which proposes a multidisciplinary approach highlighting the importance of integrate human, ecological and environmental aspects.

CHAPTER 2

GENERAL OUTLINE OF THE MASTER THESIS AND RELATION BETWEEN CHAPTERS

This master thesis aims to identify and characterized multidrug-resistance Gram-negative bacteria from skin of amphibians collected at the Brazilian Atlantic Forest.

For this purpose, the specific objectives were:

(i) To isolate and identify Gram-negative bacteria from skin samples
(ii) To determine susceptibility profiles of isolates
(iii) To perform whole-genome sequencing (WGS) of resistant bacterial strains

CHAPTER 3

The first report of clinical isolates as part of cutaneous microbiota from *Phyllomedusa distincta* (Anura: Phyllomedusidae) in the Brazilian Atlantic Forest.

CHAPTER 4

Report of a novel variant of AmpC β-lactamase in *Enterobacter huaxiensis* isolated from *Phyllomedusa distincta* (Anura: Phyllomedusidae) in the Brazilian Atlantic Forest.

CHAPTER 5

Description of a novel species, *Pseudomonas brasiliensis* isolate from skin frog *Scinax fuscovarius* (Anura:Hylidae) in Southeast Brazil

CHAPTER 3

Draft Genome of *Enterobacter* spp., harboring AmpC β-lactamases isolated from cutaneous bacteria of *Phyllomedusa distincta* (Anura: Phyllomedusidae) in the Brazilian Atlantic Forest

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Abstract

The occurrence of antimicrobial resistance genes in wild animals has been increasingly worldwide in parallel to the situation in human and veterinary medicine, representing a risk for humans and animals. The aim of this study was to report first drafts genome sequences of isolates of *Enterobacter bugandensis* (4Pd7) and *E. huaxiensis* (4Pd9), recovered from skin of *Phyllomedusa distincta,* an endemic frog inhabiting the Brazilian Atlantic Forest. The genomes were sequenced using an Illumina MiSeq platform, and de novo genome assembly was performed using SPAdes v.3.10.1. Whole genome sequence was analyzed using bioinformatics tools from the Center of Genomic Epidemiology. This draft genome resulted in x and x bp with and protein-coding sequences respectively, revealing the presence of AmpC β-lactamases belonging to ACT family, responsible for resistance to cephalosporins. These data provide useful information for characterization of the skin frog microbiome, and to genomic analysis regarding the dissemination of antimicrobial resistance genes.

Keywords: *Enterobacter*, AmpC, *bla*$_{\text{ACT}}$, *Phyllomedusa distincta*, Brazilian Atlantic Forest.

CHAPTER 4

ACT-107, a Novel Variant of AmpC-typeβ-Lactamase in *Enterobacter huaxiensis* isolated from *Phyllomedusa distincta* (Anura: Phyllomedusidae) inhabiting the Brazilian Atlantic Forest

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Abstract

Bacteria from the *Enterobacter* genus are widely distributed in the environment, with frogs being reported as natural hosts, and described as a human pathogen. During a genomic surveillance study, we identified an AmpC-positive *Enterobacter huaxiensis* isolated from the skin of *Phyllomedusa distincta* in the Brazilian Atlantic Forest. Whole genome sequencing analysis revealed a novel variant of AmpC β-lactamase belonging to the ACT family, designated as ACT-107. This variant shows thirteen unique amino acid substitutions, five in the signal peptide sequence (Ile2, Met14, Tyr16, Gly18 and Thr20), and seven in the mature protein (Gln22, His43, Cys60, Thr157, Glu225, Ala252 and Asn310). *In silico* modeling showed that substitutions occurring in the mature chain are in the solvent-accessible surface of the protein. These mutations are not expected to affect the β-lactamase activity, as observed in their phenotypic behavior. This finding denotes that environmental bacteria may represent an unexplored reservoir of potentially transferable resistance genes, creating a further risk for the origin of novel β-lactamases.

Keywords: AmpC, β-lactamases, *bla*$_{\text{ACT-107}}$, *Phyllomedusa distincta*, Brazilian Atlantic Forest

CHAPTER 5

Pseudomonas brasiliensis sp. nov., isolated from skin frog *Scinax fuscovarius* (Anura:Hylidae) in Southeast Brazil

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Abstract

The bacterial strain SF2^{T} was isolated from skin of frog *Scinax fuscovarius* in the São Paulo University during a surveillance work in amphibians. The 16S rRNA gene sequences showed the highest similarities with *Pseudomonas putida* (99.8%), *P. monteilii* (99.8%), and *P. plecoglossicida* (99.8%).The phylogenetic trees based on multilocus sequence analyses with concatenating 16S rRNA, *gyrB, rpoD* and *rpoB* genes suggested that this strain should beafliated to group Putida. Therefore, phenotypic, phylogenetic, genomic, chemotaxonomic, and proteomic traits showed that the isolate represented a novel species of the genus *Pseudomonas*, for the name *Pseudomonas bras*iliensis sp. nov. is proposed.

Keywords: Pseudomonadaceae, multilocus sequence analyses. amphibian, Brazil

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ATTACHMENTS

A. Supplemental material for chapter 4

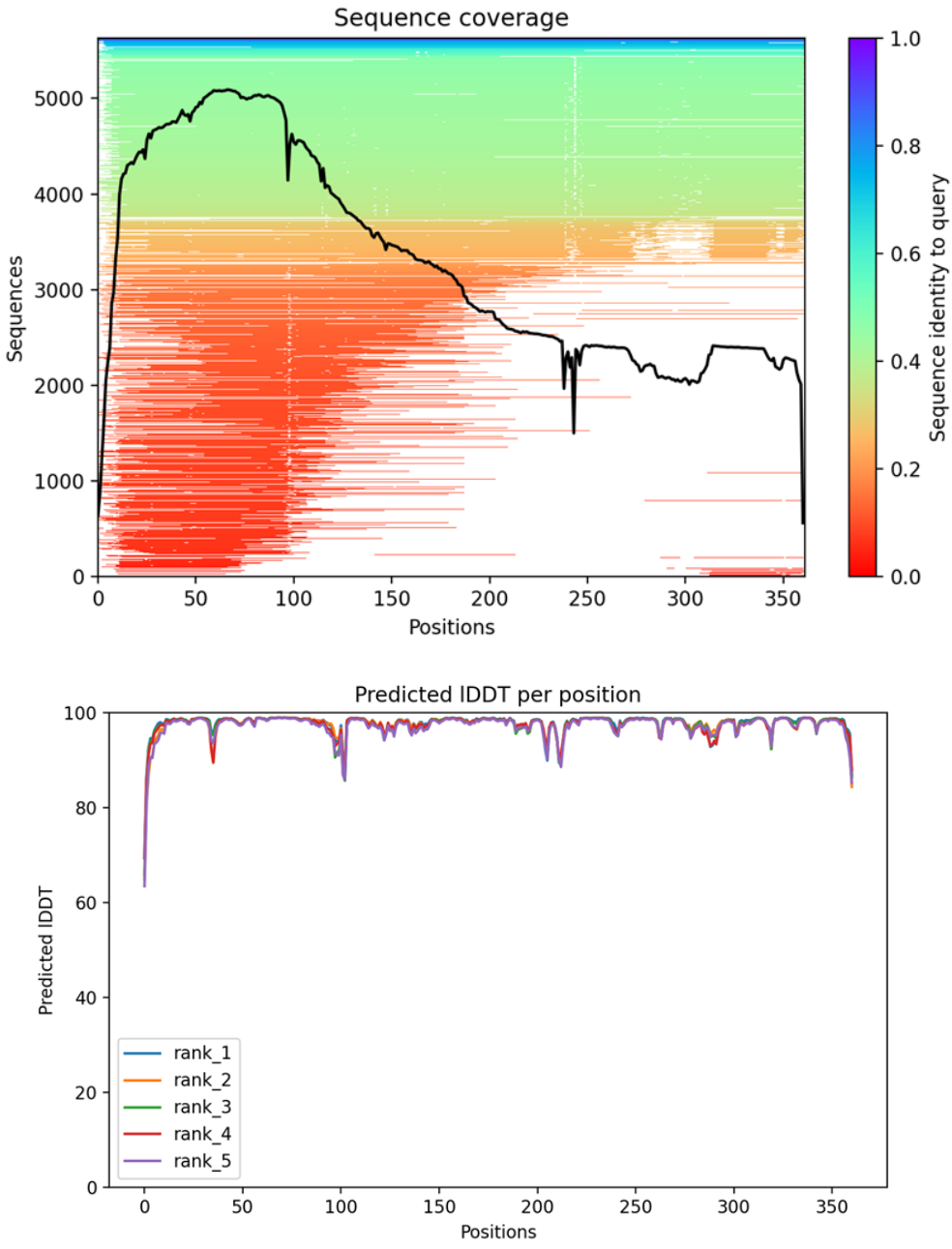

Figure S1. Results from the ColabFold implementation of AlphaFold2 prediction of the ACT-107 tertiary structure. Panel A presents the coverage depth of the multiple sequence alignment created for the predictions, showing the abundance of β-lactamase-related sequences available, while panel B presents the predicted local distance difference test (lDDT) confidence measure per position of the different models, of which the first ranked (model 5) was used for further analyses.

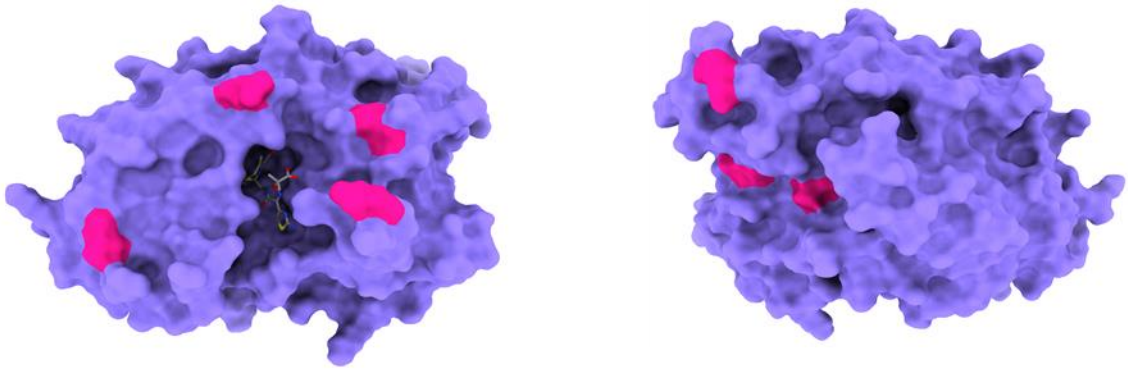

Figure S2. Surface representation of the AlphaFold2 model of ACT-107 bound to acylated ceftazidime highlighting in hot pink the positions of the unique mutations found in the mature form of this variant when compared with all ACT-type β-lactamases.

B. Supplemental material for chapter 5

Table S1. Average nucleotide identity between *Pseudomonas brasiliensis* sp. nov. SF2^T and other *Pseudomonas* spp.

Type Strain Genome Server						
Subject strain	**dDDH (d4, in %**	**C.I. (d4, in %**	**G+C content difference (in %)**	**ANIb (>95%**	**ANIm (>95%**	**Tetra (>0,999**
Pseudomonas asiatica JCM 32716T	56,5	[53.8 - 59.3]	0,7	93,46	94,48	0,99722
Pseudomonas inefficax Pseudomonas sp. JV5	54,3	[51.6 - 57.0]	0,42	92,82	94,07	0,99775
Pseudomonas oryzicola RD9SR1	42,8	[40.3 - 45.3]	0,33	90,4	91,19	0,99494
Pseudomonas anuradhapurensis RD8MR3	42,8	[40.3 - 45.4]	0,18	90,33	91,27	0,99533
Pseudomonas kurunegalensis RW1P2	41,3	[38.8 - 43.8]	1,14	89,47	90,66	0,99174
Pseudomonas monteilii DSM 14164	41,2	[38.7 - 43.8]	1,76	89,35	90,59	0,99108
Pseudomonas putida NBRC 14164	41,1	[38.7 - 43.7]	0,92	89,15	90,65	0,99561
Pseudomonas capeferrum WCS358	34,6	[32.2 - 37.2]	0,58			
Pseudomonas kermanshahensis SWRI100	34,3	[31.9 - 36.8]	1,03			
Pseudomonas plecoglossicida DSM 15088	33,6	[31.2 - 36.1]	0,28			
Pseudomonas muyukensis COW39	31,4	[29.0 - 33.9]	1,86			
Pseudomonas xanthosomatis COR54	31,2	[28.8 - 33.7]	0,94			
Pseudomonas taiwanensis DSM 21245	31,2	[28.8 - 33.7]	1,39			
Pseudomonas fakonensis COW40	31,1	[28.7 - 33.6]	1,02			
Pseudomonas entomophila L48	30,8	[28.4 - 33.3]	0,91			

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As co-author of one of the manuscripts cited above, I authorize Johana Becerra to use is a part of her master thesis presented to the Postgraduate Program in Microbiology of the Institute of Biomedical Sciences of the University of São Paulo, to obtain the title of Master of Science (MSc.).

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APPENDICES

A. Articles published or submitted as co-author during the MSc.

1. "Detecting KPC-2 and NDM-1 co-expression in *Klebsiella pneumoniae* complex from human and animal hosts in South America" by Felipe Vásquez-Ponce, Karine Dantas, **Johana Becerra**, Gregory Melocco, Fernanda Esposito, Brenda Cardoso, Larissa Rodrigues, Keila Lima, Aline V. de Lima, Fábio P. Sellera, Renata Mattos, Lucas Trevisoli, Marco A. Vianello, Thais Sincero, Jose Di Conza, Eliana Vespero, Gabriel Gutkind, Jorge Sampaio, and Nilton Lincopan.

B. Books chapter published or submitted as author during the MSc.

1. "Anfíbios como fonte não convencional de moléculas com potencial antimicrobiano" by**Johana Becerra**, Faride Lamadrid-Feris, Raul Ferreira, Jorge Arboleda-Valencia, Felipe Vásquez-Ponce, Gabriel Padilla, and Nilton Lincopan.

C. Congress and symposium presentation during the MSc.

1. "Bactérias potencialmente benéficas na microbiota da pele de anuros são moduladas por fatores intrínsecos do hospedeiro" by Faride Lamadrid-Feris, **Johana Becerra**, and Fernando Ribeiro Gomes. Simposio de Fisiologia Animal, Instituto de Biociências, Universidade de São Paulo, 2019.

2. "Bacterias con potencial regulador de *Aeromonas hydrophila* aislados de la piel de *Phyllomedusa distincta* (Anura: Phyllomedusidae)" by Faride Lamadrid-Feris, **Johana Becerra**, and Fernando Ribeiro Gomes. XXV Congresso Latinoamericano de Microbiologia (ALAM), 2021.

3. "Detection and genomic analysis of amphibian Gram-negative multidrug-resistant bacteria" by **Johana Becerra**, Faride Lamadrid-Feris, and Nilton Lincopan. 31º Congresso Brasileiro de Microbiologia, 2021.

4. "Inhibition of WHO Critical Priority Multidrug-Resistant Pathogens by Cutaneous Bacteria of *Phyllomedusa distincta* (Anura: Phyllomedusidae)" by **Johana Becerra**, Faride Lamadrid-Feris, Fernando Gomes, Raúl Ferreira, Gabriel Padilla, and Nilton Lincopan. 31º Congresso Brasileiro de Microbiologia, 2021.