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Multiple antibiotic-resistance of *Enterococcus* isolated from coastal water near an outfall in Brazil

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Marine outfalls contribute to the environmental protection of coastal zones. However, these structures may serve as vehicles for microbiological contamination. This study aimed to investigate the occurrence of antimicrobial-resistant bacteria in water samples collected from 67 stations located in nearby areas of the ocean outfall in Fortaleza, Brazil. 81 *Enterococcus* strains were isolated, identified and distributed in the following groups of species: *Enterococcus faecalis* (n = 37; 45.7%), *Enterococcus faecium* (n = 30; 37%), *Enterococcus mundtii* (n = 9; 11.1%), *Enterococcus raffinosus* (n = 2; 2.5%), *Enterococcus dispar* (n = 2; 2.5%) and *Enterococcus durans* (n = 1; 1.2%). Antimicrobial resistance was observed in 47 (58%) of the strains, and the most predominant profile was the concurrent resistance to ampicillin, clindamycin, penicillin and vancomycin. In 31 strains were detected phenotypically, plasmid resistance factors. The data reported in this study should serve as an alert to public health authorities, since they suggest that the area near the submarine outfall in Fortaleza may contribute to antimicrobial-resistant enterococci spread.

Key words: Enterococci, seawater, multidrug-resistant bacteria, public health.

INTRODUCTION

The coexistence of different interests in coastal areas, that is, the presence of highly populated cities, recreational areas and extensive shellfish farming (Scroccaro et al., 2010) justifies the construction of structures which contribute to mitigate the impact caused by adding residual water into the aquatic environment. Thus, marine outfalls must be considered part of an integrated environmental protection system for coastal zones (Mendonça et al., 2013). However, these structures need to be constantly monitored, once they serve as vehicle to pollution due to organic enrichment and microbiological contamination (Gubitoso et al., 2008).

Among the bacteria constantly associated to domestic sewage, those which belong to the *Enterococcus* genus

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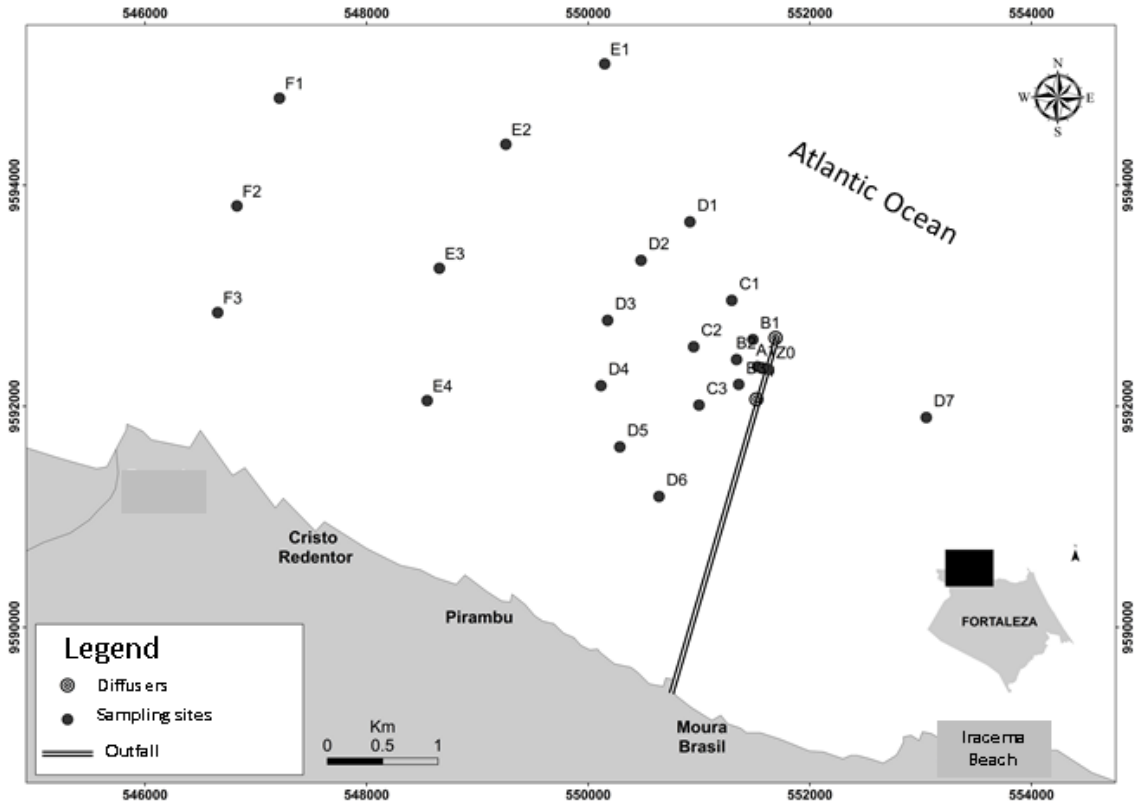

Figure 1. Water samples from multiple collection points around the marine outfall in Fortaleza, Ceara, Brazil.

are certainly noteworthy. These micro-organisms are ranked as excellent indicators for saline waters, since they have a higher survival time levels and greater resistance as compared to the *Escherichia coli* species and other thermal tolerant coliforms (Dufour, 1994). In addition, their ability to survive in extreme ranges of temperature, pH and salinity must be highlighted, as well as their resistance to detergent action, which prevents their removal from the environment (Hayes et al., 2003).

The presence of enterococci in recreational waters, sediments and beach sand affects the quality of these habitats and poses a risk to bathers' health (Erdem-Kimiran et al., 2007). Besides, beaches that are domestic waste recipients contribute to the spread of microorganisms that carry antibiotic-resistant genes (Iversen et al., 2004).

The main objective of this article was to identify strains of *Enterococcus* spp. in water samples collected near the ocean outfall in Fortaleza, Ceara, Brazil, determine the antimicrobial susceptibility profile, and investigate if the antimicrobial resistance was plasmid coded.

MATERIALS AND METHODS

Sampling sites

Water samples from 67 stations divided into surface (AS, BS, CS, DS, ES, FS), middle (AM, BM, CM, DM, EM, FM), bottom (AB, BB, CB, DB, EB, FB) and raw sewage (RS) were analyzed. All stations are located in a nearby area around the marine outfall in Fortaleza, Ceara, Brazil (Figure 1). Samples were collected in Van Dorn bottles and stored until bacteriological analysis in sterile amber glasses with 1 L capacity.

Isolation and identification of *Enterococcus*

Seawater samples were dilluted (1:9) in 1% saline solution and inoculated using Azide Broth (Difco), incubated at 35°C/48 h. After the incubation period, aliquots from positive (turbid) tubes in the Azide Broth were taken and plated on m-Enterococcus Agar (Difco), incubated at 35°C/48 h. Colonies with similar characteristics with the genus *Enterococcus* were isolated on Brain Heart Infusion Agar (BHI-Difco) and subjected to characterization by Gram staining. All isolates identified as Gram-positive cocci (n=81) were identified to the species level in accordance with Carvalho et al. (2004). The strains were maintained in BHI Agar (pH 7.5) at 18-20°C until antibiogram procedures.

Antibiogram

Antimicrobial susceptibility was verified by disk diffusion method using Müeller-Hinton agar (Difco) (CLSI, 2010). The following antimicrobials were tested: Ampicillin (Amp 10 µg), Penicillin (Pen 10 U.I), Gentamicin (Gen 10 µg), Streptomycin (Str 10 µg), Tetracycline (Tcy 30 µg), Chloramphenicol (Chl 30 µg), Clindamycin (Cli 2 µg) and Vancomycin (Van 30 µg). For this procedure, the cell concentration for all strains was adjusted to a turbidity level similar

Table 1. Distribution of *Enterococcus* species in sampling sites from the marine outfall (Fortaleza, Brazil).

Specie	Number	Sampling site (number of isolates)
E. faecalis	37	RS (n=22), A1S (n=2), A1B (n=2), B2M (n=2), B2B (n=2), A1M (n=1), B1S (n=1), B3S (n=1), C3B (n=1), C4B (n=1), D2M, F3S (n=1)
E. faecium	30	RS (n=7), F3S (n=3), A1S (n=2), A1M (n=2), B1S (n=2), B2S (n=2), B2M (n=2), C2M (n=2), D2M (n=2), F3M (n=2), A1B (n=1), B2B (n=1), C1M (n=1), C2B (n=1)
E. mundtti	9	B1M (n=3), RS (n=1), A1B (n=1), B1S (n=1), B3S (n=1), F2B (n=1), F1S (n=1)
E. raffinosus	2	B1M (n=1), B2S (n=1)
E. dispar	2	A1M (n=1), D2M (n=1)
E. durans	1	C2M (n=1)

*RS: Raw sewage. S: surface. M: middle. B: bottom.

Table 2. Number of antibiotic-resistant *Enterococcus* strains isolated from water samples collected in the nearby area around the marine outfall (Fortaleza, Brazil).

Specie	Number	Resistance to							
		Cli	Van	Pen	Tcy	Str	Amp	Gen	Chl
E. faecalis	37	22	14	13	12	12	11	12	6
E. faecium	30	16	9	8	7	7	7	5	2
E. mundtti	9	5	2	1	3	3	1	-	-
E. raffinosus	2	2	2	1	-	-	1	-	1
E. dispar	2	-	-	-	-	-	-	-	-
E. durans	1	-	-	-	-	-	-	-	-
Total	81	45	27	23	22	22	20	17	9

* Cli: Clindamycin 2 µg; Van: Vancomycin 30 µg; Pen: Penicillin 10 U.I; Tcy: Tetracycline (Tcy 30 µg); Str: Streptomycin 10µg; Amp: Ampicillin 10 µg; Gen: Gentamicin 10 µg; Chl: Chloramphenicol 30 µg.

to MacFarland 0.5 scale. The inoculation procedure for plates containing Müeller-Hinton medium was made using swabs, followed by application of the antimicrobial discs. All plates were incubated at 35°C for 24 h. Zones of inhibition were measured using a digital caliper (Digimess) and each strain behavior was classified as sensitive, intermediate or resistant, according to CLSI (2010) recommendations. The *Enterococcus faecalis* ATCC 29212 strain was used as a control. Multiple antibiotics resistance (MAR) index was determined according to Krumperman (1983).

Plasmid curing

Multiple resistant strains were selected and submitted to plasmid curing according to Molina-Aja et al. (2002) with modifications. We use Luria-Bertani broth (LB), supplemented with 0.85% NaCl and acridine orange at 50 µg/mL. Strains grown under constant shaking in LB medium for 24 h at 30°C were once again subjected to antibiotic susceptibility testing (described above) against the antimicrobials to which they were resistant. Resistance was classified as plasmid dependent when affected by plasmid curing.

RESULTS

The identification of 81 isolates from 20 sampling sites revealed the presence of six species: *Enterococcus faecalis* (n = 37; 45.7%), *E. faecium* (n = 30; 37%), *Enterococcus mundtii* (n = 9; 11.1%) (Table 1).

The antimicrobial susceptibility profile test showed that 53 (65.4%) strains were resistant to at least one antimicrobial, and the number of clindamycin-resistant isolates was high and should be highlighted (n = 45, 55.5%) (Table 2).

Twenty four multi-resistant profiles were detected from a total of 47 (58%) strains, the most predominant being Amp+Cli+Pen+Van (n=8) (Table 3). The species with the highest number of isolates with multiple-resistant profiles was *E. faecalis* (n=26), followed by *E. faecium* (n=13), *Enterococcus mundtii* (n=6), *Enterococcus raffinosus* (n=1) and *Enterococcus dispar* (n=1). MAR levels ranged from 0.25 to 0.87 (Table 3)

In 31 strains were detected phenotypically, plasmid resistance factors. Resistance to at least two of the following antimicrobials was verified in 27 (57.4%) of the multiple-resistant strains: Cli, Van, Pen, Chl, Str, Tet, Amp and Gen (Table 4).

DISCUSSION

In Fortaleza, there is a large number of Enterococci in the vicinity of outfalls and nearby areas. In this study, the

Table 3. Multiple resistance profiles to antimicrobials by *Enterococcus* strains isolated from water samples collected in the nearby area around the marine outfall (Fortaleza, Brazil).

Profile	n	*E. faecalis* n=26	*E. faecium* n=13	*E. mundtti* n=6	*E. raffinosus* n=1	*E. dispar* n=1	MAR
Amp+Cli+Pen+Van	8	3	3	2			0.5
Amp+Cli+Str+Pen+Tcy+Van	3	1		1	1		0.75
Amp+Cli+Chl+Str+Pen+Tcy+Van	3	2	1				0.87
Amp+Cli+Chl+Str+Pen+Van	2	1	1				0.75
Cli+Str+Gen+Tcy	3	2	1				0.5
Cli+Str+Gen+Tcy	3	2	1				0.5
Cli+Str+Gen	3	2	1				0.37
Cli+Est+Tet	3	1	1	1			0.37
Cli+Van	3	1	1	1			0.25
Amp+Cli+Gen+Pen+Tcy+Van	2	2					0.75
Cli+Str	2	2					0.25
Cli+Gen	2	1	1				0.25
Cli+Tcy	2	1	1				0.25
Amp+Cli+Str+Gen+Pen+Tcy+Van	1	1					0.87
Amp+Cli+Str+Pen+Van	1	1					0.62
Cli+Gen+Pen+Tcy+Van	1	1					0.62
Cli+Chl+Gen+Tcy	1	1					0.5
Cli+Gen+Tcy	1	1					0.37
Cli+Chl+Van	1					1	0.37
Cli+Pen+Van	1		1				0.37
Str+Gen+Tcy	1		1				0.37
Chl+Tcy	1	1					0.5
Pen+Van	1	1					0.5
Chl+Gen	1			1			0.5

*n: number of strains. Amp: Ampicillin 10 µg; Cli: Clindamycin 2 µg; Pen: Penicillin 10 U.I; Van: Vancomycin 30 µg; Str: Streptomycin 10µg; Tcy: Tetracycline (Tcy 30 µg); Chl: Chloramphenicol 30 µg; Gen: Gentamicin 10 µg. MAR: Multiple Antibiotic Resistance index.

species *E. faecalis* and *E. faecium* were the most frequently isolated. This result is similar to those obtained by Graves and Weaver (2010), who reported a diversity of ten species of *Enterococcus* (*E. faecalis* - 30.6%; *Enterococcus pseudoavium* - 24%, *Enterococcus casseliflavus* - 12.8%; *E. faecium* - 11.2%, *E. mundtii* 7.9%, *Enterococcus gallinarum* - 6.2%; *E. dispar* - 3.7%; *Enterococcus hirae* - 2.1%, *Enterococcus durans* – 0.8% and *Enterococcus flavescens* - 0.8%) in water samples in a wetland close to College Station, Texas. According to the authors, the distribution of these species depends on environmental factors and specific points and sources of the sewer system. This statement was confirmed in this study, since 30 (37%) strains were isolated from the station corresponding to the raw sewage (RS) and only 8 (9.9%) were derived from more distant stations (F1S, FSB, F3S and F3M) from the RS.

The relationship between water quality and sewage discarding via submarine outfall has been discussed. In Tijuana-Mexico, Gersberg et al. (2008) makes statistical comparisons of the bacterial water quality - including enterococci densities of the ocean, both before and after discharge of sewage to the South Bay Ocean Outfall (SBOO). The authors observed that only four (of the 11 total) showed significant improvement, that is, decreased frequency of incidence of bacterial indicator thresholds, after SBOO discharge began.

Regarding antimicrobial resistance data, values similar to those presented in this study were reported by Oliveira and Piñata (2008) when they evaluated the antimicrobial drug resistance of 160 strains of *Enterococcus* from two recreational beaches in São Paulo, Brazil. The authors found a 51.9% rate of antimicrobial resistance, and showed the problem of the discharge of domestic sewage on beaches.

In the present study, the highest percentages of resistance were observed for Cli, Van, Pen, Tcy and Str (Table 2). Moore et al. (2010), researching antimicrobial resistance in bacteria in the waters of rivers and streams in Northern Ireland, observed Cli, Van, Pen and Tcy

Table 4. Multiple resistance profiles of *Enterococcus* strains isolated from water samples collected in the nearby area around the marine outfall (Fortaleza, Brazil) before and after plasmid curing.

Collection station	Antimicrobial resistance profiles			
	Before plasmids curing	**n**	**After plasmids curing**	**n**
AS	Cli+Van	1	-	1
AM	Cli+Chl+Van	1	Cli	1
AB	Cli+Str+Tcy	1	Cli+Str+Tcy	1
BS	Cli+Van	1	Cli	1
BM	Cli+Van	1	Cli	1
BM	Cli+Pen+Van	1	Cli	1
BB	Gen+Van	1	Gen	1
DM	Tcy+Chl	1	Tcy	1
FS	Tcy+Cli	1	Amp+Cli+Pen+Van	1
FS	Tcy+Cli	1	Amp+Cli+Pen+Van	1
FS	Amp+Cli+Pen+Van	1	Amp+Cli+Pen+Van	1
FB	Amp+Cli+Pen+Van	1	Tcy+Cli	1
FB	Amp+Cli+Pen+Van	1	Tcy+Cli	1
FB	Amp+Cli+Pen+Van	1	Amp+Cli+Pen+Van	1
FM	Amp+Cli+Pen+Van	1	Amp+Cli+Pen+Van	1
FM	Amp+Cli+Pen+Van	1	Amp+Cli+Pen+Van	1
	Cli+Gen	2	Cli	6
	Cli+Str	2	Van	1
	Pen+Van	1	Cli+Tcy	2
	Cli+Str+Gen	3	Cli+Str	1
	Str+Gen+Tcy	2	Cli+Str+Tcy	3
	Str+Tcy+Van	1	Cli+Pen+Van	2
	Cli+Str+Tcy	1	Str+Gen+Tcy	2
	Cli+Str+Gen+Tcy	3	Cli+Str+Gen	1
RS	Amp+Cli+Pen+Van	2	Amp+Cli+Pen+Van	8
	Cli+Chl+Gen+Tcy	1	Amp+Cli+Pen+Tcy+Van	2
	Amp+Cli+Str+Pen+Van	1		
	Cli+Gen+Pen+Tcy+Van	1		
	Amp+Cli+Str+Pen+Tcy+Van	3		
	Amp+Cli+Gen+Pen+Tcy+Van	2		
	Amp+Chl+Cli+Str+Pen+Van	1		
	Amp+Cli+Chl+Str+Pen+Van	1		
	Amp+Cli+Chl+Str+Pen+Tcy+Van	3		
	Amp+Cli+Str+Gen+Pen+Tcy+Van	1		

*S: Surface. M: Middle. B: Bottom. RS: Raw Sewage. n: number of strains. Cli: Clindamycin 2 µg; Van: Vancomycin 30 µg; Chl: Chloramphenicol 30 µg; Str: Streptomycin 10 µg; Tcy: Tetracycline (Tcy 30 µg); Pen: Penicillin 10 U.I; Gen: Gentamicin 10 µg; Amp: Ampicillin 10 µg.

resistant profiles. According to these authors, the resistance profile expressed by environmental strains allows the identification of the type of polluting source that the ecosystem has been receiving. Mudryk et al. (2010) showed a 27% resistance to Cli in beach and sediment isolates from the National Park at the South Coast of the Baltic Sea, a lower percentage than the one detected in this study. Cli belongs to the family of Lincosamides, and according to Moellering (1991) resistance to such antimicrobial is common in enterococci, which may be considered a case of intrinsic resistance, with interspecies associations. For Lüthje and Schwarz (2007), bacterial resistance to lincosamide is related to efflux mechanisms and mutations.

The susceptibility of enterococcal isolates to clindamycin was reported by Schmitz et al. (1999) - only 4.4 of all *E. faecalis* strains from 20 European university hospitals were clindamycin-susceptible. Thus, these authors call attention to the fact that lincosamides cannot be considered as a therapeutic option for enterococcal infec-

tions.

Vancomycin was the second antimicrobial agent to which the strains showed a higher resistance (34%). Iversen et al. (2002) observed that 60% of the residual water samples from four sewage treatment plants in Sweden, 36% of hospital sewage effluent and 19% of those of treated sewage had vancomycin-resistant strains. Similarly, Talebi et al. (2007) detected resistance to Van in 19% of *Enterococcus* strains isolated in three sewage treatment plants in Iran. For Whitman et al. (2003), the presence of resistant enterococci, including Vancomycin Resistant *Enterococcus*- VRE, in marine ecosystems and hospital waste is an indication of fecal contamination. According to the Centers for Disease Control and Prevention- CDC (2002), resistance to this drug is relatively recent, occurring primarily by the production of peptidoglycan precursors in the cell wall associated with vancomycin, preventing its action in blocking cell wall synthesis (Lai et al., 1998).

The high levels of multidrug resistance (n=47; 58%) (Table 3) in the present study should be highlighted. These results are similar to the ones of Costa et al. (2006), who found multidrug resistant characteristics in 46% of *Enterococcus* isolates derived from 14 treatment plants in Portugal. In addition, Arvanitidou et al. (2001) found multidrug resistance values in 33 (5%) of *Enterococcus* isolates from coastal waters in northwestern Greece, and 20 distinct multidrug resistance profiles. For Xu et al. (2007), selective pressure experienced by bacteria force them to adapt quickly, especially by horizontal gene transfer, and their resistance to several antimicrobials is now widely recognized.

Hayes et al. (2004) found similar results with the ones in this study, distributed as follows: Cli+Str+Tcy and Cli+Str+Pen+Tcy. Similar to the results presented here, the species with the most frequent resistance phenotype were: *E. faecalis* (53.2%) and *E faecium* (31.4%). These statements suggest that enterococci isolated from the marine environment may become multi-resistant to antibiotics used in human medicine.

MAR index obtained in this study (Table 3) indicate that multi resistance to drugs was predominant in all isolates. Similar data was verified by Son et al. (1999), who reported MAR rates ranging from 0.2 to 0.9.

Another finding worth mentioning is the frequency of isolates (37%) with plasmid-mediated resistance (Table 4). From 31 strains with plasmid resistance expression (phenotypic detection), 24 were derived from the station corresponding to the raw sewage (RS). This suggests that environments polluted by sewage, that is, rich in organic matter, may be vehicles of bacteria carrying antibiotic-resistant plasmids. For McBride et al. (2007), the presence of this type of mobile genetic element is common in enterococci, as they make up a substantial fraction of their genome, and responsible for much of the horizontal gene transfer. The same authors found seven resistance profiles related to plasmids in 88 *E. faecalis* isolates.

It is noted that the isolates from the same sampling site showed different profiles before and after the curing (Table 4). According to Boehm et al. (2002), the transportation of fecal bacteria in the sewage to the surf zone depends on the following conditions: oceanographic (stratification of the water column), wave, tidal range and currents. Thus, this contamination in marine waters might contribute to the establishment of resistance routes for environmental bacteria (Meirelles-Pereira et al. 2002).

Enterococci are prone to acquiring resistance to antibiotics, either by mutation or by horizontal transfer of mobile genetic elements (plasmids and transposons) (Hasmann et al., 2005). The reasons for the high number of plasmids in the resistance levels among several species of *Enterococcus* are still unknown (Jensen et al., 2010). Therefore plasmid detection in environmental bacteria may represent a tool to compare a large number of aquatic environments, promoting a better understanding of their development and ecology (Meireles-Pereira et al., 2002).

The data reported in this study should serve as an alert to public health authorities, since results suggest that the area near the submarine outfall in Fortaleza may contribute to antimicrobial-resistant enterococci spread. Furthermore, it is worth noting, the relationship between the content of the sewages disposal thrown in marine waters and the occurrence of plasmid-carrying, antibiotic-resistant *Enterecoccus* in the same environment.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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