

Multiple antimicrobial resistance in *Enterobacteriaceae* isolated from a Sea Lion (*Otaria flavescens*) specimen from Isla de Lobos, Uruguay: a case report

Múltipla resistência antimicrobiana em *Enterobacteriaceae* isolada de um espécime de Leão Marinho (*Otaria flavescens*) da Isla de Lobos, Uruguai: um relato de caso

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ABSTRACT

The rise of antimicrobial resistance is a growing concern worldwide, however the role that wildlife plays as a reservoir for antimicrobial resistant bacteria and the effect antimicrobial resistance has on them remain relatively unknown. We isolated and identified pathogenic bacteria from samples obtained from a juvenile sea lion (*Otaria flavescens*) necropsy from Isla de Lobos, Uruguay and tested the Enterobacterial strains for the presence of antimicrobial resistance. Swabs were taken from the larynx, esophagus, trachea, duodenum, and bladder. From a total of five isolated Gram-negative strains, three bacterial species were identified: *Escherichia coli*, *Escherichia ferrusoni* and *Proteus mirabilis*. All isolates expressed a resistance phenotype to Amoxicillin/clavulamic, Sulfamethoxazole, Tetracycline, Doxycycline and Streptomycin, while three of the five isolates showed resistance against Ciprofloxacin and two against Enrofloxacin and Cefovecin. These results provide relevant information on the presence of antimicrobial resistant bacteria in marine mammals in

Uruguay, demonstrating the need to deepen scientific knowledge of the dynamics of antimicrobial resistance.

Keywords: multiple antibiotic resistance, wildlife antimicrobial resistance, one health, *Escherichia fergusonii*

RESUMO

O aumento da resistência antimicrobiana é uma preocupação crescente em todo o mundo, no entanto, o papel que a vida selvagem desempenha como reservatório de bactérias resistentes aos antimicrobianos e o efeito da resistência antimicrobiana sobre elas permanecem relativamente desconhecidos. Isolamos e identificamos bactérias patogênicas de amostras obtidas de uma necropsia de um leão marinho juvenil (*Otaria flavescens*) de Isla de Lobos, Uruguai e testamos as cepas de enterobactérias quanto à presença de resistência antimicrobiana. Swabs foram retirados da laringe, esôfago, traqueia, duodeno e bexiga. De um total de cinco cepas Gram-negativas isoladas, três espécies bacterianas foram identificadas: *Escherichia coli*, *Escherichia fergusonii* e *Proteus mirabilis*. Todos os isolados expressaram um fenótipo de resistência à Amoxicilina/clavulâmico, Sulfa-trimetoprim, Tetraciclina, Doxiciclina e Estreptomicina, enquanto três dos cinco isolados apresentaram resistência contra Ciprofloxacina e dois contra Enrofloxacin e Cefovecina. Esses resultados fornecem informações relevantes sobre a presença de bactérias resistentes aos antimicrobianos em mamíferos marinhos no Uruguai, demonstrando a necessidade de aprofundar o conhecimento científico da dinâmica da resistência antimicrobiana.

Palavras-chave: resistência a múltiplos antibióticos, resistência antimicrobiana em animais selvagens, uma saúde, *Escherichia fergusonii*

1 INTRODUCTION

The growing presence of antimicrobial resistance (AMR) is a pressing issue that if left unaddressed may compromise human and animal health, possibly leading to a return to the pre-antibiotic era (Camou et al., 2017). The United Nations' Food and Agriculture Organization (FAO), the World Organization for Animal Health (OIE) and the World Health Organization (WHO) jointly called for the prevention and control of sanitary risks in the human-animal-environment interface, a concept called "One Health", which established the fight against AMR as a top priority. Since then, the number of multi-resistant bacterial strains identified has increased continuously (Ahasana et al., 2017). While the presence of AMR bacteria in wildlife has been known for some time, our understanding of the scope and scale of the issue are inadequate due to a lack of monitoring and documentation (Van den Honert et al., 2018).

Enterobacteriaceae are common commensal intestinal flora which can be reservoirs of antibiotic resistance not only in humans (Bonelli et al., 2014), but also reptiles (Ahasana et al., 2017) birds, fish (Lee et al., 2018; Wu et al., 2018), and marine mammals (Wallace et al., 2013). Several genera of *Enterobacteriaceae* have pathogenic potential and are implicated in serious infections for humans and animals, such as acute gastroenteritis, urinary tract infections and respiratory tract infections (Kresken et al., 2016; Najjuka et al., 2016; Wright, 2010). For this reason,

Enterobacteriaceae are a public health concern (Fariñas and Martínez-Martínez, 2013), including the emerging pathogen *Escherichia fergusonii*. Furthermore, the resistome of Enterobacteriaceae and other Gram-negative bacteria possess incipient resistance to practically all available drugs (CDC 2013; Golkar et al., 2014).

Aquatic environments can be contaminated from a wide variety of sources, including but not limited to urban surface seepage and coastal effluent discharges (Goñi-Urriza et al., 2000). Proximity to these sources of contamination increase the likelihood that marine life may be serving as vectors and reservoirs AMR bacteria. In contrast, marine mammals not exposed to anthropic pressure have much lower, and often a near-zero incidence rate of AMR bacteria (Wheeler et al., 2012), indicating that anthropogenic contamination is likely the primary source of AMR in marine microorganisms (Allen et al., 2010; Martinez, 2008). Thus, characterizing the impact of AMR on wild populations and the role of wildlife as reservoirs of multi-resistant bacterial flora, as well as bioindicators of the environmental health are paramount (Bonnedahl and Järhult, 2014; Dolejska et al., 2007; Koenig et al., 2011; Sayah et al., 2005; Sousa et al., 2014). Present information is insufficient to characterize AMR bacteria in wildlife on a global scale. Thus, this case study serves as an illustration of AMR in the region and the emergence of a pathogen novel to marine mammals.

2 MATERIALS AND METHODS

Isla de Lobos sits 8 km from the coast of Punta del Este, Uruguay (35°01'28"S 54°52'59"W). The island hosts various marine animal species, including reptiles, birds, and mammals—the latter represented by South American sea lions (*Otaria flavescens*) and South American fur seals (*Arctocephalus australis*). The island is the largest focal concentration of Pinnipeds in South America with an estimated 400,000 individuals (Ponce de León and Pin, 2006), and its proximity to the relatively densely populated coast makes it susceptible to anthropic effects.

Samples were taken from the carcass of a six-month-old female sea lion (*Otaria flavescens*) 18 hours postmortem. During the necropsy, samples of the bladder, esophagus, larynx, trachea, and two from the jejunum were taken using sterile agar gel transport swabs. Samples were taken to the lab and refrigerated at 4° C until their processing (within 24 hours of sampling). The swabs were seeded in Tryptic Soy Agar (TSA) and incubated at 37°C for 18-24 h, followed by gram staining. Some isolates had small to medium-sized colonies, some white and some butter-colored, all with a rounded shape and smooth edges. Gram-negative small pleomorphic bacilli, characteristic of Enterobacteriaceae, could be observed in the Gram stain of all isolates. Very small Gram-positive cocci were isolated from the trachea, though these isolates were not included in the study. In the isolates of the jejunum sample, unlike the others, the growth of two types of colonies was observed.

One medium sized colony, similar to those isolated in the other plates and another with swarming production and a deep smell, characteristics associated with the genus *Proteus sp.*

The cultured bacteria were identified by PCR amplification of 16s rRNA followed by sequencing. Genomic DNA extraction was performed using a boiling PBS + 0.05% Tween-20 protocol (Ghatak et al., 2013). The purity of the extracts was evaluated by spectrophotometry using a NanoDrop (Thermo Scientific). Then a 844 bp fragment was amplified using primers 63F (5' 'CAGGCCTAACACATGCAAGTC3') and 907R (5' 'CCGTCAATTCCTTTRAGTTT3') (Martínez-Rosales et al., 2011) in 25 µL reactions: 12.5 µL of MangoMix (Bioline®), 1 µL of each primer at a concentration of 10 µM, 9.5 µL of sterile mQ H₂O and 1 µL of DNA (approx. 150 ng/µL). The cycling conditions were: initial denaturation at 94° C for 2 minutes followed by 30 cycles of 40 seconds at 94° C, 50 seconds at 58° C and 1 minute at 72° C ending with a final extension of 1 minute at 72° C. Presence of the amplified fragment was confirmed by gel electrophoresis. PCR products were purified and sequenced by MacroGen Inc. (Seoul, South Korea). Sequences were compared with the same gene and bacterial genus found in the Genbank database (www.ncbi.nlm.nih.gov). Strains were identified using EZBioCloud (www.ezbiocloud.net).

Antimicrobial susceptibility was analyzed by antibiogram following the Kirby-Bauer Disc diffusion method under the recommendations of the Clinical & Laboratory Standards Institute (CLSI). At least one representative from each of the main antimicrobial families (Beta-lactams, Aminoglycosides, Macrolides, Fluoroquinolones, Tetracyclines, Sulfonamides and Diaminopyrimidines) was selected for the test whose spectrum of action includes the isolated strains.

3 RESULTS

DNA sequences from the bladder and esophagus isolates corresponded to *Shigella flexneri* and *Escherichia fergusonii* strains with 99% identity, respectively. The larynx isolate and jejunum isolate 1 were confirmed as *E. fergusonii* with 100% identity in all databases, and finally jejunum isolate 2 was identified as *Proteus mirabilis* with 100% identity.

All isolated strains expressed resistance phenotype to Amoxicillin / clavulamic, sulfamethoxazol / trimethoprim, Tetracycline, Doxycycline and Streptomycin. Additionally, the bladder, esophagus, and larynx strains showed resistance against Ciprofloxacin and finally the esophagus and larynx isolates showed resistance against Enrofloxacin and Cefovecin. None of the isolated strains were resistant to Gentamicin (Table 1).

Table 1. Results of the antibiograms performed on the bacterial isolates from *Otaria flavescens*. R: Resistant; S: Sensitive.

Sample origin	Bladder	Esophagus	Larynx	Jejunum (isolate 1)	Jejunum (isolate 2)
Bacteria Species	<i>Shigella flexneri</i>	<i>Shigella flexneri</i>	<i>Escherichia fergusonii</i>	<i>Escherichia fergusonii</i>	<i>Proteus mirabilis</i>
Amoxicillin and Clavulamic acid	R	R	R	R	R
Cefovecin	S	R	R	S	S
Ciprofloxacin	R	R	R	S	S
Enrofloxacin	S	R	R	S	S
Tetracycline	R	R	R	R	R
Doxycycline	R	R	R	R	R
Azithromycin	S	S	S	S	R
Streptomycin	R	R	R	R	R
Gentamicin	S	S	S	S	S
Sulfa-Trimethoprim	R	R	R	R	R

4 DISCUSSION

Remarkably, all the isolated strains of Gram-negative Enterobacteriaceae manifested phenotypic resistance to five or more of the ten antimicrobials to which they were exposed. The selected antimicrobials are frequently used in human medicine such as Amoxicillin/Clavulamic Acid and Sulfamethoxazol/Trimethoprim (Durand et al., 2018), as well as others widely used in veterinary medicine such the tetracycline family (Tetracycline and Doxycycline) and the aminoglycoside streptomycin (Hao et al., 2016). Evidence of resistance to cefovecin, the third-generation cephalosporin, was revealed in some strains. The only antimicrobial against which no resistance was found in this trial was Gentamicin. This widespread antimicrobial resistance may be unsurprising considering the local economy depends, in part, on livestock production.

These samples represent the first documentation of *E. fergusonii* in marine mammals. According to the most recent review about *E. fergusonii* in animal species (Gaastra et al., 2014), *Otaria flavescens* and other marine mammal species are not carriers. *Escherichia fergusonii* is an uncommon bacterial species that is emergent in humans and animals, and is generally associated

with cases of urinary tract infection, bacteremia, diarrhea, pancreatic carcinoma, endophthalmitis, and pleurisy in humans (Wragg et al., 2009). The importance of this bacterium for *Otaria flavescens* and the role of marine mammals in the epidemiology of this infection in humans is unclear and should be further studied.

These are the first results of this kind in *Otaria flavescens*, as well as the first in Uruguay. Studies in other countries have found multiple antibiotic resistance in harbor seals (*Phoca vitulina*) and harbor porpoises (*Phocoena phocoena*) from the Salish Sea on the West Coast of the United States (Norman et al., 2021), and from fecal samples of *Arctocephalus australis* and *Arctocephalus tropicalis* of the Southeastern Coast of Brazil (Santestevan et al., 2015). It is unclear if the resistance was accumulated via effluents of antibiotics and other contaminants, or of the inter-species transfer of the AMR bacteria themselves. In either case, the source of AMR was likely anthropogenic.

The release of antimicrobials, disinfectants, and other compounds, such as heavy metals into the natural environment can favor the evolution of resistant bacteria (Baker-Austin et al., 2006). These compounds are found in water and soil in different concentrations depending on the source and their behavior in terms of degradation rate and adsorption to solids (Kümmerer, 2009). Municipal wastewater contains a variety of pollutants, including pharmaceuticals and personal hygiene products. Similarly, hospital waste contains high concentrations of antimicrobials and disinfectants, and industrial waste can include heavy metal compounds. Despite the roles these effluents play in the creation and spread of AMR, little research exists on the dynamics of antimicrobials and antibiotic-resistant strains and how these impact human and animal health on a broad scale (Brahmi et al., 2018). This illustrates the existence of bacteria resistant to antimicrobials in natural environments with which humans have close contact, and hints at the use of these species as bioindicators of that system.

In conclusion, this is the first report of *Escherichia fergusonii* in *Otaria flavescens*, which has important implications for public health as a pathogen and ought to be further investigated. In addition, this is the first report of the presence of multi-resistant bacteria in *Otaria flavescens* in Uruguay, the most important focal concentration of Pinnipeds in South America. This study highlights the role wildlife play in AMR, a research thread that continues to lag behind our knowledge of AMR in human and agricultural animals; to ensure that infectious diseases can continue to be treated with effective and safe drugs (Torres et al., 2020) further research is needed.

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