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Resistance to antimicrobial agents among enterococci isolated from fecal samples of wild marine species in the southern coast of Brazil

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ABSTRACT

The purpose of this study was to evaluate species distribution, antimicrobial resistance profiles, and presence of resistance genes in enterococci isolated from fecal samples of wild marine species, including seabirds (n = 12), sea turtles (n = 8), and mammals (n = 3) found alive or dead in southern coast of Brazil. Enterococci were classified based on phenotypic and genotypic characteristics, tested for antibiotic susceptibility, and the presence of *tet*(S), *tet*(M), *tet*(L), *mrs*C, and *erm*(B) genes by PCR. *Enterococcus faecalis* and *Enterococcus faecium* were the most common species. Single (37.09%), double (25.80%), and multiple (16.12%) antibiotic resistance patterns were observed. Resistance to rifampicin occurred most frequently. The *msr*C, *tet*(M), and/or *tet*(L) genes were detected in 60.15%, 73.07%, and 23.07% of the resistant strains, respectively. In conclusion, the presence of antibiotic resistant strains in these species could be related to food web interactions and aquatic pollutants or linked to environmental resistome.

1. Introduction

Biological fluids contaminated with antimicrobials or antimicrobial-resistant microorganisms from human and animal origins, such as urine and feces, are being released into the sewage — particularly as wastewater from hospitals and intensive agricultural installations (Baquero et al., 2008). These effluents provide the pathway for introduction of resistant bacteria into seawater, thereby contaminating the species inhabiting these environments (Kummerer, 2009a). The impact created by the presence of antimicrobial agents in the aquatic environment and the frequency with which these resistance genes are transferred remains a subject of debate (Kummerer, 2009b).

Past studies conducted on marine species have discussed drug-resistant bacteria arising from marine environments. Al-Bahry et al. (2009) isolated antibiotic-resistant bacteria from the eggshell layers, albumen, and yolk of green turtle (*Chelonia mydas*) eggs. The study indicates that green turtle populations were subjected to polluted effluents during some of their migratory routes and feeding habitats. Rose et al. (2009) reported widespread antibiotic resistance in marine vertebrates off the northeastern coast of the United States. Stewart et al. (2014) isolated antibiotic-resistant bacteria from fecal and blowhole swabs of wild bottlenose dolphins (*Tursiops truncatus*).

Enterococci are considered commensal microbiota of the oral cavity, genitourinary, and gastrointestinal tract of humans and other species that are widely distributed in nature. This genus has the ability to survive adverse environmental conditions, including extreme temperatures (10–45 °C), an extreme range of pH (4.5–10.0), and high salinity (6.5%) (Teixeira et al., 2011). The species most frequently encountered in the gastrointestinal tract are *Enterococcus faecalis*, *E. faecium*, *E. hirae*, *E. durans*, *E. casseliflavus*, *E. gallinarum*, and *E. mundtii* (Poeta et al., 2005; Layton et al., 2010; Marinho et al., 2013; Lebreton et al., 2014). The occurrence of these different species appears to vary according to the host and its age, diet, underlying diseases, and prior antimicrobial therapy (Lebreton et al., 2014).

The resistance to several classes of antimicrobial agents is a remarkable characteristic of enterococcal isolates (Kristich et al., 2014). Many species are recognized for their capacity to acquire and transfer resistance and virulence genes, which provides selective advantages for survival and dispersal in the environment (Gilmore et al., 2013). As a result, the presence of the enterococci has been investigated and monitored in a variety of habitats, providing important information regarding the

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interactions and environmental disturbances related to enterococci (Barros et al., 2011; Dada et al., 2013; Ahmad et al., 2014; Radhouani et al., 2014).

To date, few studies have examined the presence of enterococci in wild marine species, likely due to the migratory habits of many species and the inherent difficulty in obtaining samples (Johnson et al., 1998; Thornton et al., 1998; Lisle et al., 2004; Lockwood et al., 2006; Barros et al., 2011; Marinho et al., 2013; Spence et al., 2014; Santestevan et al., 2015). Therefore, studies of wild marine species can provide information that can serve to protect these species and the marine environment. The relevance of investigating the enterococci genus stems from their role as components of the microbiota of a number of species, and their direct influence on animal health and ecology.

The coast of Brazil presents a large variety of ecosystems of high environmental relevance that serve as a habitat for a diversity of marine species, both endemic and exotic. The diversity of marine species in Brazilian coast is primarily influenced by three current systems, which increase nutritional support and subsequently contributes to the establishment of high species richness (Mäder et al., 2010). Migratory seabirds, sea turtles, sea lions, dolphins, whales, and other marine species become stranded on the Brazilian coast either in poor health or dead (Dantas et al., 2013; Ceclimar, 2015).

The purpose of this study was to evaluate species distribution, antimicrobial resistance profile, and the presence of resistance genes in enterococci isolated from fecal samples of wild marine species including hawksbill turtles (*Eretmochelys imbricata*), green turtles (*Chelonia mydas*), Magellanic penguins (*Spheniscus magellanicus*), snowy-crowned tern (*Sterna trudeaui*), white-backed stilt (*Himantopus melanurus*), humpback whale (*Megaptera novaeangliae*), dwarf minke whale (*Balaenoptera acutorostrata*), and Risso's dolphin (*Grampus griseus*) found off the southern coast of Brazil.

2. Materials and methods

2.1. Sample collection

Twenty-three wild marine species were used in this study. These included seabirds (n = 12), sea turtles (n = 8), and mammals (n = 3) that were discovered live or dead along the north coast of Rio Grande do Sul, Southern Brazil, from Itapeva Beach, Torres (29°21′32.2″S; 49°44′10.3″W) to Dunas Altas Beach, Palmares do Sul (30°23′58.75″S; 50°17′24.73″W), between October 2012 and April 2014 (Table 1).

Animals were classified into two categories, Code 1 (live animals) and Code 2 (carcasses in good condition, characterized by having normal appearance, little scavenger damage, fresh odor, lack of bloating, and firm muscle and blubber, among other features) as described by Geraci and Lounsbury (2005). Fifteen animals, including penguins (n = 8) and sea turtles n = 7) were classified as Code 1. Eight animals, including penguins (n = 2), seabirds (n = 2), whales (n = 2), sea turtle (n = 1) and dolphin (n = 1) were classified as Code 2.

Fecal samples were collected from these animals by cloacal or rectal swabs. Swabs were stored in Stuart transport medium and transported to the laboratory for microbiological analyses. The sampling of wild marine species was performed in accordance with regulations established by the System Authorization and Information on Biodiversity (SISBIO) n° 20185–4, code number 62966211.

Necropsies were performed on all dead animals (Code 2) using conventional methods in the Center for Coastal Studies, Limnology and Marine (CECLIMAR) according to protocol for sample collection described by Geraci and Lounsbury (2005). Since no animals exhibited signs of infection and their stomachs were empty, the necropsies concluded that all animals studied died of natural causes.

2.2. Isolation and identification of enterococci

Swabs containing fecal samples were inoculated in 9 mL of azide dextrose broth (Himedia, Mumbai, India) and incubated for 24 h at 37 °C. Aliquots of 1 mL were placed in 9 mL of saline water, and initial samples were further diluted 10-fold to obtain a final dilution factor of 1/1000. From each dilution, 100 μL was inoculated in brain heart infusion (BHI) agar plates (Himedia, Mumbai, India) supplemented with 6.5% NaCl, before being incubated as previously described (Prichula et al., 2013; Santestevan et al., 2015).

Twenty-five colonies were randomly selected from each fecal sample, since enterococci are present in high concentrations in feces, typically between 10^5 and 10^7 CFU/g. Phenotypic criteria, such as size/volume, shape, color, gram staining, catalase production, growth capacity at 45 °C, and bile aesculin reaction were used to separate the enterococci group and the non-enterococcal strains (Teixeira et al., 2011). Selected pure colonies were stored at −20 °C in a 10% (*w*/*v*) solution

Table 1
Details of the wild marine species analyzed in this study.

	Sample[1]	Specie	Age	Sex[2]	Code[3]	Location
Sea turtles	HT1	*Eretmochelys imbricata*	young	I	1	Tramandaí
	HT2	*Eretmochelys imbricata*	young	I	1	Capão da Canoa
	GT1	*Chelonia mydas*	young	I	1	Tramandaí
	GT2	*Chelonia mydas*	adult	I	1	Palmares do Sul
	GT3	*Chelonia mydas*	young	I	2	Cidreira
	GT4	*Chelonia mydas*	young	I	1	Magistério
	GT5	*Chelonia mydas*	young	I	1	Litoral médio leste
	GT6	*Chelonia mydas*	young	I	1	Nova Tramandaí
Seabirds	MP1	*Spheniscus magellanicus*	young	F	1	Cidreira
	MP2	*Spheniscus magellanicus*	young	M	1	Xangri-lá
	MP3	*Spheniscus magellanicus*	young	F	1	Arroio do Sal
	MP4	*Spheniscus magellanicus*	young	M	1	Torres
	MP5	*Spheniscus magellanicus*	young	I	2	Cidreira
	MP6	*Spheniscus magellanicus*	adult	I	1	Capão da Canoa
	MP7	*Spheniscus magellanicus*	young	I	1	Imbé
	MP8	*Spheniscus magellanicus*	young	M	1	Cidreira
	MP9	*Spheniscus magellanicus*	young	F	2	Cidreira
	MP10	*Spheniscus magellanicus*	young	I	1	Torres
	ST1	*Sterna trudeaui*	adult	M	2	Arroio do Sal
	WS1	*Himantopus melanurus*	adult	F	2	Xangri-lá
Mammals	DMW1	*Balaenoptera acutorostrata*	young	M	2	Tramandaí
	HW1	*Megaptera novaeangliae*	puppy	M	2	Palmares do Sul
	RD1	*Grampus griseus*	adult	F	2	Balneário Pinhal

[1]HT — Hawksbill Turtle; GT — Green Turtle; MP — Magellanic Penguin; ST — Snowy-crowned Tern; WS — White-backed Stilt; HW — Humpback Whale; DMW — Dwarf Minke Whale; RD — Risso's Dolphin. [2]I: indeterminate sex; M: male, F: female. [3]Code based on Geraci and Lounsbury (2005).