Colistin-Resistant Enterobacter kobei carrying mcr-9.1 and blaCTX-M-15 infecting a critically endangered Franciscana dolphin (Pontoporia blainvillei), Brazil

Danny Fuentes-Castillo[1,2], Fábio Sellera[2], Daphne Goldberg[3], Herrison Fontana[2], Fernanda Esposito[4], Brenda Cardoso[5], Joanna Ikeda[6], Anneliese Kyllar[7], José Luiz Catão-Dias[8], and Nilton Lincopan[1]

[1]University of São Paulo
[2]University of Sao Paulo
[3]Londrina State University
[4]School of Pharmaceutical Sciences, University of São Paulo
[5]Department of Microbiology, Institute of Biomedical Sciences, Universidade de São Paulo
[6]Affiliation not available
[7]UERJ
[8]Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo

August 23, 2020

Abstract

The emergence of mobile mcr genes mediating resistance to colistin is a critical public health issue that has hindered the treatment of serious infections caused by multidrug-resistant pathogens in humans and other animals. We report the emergence of the mcr-9.1 gene in a polymyxin-resistant extended-spectrum β-lactamase (ESBL)-producing Enterobacter kobei infecting a free-living Franciscana dolphin (Pontoporia blainvillei), threatened with extinction in South America. Genomic analysis confirmed a wide resistome with additional presence of genes conferring resistance to clinically relevant β-lactam [blaCTX-M-15, blaACT-9, blaOXA-1 and blaTEM-1B], aminoglycoside [aac(3)-IIa, aadA1, aph(3'')-Ib and aph(6)-Id], trimethoprim [dfrA14], tetracycline [tetA], quinolone [aac(6')-Ib-cr and qnrB1], fosfomycin [fosA], sulphonamide [sul2], and phenicol [catA1 and catB3] antibiotics. The identification of mcr-9.1 in a CTX-M-15-producing pathogen infecting a critically endangered animal is worryingly, due to the restricted therapeutic options, and should be interpreted as a sign of further spread of critical-priority pathogens and their resistance genes in threatened ecosystems.

INTRODUCTION

The global emergence and rapid dissemination of mobile phosphoethanolamine transferase *mcr* genes, responsible for transferable colistin resistance in Enterobacterales, is a public health concern (El-Sayed Ahmed et al., 2020; Wang et al., 2020). In this regard, since the first report of the *mcr-1* gene, in 2016, novel alleles including *mcr-2* , *mcr-3* , *mcr-4* ,*mcr-5* , *mcr-6* , *mcr-7* , *mcr-8* , *mcr-9* and*mcr-10* have been globally identified (El-Sayed Ahmed et al., 2020; Liu et al., 2016; Wang et al., 2020). Worryingly, the occurrence of *mcr* genes has been documented in critical-priority extended-spectrum β-lactamase (ESBL)-producing pathogens, most isolated from humans and food-producing animals (El-Sayed Ahmed et al., 2020; Liu et al., 2016; Wang et al., 2020). In this study, we report the emergence of *mcr-9.1* in an ESBL-producing *Enterobacter kobei*infecting a free-living Franciscana dolphin (*Pontoporia blainvillei*), threatened with extinction, in Brazil. Additionally, an epidemiological landscape on global distribution of MCR-9-producing

Enterobacterales circulating at human-animal interface is presented.

MATERIALS AND METHODS

In December 2019, a female neonate Franciscana dolphin was found stranded alive in Mambucaba beach, in Angra dos Reis (-23.027184, -44.518130), located in the Southern coast of Rio de Janeiro state, Brazil (Figure S1). The animal was rescued by the staff of the Santos Basin Beach Monitoring Project (PMP-BS), presenting with excoriations on the head and with part of the umbilical cord. The dolphin was closely monitored, receiving intensive care and bottle-feeding with a special dolphin formula every 3 hours. However, after 11 hours in captivity, the animal began to exhibit clinical signs of shock with subsequent death. In order to determine the main causes of death, necropsy was performed, where histopathological analysis of fixed lung tissue revealed severe pneumonia. Additionally, bacteriological culture of respiratory exudate collected through the spiracle was positive for Gram-negative bacilli.

Antimicrobial susceptibility testing was performed by the disc diffusion method (CLSI, 2020), including amoxicillin/clavulanic acid, aztreonam, cefotaxime, ceftriaxone, cefepime, cefoxitin, ceftiofur, ciprofloxacin, enrofloxacin, chloramphenicol, fosfomycin, amikacin, gentamicin, ertapenem, imipenem, meropenem, sulfamethoxazole/trimethoprim and tetracycline. In addition, colistin susceptibility testing was performed by broth microdilution method (http://www.eucast.org). ESBL production was screened by the double-disc synergy test (DDST; CLSI, 2020).

Genomic DNA was extracted and used to construct a paired-end library, which was sequenced using the NextSeq 550 platform (Illumina, San Diego, CA, USA), using paired-end reads (150 bp). *De novo* genome assembly and contig annotation was carried out using CLC Genomics Workbench 12.0.3. Prediction of bacterial species, resistome and plasmidome was performed using fast K-mer algorithm KmerFinder 3.2 (Larsen et al, 2014), ResFinder 3.2 (Zankari et al. , 2012), and PlasmidFinder 2.1 (Carattoli et al. , 2014) databases, respectively (http://www.genomicepidemiology.org/).

RESULTS AND DISCUSSION

The Gram-negative bacilli were identified as belonging to the*Enterobacter cloacae* complex (E11R strain) by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF). The E11R strain displayed a multidrug-resistant (MDR) profile (Magiorakos et al., 2012) to amoxicillin/clavulanic acid, aztreonam, cefotaxime, ceftriaxone, cefepime, cefoxitin, ceftiofur, ciprofloxacin, enrofloxacin, chloramphenicol, fosfomycin, gentamicin, sulfamethoxazole/trimethoprim and tetracycline; remaining susceptible to ertapenem, imipenem, meropenem and amikacin. Furthermore, E11R strain exhibited resistance to colistin (MIC, 16 μg/mL), whereas ESBL production was detected by the DDST.

Genomic analysis identified the E11R strain as *E. kobei* , confirming a wide resistome, with genes conferring resistance to colistin [*mcr-9.1*], β-lactams [*bla* $_{\text{CTX-M-15}}$, *bla* $_{\text{ACT-9}}$,*bla* $_{\text{OXA-1}}$ and *bla* $_{\text{TEM-1B}}$], aminoglycosides [*aac(3)-IIa* , *aadA1* , *aph(3")-Ib*and *aph(6)-Id*], trimethoprim [*dfrA14*], tetracycline [*tetA*], quinolones [*aac(6')-Ib-cr* and*qnrB1*], fosfomycin [*fosA*], sulphonamide [*sul2*], and phenicols [*catA1* and *catB3*]. IncHI2 and IncHI2A replicons were detected, and analysis of the genetic environment confirmed that *mcr-9.1* was flanked by IS*903B*and IS*26* upstream and downstream, respectively (Figure 1) (Kieffer et al., 2019; Lin et al., 2020; Tyson et al., 2020; Yuan et al., 2019).

In the last years, colistin has been used as a last-resort for the treatment of infections caused by multidrug-resistant and/or carbapenem-resistant Gram-negative bacteria (El-Sayed Ahmed et al., 2020). However, the previous and extensive use of colistin in production animals, as a growth promoter or for prophylaxis, has been recognized as a responsible factor for the emergence and rapid dissemination of mobile colistin resistance (*mcr*) genes (Rhouma, Beaudry, & Letellier, 2016). In this respect, since the description of *mcr-1* , nine additional *mcr* homologues have been described, with several gene variants occurring worldwide (El-Sayed Ahmed et al., 2020; Wang et al., 2020).

The *mcr-9.1* allele was identified for the first time in*Salmonella* Typhimurium isolated from a human patient (Carroll et al., 2019), and currently has been reported worldwide with a rapid dissemination among Enter-

obacterales from human, food, poultry, swine and horse samples (Figure 2) (Börjesson et al., 2020; Carroll et al., 2019; El-Sayed Ahmed et al., 2020; Faccone et al., 2020; Khalifa et al., 2020; Li et al., 2020; Ling et al., 2020; Osei Sekyere, Maningi, Modipane, & Mbelle, 2020; Saidenberg et al., 2020; Wang et al., 2020). Recently, two novel variants, *mcr-9.2* and *mcr-9.3* , have been identified in *Enterobacter hormaechei subsp. xiangfangensis*(GenBank accession number: MN164032.1) and*Klebsiella pneumoniae* (GenBank accession number: MT505326.1) isolates, respectively. The environmental dissemination of critical priority pathogens has been considered a serious threat to ecosystem maintenance (de Carvalho et al., 2020; Sevilla et al., 2020). The exposure to polluted environments could also substantially increase the risk for marine populations acquire such bacteria (Power et al., 2016). Specifically in Brazilian coast, the occurrence of MCR-type, ESBL and/or carbapenemases has been documented in recreational waters (Campana, Montezzi, Paschoal, & Picão, 2017; Fernandes et al., 2017; Paschoal et al., 2017; Sellera et al., 2017a), beach sand samples (Furlan, dos Santos, Ramos, Gallo, & Stehling, 2020), and mangrove waters (Sacramento et al., 2018). More critically, their occurrence colonizing or infecting marine host have begun to be documented (Goldberg et al., 2019; Sellera et al., 2017b; Sellera et al., 2018).

In this study, we report the emergence of *mcr-9.1* in an ESBL-producing *E. kobei* isolated from an infected free-living dolphin. In this regard, the Franciscana dolphin is considered the most threatened small cetacean in the southwestern Atlantic Ocean, which includes the coasts of Brazil, Uruguay, and Argentina (Sucunza, Danilewicz, Cremer, Andriolo, & Zerbini, 2018). Due to their coastal habits, these animals have been frequently exposed to different degrees of anthropogenic impacts, including fisheries by catch and habitat degradation (Sucunza, Danilewicz, Cremer, Andriolo, & Zerbini, 2018). Consequently, this species is currently listed as vulnerable to extinction at both global and regional levels (Zerbini, Secchi, Crespo, Danilewicz, & Reeves, 2017). Therefore, the environmental dissemination of antibiotic-resistant critical-priority pathogens may have serious implications for endangered wild animals. In this regard, while occurrence of CTX-M-15-producing *E. coli* has been reported in captive dolphins (Manageiro et al., 2015), we demonstrated that this type of pathogen can also threaten free-living dolphins. Particularly,*E. kobei* has never been isolated from animal infections. In fact,*E. kobei* is member of the *E. cloacae* complex that includes other five species (*i.e.,* *E. asburiae* , *E. cloacae* ,*E. dissolvens* , *E. hormaechei* and *E. nimipressuralis*) recognized as important nosocomial pathogens (Mezzatesta, Gona, & Stefani, 2012).

In summary, we report the emergence of MCR-9-producing bacteria in marine wildlife. Considering that oceanic environments and human and animal health are strictly connected, the dissemination of clinically important MDR pathogens in marine ecosystems must be viewed as serious One Health problem. Finally, since multidrug-resistant pathogens have begun to be associated with fatal cases of infections in endangered animals (Fuentes-Castillo et al, 2020), continued surveillance of MCR- and ESBL-producing bacteria in marine ecosystems should be globally performed for a better comprehension of the transmission pathways and clinical impacts on marine wildlife.

DATA AVAILABILIT Y STATEMENT

The whole genome nucleotide sequence of the *E. kobei* E11R isolate is available in the GenBank database under accession number PRJNA615090.

ACKNOWLEDGEMENTS

This work was supported by Bill & Melinda Gates Foundation, Grand Challenges Explorations Brazil – New approaches to characterize the global burden of antimicrobial resistance [grant OPP1193112]; Fundação de Amparo à Pesquisa do Estado de São Paulo [FAPESP 19/15578-4]; Conselho Nacional de Desenvolvimento Científico e Tecnológico [CNPq 462042/2014-6, 312249/2017-9 and 433128/2018-6]; Comisión Nacional de Investigación Científica y Tecnológica [CONICYT BCH 72170436]; and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior [CAPES 88882.333054/2019-01, 88887.506496/2020-00]. N. L. and J.L C-D are research fellows of CNPq (312249/2017-9 and 304999/2018-0). We thank Cefar Diagnóstica Ltda. (São Paulo, Brazil) and CEFAP-GENIAL facility for kindly supplying antibiotic discs for susceptibility testing and Illumina sequencing, respectively. The Santos Basin Beach Monitoring Project (Projeto de Monitoramento

de Praias da Bacia de Santos - PMP-BS) is one of the monitoring programs required by Brazil's federal environmental agency, IBAMA, for the environmental licensing process of oil production and transport by Petrobras at the pre-salt pole (25°05'S 42°35'W to 25°55'S 43°34'W), between 2100 m and 2300 m isobaths.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required for this specific study.

CONFLICT OF INTERESTS

No potential conflict of interest was reported by the authors.

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FIGURE LEGENDS

FIGURE 1. Genetic context of the *mcr-9.1* gene in the colistin-resistant *Enterobacter kobei* strain E11R. IS*903B*and IS*26* elements were found upstream and downstream of*mcr-9.1* in a similar way that in MCR-9-producing*Salmonella* Saintpaul [CVM N16S133 (CP049986.1), NY-N14748 (CP048926.1), CVM N40391 (CP049983.1) and CVM N52030 (CP049981.1)],*S.* Heidelberg [CVM N16S321 (CP049313.1), CVM N58631 (CP049307.1) and CVMN53023 (CP049310.1)], and *S.* Albany [CVM N18S2238 (CP049312)] strains isolated from ground turkey; *S.*Johannesburg [CVM N58011 (CP049309)] strain isolated from chicken breast; and *E. coli* [CVM N18EC0432 (CP048293.1)] strain isolated from chicken wings, in the United States of America (Tyson et al., 2020).

FIGURE 2. Global distribution of MCR-9-positive Enterobacterales. The occurrence of MCR-9- producing Enterobacterales (*i.e., Citrobacter freundii* , *Enterobacter cloacae* ,*Enterobacter hormaechei* , *Enterobacter kobei* ,*Escherichia coli* , *Klebsiella oxytoca* , *Klebsiella pneumoniae* , *Klebsiella quasipneumoniae* , *Klebsiella variicola* , *Providencia alcalifaciens* , and *Salmonella enterica*) has been reported in Argentina, Belgium, Brazil, China, Denmark, Egypt, France, Germany, Japan, Montenegro, Poland, Qatar, Romania, Serbia, Slovenia, South Africa, Spain, Sweden, and the United States of America, from human and non-human sources Data search was updated in the PubMed database on July 20, 2020.

FIGURE 1.

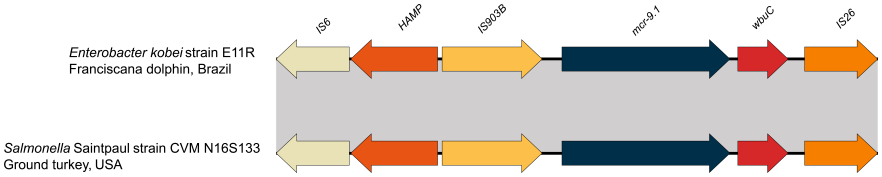

FIGURE 2.