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Extended-spectrum-β-lactamase (CTX-M)-producing *Escherichia coli* in wild fishes from a polluted area in the Atlantic Coast of South America

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ABSTRACT

The presence of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* in oceanic ecosystems constitutes an emerging public health risks in the marine environment. In this study, we report for the first time the identification of ESBL (CTX-M)-producing *E. coli* in wild fishes from a polluted area in the South Atlantic coast of Brazil, where a genomic analysis confirm the presence of livestock and human *E. coli* lineages belonging to sequence types (STs) ST744 and ST746, which carried clinically relevant resistance genes for human and veterinary antibiotics, and heavy metals. These findings reveal the presence of multidrug-resistant (MDR) bacteria in the gut microbiota of wild fishes living in polluted coastal waters, alerting that microbial contamination by bacteria related directly and indirectly to human or animal activities could affect the safety of the seafood supply, as well as the commercial and recreational use of coastal marine waters.

The increasing rates of land-based anthropogenic pollution in marine ecosystem have become an important factor to promote the spread of multidrug resistant (MDR) bacteria to aquatic animals (Foti et al., 2009; Ahasan et al., 2017; Fernandes et al., 2017). In this way, the rapid dissemination of extended-spectrum β-lactamases (ESBL)-producing Enterobacteriaceae in marine coastal areas is worrisome, because enterobacterial species (most *Escherichia coli*) are also a constituent of the gut microbiota of fishes (Brahmi et al., 2018; Gerzova et al., 2014). Thus, the consumption of infected or colonized fishes could has public health implications (Heuer et al., 2009), constituting a further risk to other marine predators (L'Abée-Lund and Sørum, 2001). In this study, we report for the first time the occurrence of broad-spectrum cephalosporin-resistant *E. coli* isolates carrying *bla*_{CTX-M}-type genes in coastal fishes from an urban polluted area of the South Atlantic Ocean of Brazil, highlighting a potential source of dissemination of these organisms for marine ecosystems, with additional consequences for seafood safety and quality.

Between November and December 2016, during a local surveillance study conducted to monitor the occurrence of clinically significant MDR bacteria in marine environments, we have followed the route of artisanal licensed fishers who used seine net to caught marine fishes in

beaches of the southeast coast of Brazil, for own consumption. A total of 42 different living wild fish samples were collected throughout cloacal swabs, including Florida pompano (*Trachinotus carolinus*, *n* = 13), common snook (*Centropomus undecimalis*, *n* = 6), white mullet (*Mugil curema*, *n* = 6), smooth weakfish (*Cynoscion leiarchus*, *n* = 6), Atlantic croaker (*Micropogonias undulatus*, *n* = 3), marine catfish (*Genidens genidens*, *n* = 2), Brazilian mojarra (*Eugerres brasiliensis*, *n* = 2), Southern kingcroaker (*Menticirrhus americanus*, *n* = 2), Atlantic bonito (*Sarda sarda*; *n* = 1), and Atlantic spadefish (*Chaetodipterus faber*, *n* = 1).

Samples were streaked onto MacConkey agar plates supplemented with ceftriaxone (2 μg/mL), colistin (2 μg/mL), or meropenem (2 μg/mL). In this regard, two ceftriaxone-resistant *E. coli* strains (ICBEPX6 and ICBEPX8) were isolated from Florida pompano fishes and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF). The minimum inhibitory concentration (MIC) was determined by E-test strips (CLSI, 2017), where both isolates displayed resistance to ceftiofur (MIC > 32 μg/mL), cefotaxime (MIC > 32 μg/mL) and ceftriaxone (MIC > 32 μg/mL), whereas ICBEPX6 displayed additional resistance to ciprofloxacin (MIC > 32 μg/mL), nalidixic acid (MIC > 64 μg/mL), gentamicin (MIC > 128 μg/mL),

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Table 1
Characteristics of CTX-M-producing *Escherichia coli* strains isolated from Florida Pompano (*Trachinotus carolinus*), in the Atlantic Coast of Southeastern Brazil.

Description	Broad-spectrum cephalosporin-resistant <i>E. coli</i> strain	
	ICBECPX6	ICBECPX8
Fish specie	<i>Trachinotus carolinus</i>	<i>Trachinotus carolinus</i>
Coastal location (latitude, longitude)	– 23.984994, – 46.310007	– 23.978029, – 46.317558
Genome size (bp)	4,865,091	4,444,418
G + C content (%)	50.4	50.8
Serotype	O89:10	O112:H18
<i>fimH</i> -type	H54	H54
Phylogroup	A	A
ST/CC ^a	744/–	746/–
wgMLST ^b	66,895	65,771
cgMLST ^c	61,656	60,737
Resistome		
Aminoglycosides	<i>aadA5</i> , <i>strA</i> , <i>aph(4)-Ia</i> , <i>strB</i> , <i>aac(3)-IVa</i>	–
β-Lactams	<i>bla_{CTX-M-2}</i> , <i>bla_{TEM-1B}</i>	<i>bla_{TEM-1B}</i> , <i>bla_{CTX-M-55}</i>
Macrolides	<i>mphA</i> , <i>mphB</i>	–
Quinolones	<i>GyrA</i> (Ser83Leu, Asp87Asn), <i>ParC</i> (Ser88Ile, Ala-56-Thr)	–
Phenicol	<i>catA1</i> , <i>floR</i>	–
Sulphonamides	<i>sul1</i> , <i>sul2</i>	–
Tetracyclines	<i>tetA</i> , <i>tetB</i>	–
Trimethoprim	<i>dfrA7</i> , <i>dfrA17</i>	<i>dfrA1</i>
Heavy metals genes	<i>terC</i>	<i>silR</i>
Plasmid (Inc-type)	IncHI2A, IncX1, IncQ1	IncFII, p0111
GenBank accession number	PDMS0000000.1	PDMT0000000.1

^a ST, sequence type; CC, clonal complex.

^b wgMLST, whole genome multilocus sequence typing.

^c cgMLST, core genome multilocus sequence typing.

and sulfamethoxazole/trimethoprim. CTX-M genes were screening by PCR, whereas direct Sanger sequencing confirmed *bla_{CTX-M-2}* and *bla_{CTX-M-55}* ESBL genes in ICBECPX6 and ICBECPX8, respectively.

Genomic DNA of *E. coli* ICBECPX6 and ICBECPX8 strains were extracted and used to construct a paired-end library, which was sequenced using the NextSeq platform (Illumina). Genome assemblies were performed using CLC Genomics Workbench version 7.0 (Aarhus, Denmark), and annotated using Prokka (www.github.com/tseemann/prokka). Main results from whole-genome sequencing analysis are summarized in Table 1. Antimicrobial resistance genes, MLST, plasmid replicons, serotype and virulence genes for *E. coli* ICBECPX6 and ICBECPX8 strains were identified using bioinformatics tools available from the Center for Genomic Epidemiology (<http://genomicsepidemiology.org/>).

Analysis from whole-genome sequencing data revealed that *E. coli* ICBECPX6 and ICBECPX8 strains belonged to serotypes O89:10-*fimH*54 and O112:H18-*fimH*54, respectively. These serotypes have been infrequently reported, however *fimH* is a type 1 fimbriae gene commonly found in biofilm-producing *E. coli* strains (Roer et al., 2017).

MLST analysis revealed that *E. coli* ICBECPX6 belonged to ST744, whereas ICBECPX8 belonged to ST746. In this regard, these bacterial lineages have been widely reported in livestock and human samples from Africa, Asia, and Europe, being frequently associated with the production of clinically significant beta-lactamases (i.e., carbapenemases and ESBL), and more recently with the plasmid-mediated colistin resistance gene, *mcr-1* (Belmahdi et al., 2016; Eisenberger et al., 2018; Gedebjerg et al., 2015; Ho et al., 2016; Tacão et al., 2017; Vignaroli et al., 2016).

The resistome analysis of ICBECPX6 revealed the presence of several antimicrobial resistance genes, conferring resistance to aminoglycosides, β-lactams, macrolides, phenicol, sulphonamides, tetracycline and trimethoprim. On the other hand, ICBECPX8 harboured resistance

genes to β-lactams and trimethoprim alone. *In silico* analysis identified mutations in the quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC* genes of *E. coli* ICBECPX6, resulting in fluoroquinolone resistance (Fu et al., 2013). Curiously, heavy metal genes, conferring resistance to tellurite (*terC*) and silver (*silR*), were also found suggesting further heavy metal contamination in the related marine environment (Łuczynska et al., 2018) (Table 1). Otherwise, both *E. coli* isolates did not present virulence genes, being classified within the non-virulent phylogenetic group A.

While *E. coli* ICBECPX6 harboured IncHI2, IncQ1 and IncX1 plasmids, *E. coli* ICBECPX8 carried IncFII and p0111 plasmids. *In silico* analyzes, using Genious 10.0.9 and blastn softwares, confirmed the presence of the *bla_{CTX-M-2}* gene on the IncHI2/ST2 plasmid carried by *E. coli* ICBECPX6, whereas in ICBECPX8 the *bla_{CTX-M-55}* gene was harboured by the IncFII plasmid (FII33:A-B -). Moreover, the transfer of the IncHI2/ST2 plasmid was successfully achieved by conjugation assay using *E. coli* C600^{STR} lineage. On the other hand, the IncFII plasmid could not be transferred.

Although, interactions between the oceans and human health are closely related, anthropogenic activities have contributed with microbial and chemical contamination, indirectly and directly affected the health of the oceans. Consequently, fishes and other marine organisms have been responsible for seafood-borne illness, where a wide variety of pathogenic organisms and toxins to humans can accumulate in the alimentary tract of shellfish and fishes, especially if the filtered water is contaminated by sewage and/or chemical pollutants (Fleming et al., 2006). Additionally, since the intensive use of antibiotics in the aquaculture industry has demonstrated that antibiotic accumulation in seafood tissues increases the potential for select MDR pathogens (Cabello, 2006), the presence of antibiotic residues from different sources (household, pharmaceutical industry, and hospital), into marine environments, could contribute with this selection pressure (Smaldone et al., 2014).

Even though the dissemination of ESBL-positive bacteria in coastal waters has been increasingly reported (Maravić et al., 2015; Fernandes et al., 2017; Jørgensen et al., 2017), the description of ESBL-producing Enterobacteriaceae isolated from gut microbiota of wild fishes has been a rare event. Indeed, the presence of ESBL producers has been only reported in coastal fishes from Portugal and Algeria, so far, being associated to SHV-12-, TEM-52- and CTX-M (-8, -9, -15, -55)-producing *E. coli* strains (Sousa et al., 2011; Brahmi et al., 2018); reinforcing that anthropogenic activities and antibiotic pressure in marine ecosystem could contribute with the selection of ESBL-positive pathogens in these environment.

In Brazil, CTX-M producing *E. coli* has been broadly disseminated among humans, food-producing animals and anthropogenically impacted aquatic environments, including rivers, lakes and coastal waters (Conte et al., 2017; Cunha et al., 2017; Fernandes et al., 2017; Fernandes et al., 2018; Nascimento et al., 2017; Rocha et al., 2016). Specifically, in this study, the CTX-M-producing *E. coli* strains were recovered from wild fishes living in coastal waters of Santos, which is the major beachfront city of the region, with the largest shipping terminal in Latin America. In this regard, this coastal area has been anthropogenically affected (Lamparelli et al., 2015), where the presence of heavy metals and microplastics have been identified as threats to the marine environment (Vedolin et al., 2018). In fact, metal concentrations in this port zone have been higher than those observed in the continental shelf of this region (Buruam et al., 2012).

Interestingly, these two *bla_{CTX-M}*-positive *E. coli* isolates were recovered from Florida pompano (*T. carolinus*). *T. carolinus* is a fast-swimming predator, widely distributed along the coastal waters of Atlantic Ocean (from Massachusetts to Brazil, and in the Gulf of Mexico) (Schrandt and Powers, 2015), and greatly appreciated for human consumption (Castro-González et al., 2013). It is difficult to determine the main reason why MDR bacteria did not colonize the other investigated species, indeed, there is scarce information regarding

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