UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
CURSO DE ESPECIALIZAÇÃO EM MICROBIOLOGIA CLÍNICA

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Pesquisa de resistência aos antimicrobianos de Enterobactérias isoladas em aves marinhas, no sul do Brasil

Porto Alegre
2020

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PESQUISA DE RESISTÊNCIA AOS ANTIMICROBIANOS DE ENTEROBACTÉRIAS ISOLADAS EM AVES MARINHAS, NO SUL DO BRASIL

Trabalho de conclusão de curso de especialização apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de Especialista em Microbiologia Clínica.

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Porto Alegre
2020

CIP - Catalogação na Publicação

Meurer, Rafael
Pesquisa de resistência aos antimicrobianos de Enterobactérias isoladas em aves marinhas, no sul do Brasil / Rafael Meurer. -- 2020.
30 f.
Orientador: Afonso Luís Barth.

Coorientadora: Priscila Lamb Wink.

Trabalho de conclusão de curso (Especialização) -- Universidade Federal do Rio Grande do Sul, Instituto de Biociências, Especialização em Microbiologia Clínica, Porto Alegre, BR-RS, 2020.

1. Resistência antimicrobiana. 2. resistência a carbapenêmicos. 3. pinguim. 4. NDM-1. 5. ST156. I. Luís Barth, Afonso, orient. II. Lamb Wink, Priscila, coorient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

RESUMO

As gaivotas (*Larus dominicanus*) possuem ampla distribuição no hemisfério sul do mundo. Os pinguim-de-magalhães (*Spheniscus magellanicus*) são migratórios, com as colônias reprodutivas na região da Patagônia (Argentina e Chile) e parte da área de alimentação no litoral brasileiro. Estas aves podem ser colonizadas por bactérias resistentes a antibióticos e, assim, carregar esses microrganismos para diferentes regiões geográficas. Este trabalho teve como objetivo avaliar o perfil de resistência aos carbapenêmicos de Enterobactérias obtidas da corrente sanguínea de pinguins e gaivotas durante um projeto de monitoramento de praias no Brasil. Um total de 74 isolados bacterianos recuperados de amostras de sangue arterial de animais mortos foram submetidos à identificação das espécies pelo sistema API (bioMérieux®) e avaliação da susceptibilidade aos antibióticos (método de disco-difusão). Um isolado apresentou resistência aos carbapenêmicos (*E. coli* 89PenNDM). Este isolado foi submetido a PCR-HRM para detecção dos genes da carbapenemase e demonstrou apresentar o gene $bla_{\text{NDM-1}}$. Experimentos de conjugação indicaram que o $bla_{\text{NDM-1}}$ de *E. coli* 89PenNDM era transmissível para *E. coli* J53. O sequenciamento do genoma completo (WGS) confirmou a presença do gene $bla_{\text{NDM-1}}$, que foi identificado em um plasmídeo conjugativo (plasmídeo IncA/C2), tanto no 89PenNDM de *E. coli* quanto em seus transconjugantes. WGS foi capaz de classificar o isolado como ST 156 e identificar muitos outros genes de resistência (sul1, sul, 2, strA, floR, tet (A)), todos carreados no mesmo plasmídeo IncA/C2. Este é o primeiro relato de *E. coli* produtora de $bla_{\text{NDM-1}}$ isolada de um pinguim (*Spheniscus magellanicus*) na costa brasileira. A presença de um gene de carbapenemase em animais selvagens é preocupante, pois podem se tornar reservatórios de bactérias multirresistentes e disseminá-las para o meio ambiente.

Palavras-chave: Resistência antimicrobiana. resistência a carbapenêmicos. animais silvestres. pinguim. NDM-1. ST156.

ABSTRACT

Seagulls (*Larus dominicanus*) inhabitan the southern hemisphere of the world. Magellanic Penguins (*Spheniscus magellanicus*) are migratory animals, with reproductive colonies in the Patagonia region (Argentina and Chile) and part of the feeding area on the Brazilian coast. These birds can be colonized by bacteria resistant to antibiotics and thus carry these microorganisms to different geographical regions. This work aimed to evaluate the carbapenemic resistance profile of Enterobacteria isolated from the bloodstream of penguins and seagulls during a beach monitoring project in Brazil. A total of 74 bacterial isolates recovered from arterial blood samples from dead animals were submitted to species identification using the API system (bioMérieux®) and antibiotic susceptibility evaluation by disc-diffusion. One isolate presented resistance to carbapenems (*E. coli* 89PenNDM). This isolate was submitted PCR-HRM to detect carbapenemase genes and proved to present the $bla_{\text{NDM-1}}$ gene. Experiments of conjugation indicated that the $bla_{\text{NDM-1}}$ of *E. coli* 89PenNDM was transmissible to *E.coli* J53. Whole genome sequencing (WGS) confirmed the presence of the $bla_{\text{NDM-1}}$ gene, which was presented in a conjugative plasmid (IncA/C2 plasmid), in both the *E. coli* 89PenNDM and its transconjugants. WGS was able to classify the isolate as ST156 and to identify many other resistance genes (eg.: sul1, sul,2, strA, floR, tet(A)), all carried in the same IncA/C2 plasmid. This is the first report of $bla_{\text{NDM-1}}$ producing *E. coli* isolated from a penguin (*Spheniscus magellanicus*) in the Brazilian seacoast. The presence of a carbapenemase gene in wildlife animals is of concern as they may become reservoirs of multidrug-resistant bacteria and disseminate them to the environment.

Keywords: Antimicrobial resistance. carbapenem resistance. wildlife. penguin. NDM-1. ST156.

SUMÁRIO

1 INTRODUÇÃO

As gaivotas (*Larus dominicanus*) possuem ampla distribuição no hemisfério sul do mundo. No Brasil, são encontradas ao longo de toda costa, principalmente, na região sul e sudeste (1, 2). A espécie vem expandindo, consideravelmente, sua população ao longo da costa do hemisfério sul (3, 4). Possui hábitos alimentares generalistas, se alimentando desde peixes até restos de animais mortos nas praias. Nos grandes centros urbanos encontraram grande disponibilidade de alimentos em lixões, sendo comum observar a espécie nidificando próximo a essas áreas (5).

Os pinguim-de-magalhães (*Spheniscus magellanicus*) são migratórios, se reproduzem e trocam de plumagem na região da Patagônia, formando colônias na Argentina e Chile entre fim de setembro e início de abril. Realizam a migração entre meados de abril e setembro para o litoral brasileiro em busca de alimentos e temperaturas mais agradáveis, assim estarão preparados fisicamente para o próximo período reprodutivo (6). Durante a migração, diversos fatores podem interromper a jornada, levando ao encalhe nas praias brasileiras, tais como a poluição marinha com petróleo e derivados (7), baixa disponibilidade de presas devido a mudanças climáticas, inexperiência dos jovens durante a primeira migração, entre outros (8).

Cada vez mais presente no contexto mundial, a resistência bacteriana aos antibióticos é muito importante de ser compreendida, uma vez que, já foram encontrados genes importantes de resistência em pinguins (9) e gaivotas (10). Entre as resistências conhecidas de Enterobactérias, podemos destacar as seguintes: *Klebsiella pneumoniae* carbapenemase (KPC), Metalo-β-lactamases (MBL) e à Colistina. Esta última foi amplamente utilizada no Brasil como aditivo zootécnico até 2016, quando o Ministério da Agricultura, Pecuária e Abastecimento (MAPA), publicou a Instrução Normativa Nº 45, de 22 de novembro de 2016 proibindo em todo território nacional a importação e fabricação da substância (11).

O surgimento de Enterobacterias resistentes aos carbapenêmicos (CRE) é um importante problema de saúde pública e seu surgimento e disseminação global são motivos de grande preocupação em todo o mundo (12, 13). Como exemplo, podemos citar as bactérias portadoras do gene NDM-1 que pode hidrolisar todos beta-lactâmicos, com exceção do aztreonam. Para infecções ocasionadas por portadores de NDM-1 restam poucas opções terapêuticas, apenas colistina e tigeciclina podem ser administrados (14).

Os reservatórios para organismos CRE estão aumentando, não apenas em hospitais, mas também na comunidade e no meio ambiente, e a disseminação de carbapenemases nos últimos anos desafiou criticamente a eficácia terapêutica dos carbapenêmicos (15). Embora

apenas alguns estudos tenham relatado a presença de CRE em animais, sua presença em animais produtores de alimentos e seu ambiente foi demonstrada. Uma condição nova e importante é a presença de tais organismos no gado, nos animais de companhia e na vida selvagem (16). Entretanto, pouco se sabe sobre sua disseminação e potencial transmissão ao ser humano (17).

É importante monitorar a susceptibilidade aos antimicrobianos em animais silvestres, pois existe fluxo de forma natural, no caso das aves migratórias ou pode haver interação indevida de humanos com animais potencialmente transmissores. A disseminação pode ser sem precedentes, podendo causar alguns surtos (18).

1.1 OBJETIVOS

1.1.1 Objetivo geral

Avaliar a suscetibilidade aos antibióticos de isolados de Enterobactérias da corrente sanguínea de aves marinhas.

1.1.2 Objetivos específicos

- Determinar o perfil de suscetibilidade aos antibióticos de Enterobactérias isoladas na corrente sanguínea de duas espécies de aves marinhas: *Larus dominicanus* e *Spheniscus magellaniscus;*
- Avaliar a presença de carbapenemases pelo método fenotípico de teste rápido Blue carba;
- Avaliar a presença de genes de carbapenemase por meio de técnicas de biologia molecular;
- Sequenciar o genoma completo de isolado bacteriano produtor de NDM-1.

CONTRIBUIÇÃO DOS AUTORES:

O autor deste TCC (Rafael Meurer) se envolveu significativamente com a realização do trabalho, contribuindo igualmente na realização dos experimentos, assim como discussão de resultados e redação do manuscrito. Com exceção do sequenciamento total do genoma que, por envolver experimentos mais complexos, foi realizado pela equipe do LABRESIS.

2 ARTIGO CIENTÍFICO

Escherichia coli carrying *bla*$_{NDM-1}$ obtained from a migratory penguin (*Spheniscus magellanicus*) in the Brazilian seacoast

Running title: Migratory penguin carrying *bla*$_{NDM-1}$ in *E. coli*

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Abstract

The reservoirs for NDM-producing *Enterobacterales* organisms are increasing, not only in hospitals, but also in the environment and in the community such as in livestock, companion animals and wildlife, challenging the therapeutic efficacy of carbapenems. This report aimed to characterize an isolate of *Escherichia coli* harboring the $bla_{\text{NDM-1}}$ gene recovered from the bloodstream of a penguin (*Spheniscus magellanicus*) during a routine beach monitoring project in Southern Brazil. A total of 74 bacterial isolates recovered from arterial blood samples from dead animals were submitted to species identification using the API system (bioMérieux®) and antibiotic susceptibility evaluation. One isolate presented resistance to carbapenems (*E. coli* 89PenNDM). This isolate was submitted PCR-HRM to detect carbapenemase genes and proved to present the $bla_{\text{NDM-1}}$ gene. Experiments of conjugation indicated that the $bla_{\text{NDM-1}}$ of *E. coli* 89PenNDM was transmissible to *E.coli* J53. Whole genome sequencing (WGS) confirmed the presence of the $bla_{\text{NDM-1}}$ gene, which was presented in a conjugative plasmid (IncA/C_2 plasmid), in both the *E. coli* 89PenNDM and its transconjugants. WGS was able to classify the isolate as ST 156 and to identify many other resistance genes (eg.: sul1, sul,2, strA, floR, tet(A)), all carried in the same IncA/C2 plasmid. This is the first report of $bla_{\text{NDM-1}}$ producing *E. coli* isolated from a penguin (*Spheniscus magellanicus*) in the Brazilian seacoast. The presence of a carbapenemase gene in wildlife animals is of concern as they may become reservoirs of multidrug-resistant bacteria and disseminate them to the environment.

Keywords: Antimicrobial resistance, carbapenem resistance, wildlife, penguin, NDM-1, ST156.

1. Introduction

The emergence of carbapenem-resistant *Enterobacterales* (CRE) is a major public health problem and its global spread in clinical settings is of great concern worldwide.[1,2] Carbapenemases are the main determinants of carbapenem resistance, and the New Delhi metallo-β-lactamase-1 (NDM-1) encoded by the $bla_{\text{NDM-1}}$ gene has drawn particular attention due to its widespread capacity and ability to hydrolyse all beta-lactams except aztreonam.[3]

The reservoirs for CRE organisms are increasing, not only in hospitals, but also in the community and in the environment which critically challenges the therapeutic efficacy of carbapenems.[4] Although only a few studies have reported the presence of CRE from wildlife animals, their presence in food-producing animals and their environment has been demonstrated.[5] However, little is known about their dissemination and potential transmission to humans.[6] The aim of this study was to characterize an isolate of *Escherichia coli* harboring the $bla_{\text{NDM-1}}$ gene recovered from the bloodstream of penguin (*Spheniscus magellanicus*) during a routine beach monitoring project in Southern Brazil.

2. Material and Methods

The Santos Basin Beach Monitoring Program ("*Projeto de Monitoramento de Praias da Bacia de Santos"*, PMP-BS) is one of the monitoring programs required by the Brazilian Federal Environmental Agency ("IBAMA") for the environmental licensing process of oil production and transport by the Brazilian Federal Petrol Company ("Petrobras") at the pre-salt province. This program collects ill and dead marine animals found in the Brazilian shoreline. Debilitated animals undergo rehabilitation treatment and the dead ones are submitted to complete necropsy. Arterial blood from dead animals are collected and inoculated into blood cultures bottles (Newprov®) and the positive bottles are subcultured onto blood agar plates for bacterial identification and further studies.

For this study, bacterial isolates recovered from arterial blood sampled were submitted to species identification using the API system (bioMérieux, Lyon, France) and antibiotic susceptibility evaluation by the disk diffusion method. Isolates with reduced susceptibility to carbapenems were further evaluated by the Blue-Carba test, a colorimetric assay for carbapenemase detection.[7] Isolates with positive result in the Blue-Carba test were submitted to species identification using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Biomérieux, France) and to carbapenemase genes (*bla*$_{\text{NDM-1}}$, *bla*$_{\text{KPC-2}}$, *bla*$_{\text{VIM-type}}$, *bla*$_{\text{GES-type}}$, *bla*$_{\text{OXA-48-like}}$, and *bla*$_{\text{IMP-type}}$) detection by multiplex high resolution melting (HRM) real-time PCR according to Monteiro et al.[8] Conjugation experiments were performed using *Escherichia coli* J53 as a recipient and minimal inhibitory concentration (MIC) of antibiotics representative of beta-lactams, aminoglycosides, tigecycline and chloramphenicol were evaluated by broth microdilution.

The genomes of the *E. coli* 89PenNDM and its transconjugants were extracted with Wizard SV Genomic DNA purification system (Promega), the library was prepared with Nextera XT DNA Library Prep Kit (Illumina) and the sequencing was performed in a MiSeq™ platform (Illumina Inc.) with MiSeq Reagent V2 (500 cycles). The obtained sequences were assembled with SPAdes V.3.1 and annotation was performed in PATRIC.[9,10] Data were analysed using webtools from the Centre for Genomic Epidemiology (http://www.genomicepidemiology.org) and detailed analyses were performed using the Geneious software.

3. Results

From 2015 to 2018, a total of 74 bacteria were obtained from arterial blood samples from 45 penguins (*S. magellanicus*) and 29 seagulls (*Larus dominicanus*). Among this collection, one isolate presented resistance to imipenem, ertapenem and meropenem. This isolate was obtained from a *S. magellanicus* in September 2018, which was found debilitated in a beach of "São Francisco do Sul" and taken to rehabilitation in "Florianópolis", both municipalities from the Brazilian state of Santa Catarina. As this *E. coli* presented positive result in the Blue-Carba test and the $bla_{\text{NDM-1}}$ gene was identified by PCR-HRM, the isolate was termed *E. coli* 89PenNDM. Broth microdilution confirmed that *E. coli* 89PenNDM was fully resistant to carbapenems and that the transconjugant presented significant increase in the MICs of carbapenems, ceftazidime, and chloramphenicol in comparison to *E. coli* J53 (Table 1).

WGS of *E. coli* 89PenNDM generated the assembly of 151 scaffolds, which resulted in an estimated draft genome of 5,186,141 bp length, with a G+C content of 50% and a total of 5,244 coding sequences. *In silico* analyses of WGS data indicated that the *E. coli* 89PenNDM belonged to the sequence type ST156 and confirmed the presence of the $bla_{\text{NDM-1}}$ gene. The WGS of the transconjugant confirmed that the plasmid harboring the $bla_{\text{NDM-1}}$ gene belonged to the IncA/C2 incompatibility family. Although it was not possible to cover the length of the plasmid sequence entirely, the sum of the scaffolds comprising the plasmid was 161,769 bp and, in addition to the $bla_{\text{NDM-1}}$, the plasmid sequences also carried genes related to resistance to sulfonamides (sul1 and sul2), tetracycline (tet(A)), aminoglycoside (aph(3')-lb, aph(6)-Id, aph(3')-VI, aac(6')-Ib-cr), rifampicin (ARR-3), macrolides (mph(A) and erm(B)), phenicols (floR and catB3), fluoroquinolone (qnrB2), ampicillin ($bla_{\text{TEM-1}}$), and oxacillin ($bla_{\text{OXA-1}}$). The *E. coli* 89PenNDM also carried two other plasmids of the incompatibility groups IncFIC(II) and IncFIB, which were not present in the transconjugant.

4. Discussion

This is the first report of the carbapenemase *bla*$_{\text{NDM-1}}$ in an *E. coli* obtained from arterial blood of a penguin (*Spheniscus magellanicus*) in the Brazilian seacoast. As the collection of the arterial blood was performed in aseptic conditions, we considered that *E. coli* 89PenNDM was probably related to bloodstream infection of the animal. *In silico* analysis of the WGS data of the *E. coli* 89PenNDM indicated that the isolate belonged to the sequence type ST156, which was previously described in an NDM-5-producing *E. coli* strain (ECCRA-119) obtained from a poultry farm in Zhejiang, China, in 2017.[11]

WGS of the *E. coli* 89PenNDM and its transconjugant indicated that the *bla*$_{\text{NDM-1}}$ gene was located in an IncA/C2 plasmid, which is a plasmid that usually carries multiple genes related to antibiotic resistance.[12-15] The sequences from the plasmid described in this study share two large regions with the plasmid pH-1238, carried by *Salmonella enterica*, subp. Enterica, serovar Covallis, obtained from a wild bird (*Milvus migrans*) in Germany. The first region (~38 Kb with 99% identity) contains plasmid partitioning genes and a restriction-modification system; the second region (~38 Kb, with 97% identity) is an antibiotic resistance island carrying several resistance genes: florR, sul2, tetA, aph(3')-lb, aph(6)-Id and mph(A). In fact, the *bla*$_{\text{NDM-1}}$ was located together with other resistance genes *bla*$_{\text{OXA-1}}$, catB3, ARR-3, sul1, aph(3')-VI and aac(6')-Ib-cr. The plasmid also carried the resistance genes qnrB2 and *bla*$_{\text{TEM-1}}$, located in unique scaffolds. Detailed analyses indicated that the *bla*$_{\text{NDM-1}}$ gene was flanked downstream by a bleomycin resistance gene, and upstream by an IS-30-like element from the ISAba125 transposase family.

Several reports describing *bla*$_{\text{NDM-1}}$ in swallows, black kites, storks, or gulls demonstrate that birds may play a role in antibiotic resistance dissemination.[5] There are no reports characterizing *bla*$_{\text{NDM-1}}$ carrying plasmids obtained from birds in Brazil. For this reason, we cannot infer whether this penguin was contaminated in Brazil or somewhere else

and brought this plasmid to the country. *S. magellanicus* inhabits the coastal zones of the extremely south of South America (Argentina, Uruguay, Chile and Falkland Islands) and migrates to Brazil, in the Atlantic Ocean, or to Peru, in the case of Pacific Ocean, after the reproductive period to search for food.

The presence of a carbapenemase gene in wildlife animals is of concern as they may become reservoirs of multidrug-resistant bacteria and, as these animals can travel long distances, may disseminate MDR bacteria in extensive areas of the environment.

Nucleotide sequence accession number

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession WTFK00000000. The version described in this paper is version WTFK01000000.

Acknowledgements

The authors would like to thank Evelin Kern Almeida, Helena de Ávila Peixoto e Silva, Marina Crispin, Cristiane Kiyomi Miyaji Kolesnikovas and Bruna Silva da Silva for technical support.

Funding

This work was supported by INPRA - Instituto Nacional de Pesquisa em Resistência Antimicrobiana - Brazil (FIPE/HCPA: 2019-0203, INCT/CNPq: 465718/2014-0 and INCT/FAPERGS: 17/2551-0000514-7).

Ethical approval

Animals were collected under ABIO 640/2015, emitted as part of the environmental licensing process required by IBAMA for oil production and transportation by Petrobras in the pre-salt province.

Author Disclosure Statement

No competing financial interests exist.

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Appendix

Table 1. Minimal inhibitory concentration (mg/L) of several antibiotics for *E. coli* 89PenNDM, Transconjugant T89PenNDM and *E. coli* J53.

Antibiotics	MIC (mg/L)		
	E. coli 89PenNDM	Transconjugant T89PenNDM	*E. coli* J53
Ertapenem	32	16	≤0.03
Imipenem	8	8	0.5
Meropenem	32	8	0.06
Ceftazidime	>256	>256	0.5
Gentamicin	2	2	2
Tigecycline	2	0.5	0.5
Amicacina	8	4	4
Chloranphenicol	>512	256	8

3 CONCLUSÃO E PERSPECTIVAS

No presente trabalho, foi realizada uma pesquisa sobre o perfil de resistência aos carbapenêmicos de Enterobactérias isoladas da corrente sanguínea de *Spheniscus magellanicus* e *Larus dominicanus*.

Com os resultados encontrados, pode-se concluir que:

- É o primeiro caso reportado do gene *bla*NDM-1 circulando em plasmídeo isolado em aves silvestres no Brasil;
- Não é possível afirmar se o pinguim trouxe o gene de resistência da colônia reprodutiva, se adquiriu no percurso da migração ou durante o período de estadia no Brasil.

É importante continuar monitorando cepas bacterianas que circulam em animais silvestres, uma vez que podem se tornar reservatórios de bactérias multirresistentes e a migração pode transportar facilmente esses agentes.

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ANEXO A – NORMAS DE PUBLICAÇÃO DA REVISTA: *MICROBIAL DRUG RESISTANCE*

Submitting Your Manuscript

When submitting your manuscript for peer review, be prepared to:

- Enter the full title of the manuscript;
- Enter the full names and institutional affiliations of ALL listed authors;
- Enter ALL listed authors' institutional email addresses;
- Identify the corresponding author;
- Enter a running (abbreviated) title of no more than 45 characters (including spaces).
- Enter 3–6 keywords or phrases;
- Provide an unstructured abstract of no more than 200 words, stating the aims, results, and conclusions drawn from the study.
- Provide the names and email addresses of at least five potential preferred reviewers familiar with the field. Please make sure preferred reviewers are not from your university or institution or with whom you have collaborated. Anyone whom the author does not want to be considered should also be named as a non-preferred reviewer. Ultimate reviewer selection is at the Editor's discretion.
- Confirm that the material has not been published or submitted for publication elsewhere.

It is critical to ensure the accuracy of ALL authors' email addresses when uploading submissions to ScholarOne to ensure the proper delivery of all email communications.

Create an Effective Title

- Manuscript titles should be brief, contain key terms, and clearly identify the purpose of the work conducted;

- Manuscript titles should not exceed 15 -18 words. Exceptions can be made with the Editor's approval;
- Manuscript titles should be direct and to the point. Remember that the journal has a global readership, so clear and concise non-vernacular language is most effective;
- Avoid the use of specific locations in the title;
- Do not use proprietary/trademarked names in the title;
- Do not use acronyms in the title unless they are universally recognized and accepted;
- **NOTE**: The title page of your submission must be included as part of your main text document (not as a separate file).

Preparation of Manuscript

Prepare text of manuscripts, figure legends, and tables in Microsoft Word, double spaced. The order of elements in each manuscript should be:

- Title page (with full manuscript title, all contributing authors and their affiliations, a short running title, a denotation of the corresponding author, and a list of 3-6 keywords;
- Abstract;
- Main text (do not embed figures or tables);
- Conclusion (if applicable; as a separate paragraph, not as part of the Discussion section);
- Acknowledgments (if applicable);
- Authorship confirmation statement (see below)
- Author(s') disclosure statement(s) (see below);
- Funding statement (see below);
- References;
- Figure legends;
- Tables;
- Supplemental files (if applicable; if the submission is accepted, the Supplemental Information will not be copyedited or typeset; it will be published as supplied.).

Manuscript Text

Maximum word count for original articles should not exceed 3,000 words. In general, the text should be organized under the headings: Introduction, Materials and Methods, Results, and Discussion. The Results and Discussion should be separate sections. Number pages consecutively; the first author's last name should appear on each page. Authors should review the style and clarity of their manuscripts with colleagues before submission. Manuscripts may be edited if needed for grammar and usage.

Use only standard abbreviations, which can be found in the AMA's Manual of Style for Authors and Editors, 10th edition or the Council of Science Editors (CSE) Style Manual, 8th edition. At first usage, spell out terms and provide abbreviations in parentheses. Thereafter, use only the abbreviations. It is not necessary to spell out standard units of measure. Use generic names for drugs if possible. If you wish to use a proprietary drug name the first time it appears, use the generic name followed by the proprietary name, manufacturer, and location in parentheses.

References

References must be prepared in Word, double spaced, and numbered consecutively as they are cited in the text (using superscript numbers). Include the reference section as part of the main text file, not as a separate file. References appearing for the first time in tables and figures must be numbered in sequence with those cited in the text where the table or figure is mentioned. Use journal abbreviations as provided by PubMed/Medline. List all authors when there are six or fewer. When there are more than six authors, list the first three, followed by et al.

If references to personal communications or unpublished data are used, they are not to be in the list of references. They should be referred to in the text in parentheses: (AB Jones, personal communication). When data from an unpublished source are given, supply the researcher's name and location. Written permission must be obtained from the author of any unpublished material cited from other laboratories. All permissions listings must be shown in the manuscript – they cannot be entered on proofs. Include among the references any articles that have been accepted but have not yet published; identify the name of publication and add "In Press." If the reference has been published online, provide the DOI number in place of the page range.

Sample style for references:

Journal article:

Ojdana D, Gutowska A, Sacha P, Majewski P, Wieczorek P, Tryniszewska E. Activity of ceftazidime-avibactam alone and in combination with ertapenem, fosfomycin, and tigecycline against carbapenemase-producing Klebsiella pneumoniae. Microb Drug Resist 2019;25:1357-1364.

Book:

Hauser AR, Rello J, eds. Severe Infections Caused by Pseudomonas aeruginosa. Boston, MA: Kluwer Academic Publishers; 2003.

Chapter in a book:

Jarvis WR. Epidemiology and control of Pseudomonas aeruginosa infections in the intensive care unit. In: Hauser AR, Rello J, eds. Severe Infections Caused by Pseudomonas aeruginosa. Boston, MA: Kluwer Academic Publishers; 2003, pp. 153–168.

Abstract:

Scacheri P, Crabtree J, Kennedy A, et al. V804 RET mutation in MEN2A: first report. J Int Med 2006;255:712 (abstract).

Proceedings:

Lavilla S, González-López JJ, Larrosa MN, Bartolomé RM, Prat G. Prevalence of the quinolone-modifying enzyme aac(6´)-Ib-cr in extended-spectrum b-lactamase-producing enterobacterial isolates in Barcelona. Abstract presented at the 18th European Congress of Clinical Microbiology and Infectious Diseases, Barcelona, Spain, April 19–22, 2008. Abstract no. P1523.

Website:

Centers for Disease Control and Prevention. National Antimicrobial Resistance Monitoring System (NARMS) Now: Human Data. U.S. Department of Health and Human Services, CDC, Atlanta, GA. 2019. Available at https://cdc.gov/narmsnow

Figure Legends

Figure legends should be uploaded as a separate Word file and double spaced. In the legend, provide explanations for any abbreviations, arrows, etc. that appear in the figure. If the figure is taken from a copyrighted publication, permission must be secured, appropriate credit must be given in the legend, and a corresponding reference must appear in the reference section. All permissions listings must be shown in the manuscript – they cannot be entered on proofs.

Tables

All tables should be prepared in one single Word file. Provide a title for each table. Cite tables in sequence in the text. Each table must stand alone, i.e., contain all necessary information in the caption, and the table itself must be understood independently of the text. Information that appears in the text should not be repeated in tables, and tables should not contain data that can be given in the text in one or two sentences. Details of experimental conditions should be included in the table footnotes. Explain abbreviations used in the body of the table in footnotes. If the table is taken from a copyrighted publication, permission must be secured, appropriate credit must be given in the legend, and a corresponding reference must appear in the reference section. All permissions listings must be shown in the manuscript – they cannot be entered on proofs.

Figures

- Submission of high resolution .TIFF or .EPS figure files is strongly recommended.
- Figures should not be embedded within the manuscript file.
- Cite figures consecutively in text within parentheses.
- A legend should be supplied for each figure and all legends numbered consecutively.

- Images should not show the name of a patient or a manufacturer.
- Do not include any illustrations as part of your text file.
- The Journal will publish color photographs, but the cost must be borne by the author.

Figure 1: summary of the manuscript submitted to the journal Microbial Drug Resistence, obtained from the online submission system.

Escherichia coli carrying blaNDM-1 obtained from a migratory penguin (Spheniscus magellanicus) in the Brazilian sea coast

Journal:	*Microbial Drug Resistance*
Manuscript ID	MDR-2020-0424
Manuscript Type:	Veterinary Microbiology
Date Submitted by the Author:	19-Aug-2020
Complete List of Authors:	Wink, Priscila; Hospital de Clinicas de Porto Alegre, de Lima-Morales, Daiana ; HCPA Meurer, Rafael; Universidade Federal do Rio Grande do Sul Barth, Afonso ; Hospital de Clínicas de Porto Alegre, Laboratório de Pesquisa em Resistência Bacteriana
Keyword:	Antimicrobial, Carbapenemases, E. Coli, Metallo-beta-lactamases, Plasmid
Manuscript Keywords (Search Terms):	Antimicrobial resistance, carbapenem resistance, wildlife, penguin, NDM-1, ST156
Abstract:	The reservoirs for NDM-producing Enterobacterales organisms are increasing, not only in hospitals, but also in the environment and in the community such as in livestock, companion animals and wildlife, challenging the therapeutic efficacy of carbapenems. This report aimed to characterize an isolate of Escherichia coli harboring the blaNDM-1 gene recovered from the bloodstream of a penguin (Spheniscus magellanicus) during a routine beach monitoring project in Southern Brazil. A total of 74 bacterial isolates recovered from arterial blood samples from dead animals were submitted to species identification using the API system (bioMérieux®) and antibiotic susceptibility evaluation. One isolate presented resistance to carbapenems (E. coli 89PenNDM). This isolate was submitted PCR-HRM to detect carbapenemase genes and proved to present the blaNDM-1 gene. Experiments of conjugation indicated that the blaNDM-1 of E. coli 89PenNDM was transmissible to E.coli J53. Whole genome sequencing (WGS) confirmed the presence of the blaNDM-1 gene, which was presented in a conjugative plasmid (IncA/C2 plasmid), in both the E. coli 89PenNDM and its transconjugants. WGS was able to classify the isolate as ST 156 and to identify many other resistance genes (eg.: sul1, sul,2, strA, floR, tet(A)), all carried in the same IncA/C2 plasmid. This is the first report of blaNDM-1 producing E. coli isolated from a penguin (Spheniscus magellanicus) in the Brazilian sea coast. The presence of a carbapenemase gene in wildlife animals is of concern as they may become reservoirs of multidrug-resistant bacteria and disseminate them to the environment.