Arquivos de Ciências do Mar

BATHING SUITABILITY AND ANTIMICROBIAL SUSCEPTIBILITY OF ENTEROCOCOS IN TROPICAL COASTAL WATERS

Balneabilidade e suscetibilidade antimicrobiana de enterococos em águas tropicais

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ABSTRACT

The bathing suitability of three beaches and the antimicrobial susceptibility profile of Enterococcus faecalis *and* E. faecium *lineages to various antimicrobials were studied weekly for 14 weeks in the city of Fortaleza, Ceará, Brazil. The water of the beaches 1, 2 and 3 was considered unsuitable for bathing (40%, 60% and 80%, respectively).* Enterococcus faecalis *was the predominant species (63.5%) in the water of the three beaches, followed by* E. faecium *(36.5%). Most lineages (36.5%) were isolated in beach 2; 34.6% were isolated in beach 1, and 28.8% in 3. High antimicrobial resistance to nalidixic acid (94.3%), tetracycline (26.4%) and amicacine (26.0%) was observed. All strains showed 100% susceptibility to ampicillin and imipenem.* Enterococcus faecalis *was also susceptible to vancomycin and* E. faecium *to penicillin. Multiple resistance profile was observed in 88.5% (MAR >0.18) of the strains, three of which were resistant to six antimicrobials. Considering the resistance increase of autochthonous bacteria observed in the marine environment, special attention should be paid to the multiple resistance profile of the isolates.*

Keywords: *bathing suitability,* Enterococcus faecalis*, beaches, contamination.*

RESUMO

A balneabilidade de três praias e o perfil de susceptibilidade de estirpes de Enterococcus faecalis *e* E. faecium *a diferentes antimicrobianos foram estudados semanalmente na cidade de Fortaleza, Ceará, durante 14 semanas. As águas das praias 1, 2 e 3 se apresentaram impróprias para balneabilidade em 40%, 60% e 80%, respectivamente.* Enterococcus faecalis *(63,5%) foi a espécie predominante em todos as praias, seguido de* E. faecium *(36,5%). Na praia 2 se isolou 36,5% das estirpes, seguido da praia 1 (34,6%) e praia 3 (28,8%). Elevada resistência foi observada para o ácido nalidíxico (94,3%), tetraciclina (26,4%) e amicacina (26,0%) e susceptibilidade a ampicilina e o imipenem.* Enterococcus faecalis *também foi susceptível a vancomicina e* E. faecium *a penicilina. Perfil de multiresistência foi observado em 88,5% das cepas (índice MAR >0,18), tendo três cepas apresentado resistência a seis antibióticos. O perfil de multiresistência dos isolados é preocupante uma vez que se tem observado o aumento da resistência em bactérias autoctones no ambiente marinho.*

Palavras-chaves: *balneabilidade,* Enterococcus faecalis*, praias, poluição.*

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INTRODUCTION

In the last years, the increase of demographic concentration in urban centers, mainly those located near lakes and coastal areas, have contributed to the increase of water-borne diseases caused by the evergrowing discharge of domestic sewage. Aquatic environments in general and beaches in particular are important for human recreation and are widely used for sports practice, sunbathing and swimming. Pollution in marine coastal ecosystems has become a serious environmental problem that affects developing as well as developed countries (Elofsson *et al*., 2003). Public health hazard monitoring involves mainly fecal coliform testing. However, the enterococci group has proved to be more sensitive as indicator of fecal contamination than *Escherichia coli* and thermotolerant coliforms for they survive longer and are more resistant in saline environments (Dufour, 1994).

Enterococci are widely distributed in the environment and can be found in soil, food, water, plants, animals, birds and insects (Blaimont *et al*., 1995). They are opportunistic pathogens responsible for most nosocomial infections and can cause endocardites and urinary tract infections in immuno-depressed individuals (Horner *et al*., 2005).

The increasing bacterial resistance to antibiotics has become a public health problem due to the fact that bacteria can be found in different niches. Among those niches, the aquatic environment is considered the most efficient for the selection of resistant populations, as well as for the exchange of resistance genes by means of mobile genetic elements, such as plasmids and transposons which encode resistance to antibiotics. The multiple drug resistance of an organism reduces the therapeutic options in both animals and humans (Alderman & Hastings, 1998).

For the above-mentioned reasons, the quantification and identification of the multiresistant lineages of the genus *Enterococcus* found in coastal waters is relevant to evaluate their contribution to the dissemination of resistance. The objetive of this study was to isolate and identify *Enterococcus faecalis* and *E. faecium* lineages from three beaches (Diários, Meireles e Mucuripe) and to assess the susceptibility profile of the isolates to various antimicrobials.

MATERIALS AND METHODS

Sample location and collection

The three beaches studied are located on the waterfront of the city of Fortaleza, Ceará State. Fourteen samples were collected and analyzed weekly from April to July 2007 at Diários Beach (1), Meireles Beach (2) and Mucuripe Beach (3) abreast of the Maceió Creek's mouth. The total number of samples analyzed amounted to 42. Water samples were collected at 1 m depth in 1000 mL sterilized amber bottles and transported to the Microbiology laboratory at the Marine Science Institute (LABOMAR- Universidade Federal do Ceará, UFC) for immediate processing. The samples were always collected between 14:00 as 15:00 h irrespective of the tide.

Enterococcus quantification and strain isolation

Most probable number (MPN) estimates were obtained by multiple-tube technique using a five tube series and Azide Dextrose Broth- Difco. After serial dilutions from 10^{-1} to 10^{-4}, the tubes were incubated at 35°C for 48 hours. The tubes with precipitation or clouding were inoculated in Agar M-*Enterococcus* (Difco) for 48 hours at 35ºC. The presence of turbidity and of characteristic strains of *Enterococcus* in the Agar M-*Enterococcus* medium was considered positive for bacterial growth. For the determination of the MPN of *Enterococcus* mL^{-1} the Hoskins tables were used (95% confidence limit). The colonies with characteristics of *Enterococcus* in Agar M-*Enterococcus* were inoculated in Brain Heart Infusion Agar (BHI) (Oxoid) for biochemical identification. Morphological (Gram staining reaction) and biochemical tests (catalase production, arginine utilization, growth at 10ºC and 45ºC, growth in 6.5% NaCl, pH 9.6 and in 0.04% telurite) were used for bacterial identification (Silva et al., 2007). The same tests were performed on a strain of *Enterococcus faecalis A*TCC 25922.

Antibiogram

The agar diffusion disk test was used to evaluate the bacterial susceptibility to antimicrobial agents following the Clinical and Laboratory Standards Institute guidelines - CLSI (2007). The inoculum density corresponded to 0.5 in the McFarland scale and was checked by using a spectrophotometer (Micronal, mod. B542). The suspension was seeded with sterile swabs in plates containing Müeller-Hinton Agar. Once the inoculum had been absorbed, antimicrobial disks were applied aseptically and incubated at 35°C for 24 hours. After incubation, the diameters of the inhibition zones were measured with a caliper. All the experiments

were set up in duplicate, and *Enterococcus faecalis* ATCC 25922 were used as standard quality control strains. The following antibiotics were tested: nalidixic acid - NAL (30 µg), amicacin - AMI (30 µg), ampicillin - AMP (10 µg), cefalotin - CFL (30 µg), ceftriaxon - CRO (30 µg), erythromycin - ERI (15 µg), gentamicin - GEN (10 µg), imipenem - IMP (10 µg), penicillin - PEN (10 µg), tetracycline - TET (30 µg) and vancomycin - VAN (30 µg).

Multiple Antimicrobial Resistance

When applied to a bacterial isolate, multiple antimicrobial resistance (MAR) is defined as a/b, where *a* is the number of antibiotics to which the isolate is resistant, and *b* the number of antibiotics to which the isolate was exposed. Values higher than 0.2 indicate multiple resistance (Krumperman, 1983).

Statistical analysis

The antibiogram results were expressed in a binomial matrix were 1 is resistance and 0 is sensitivity to the antimicrobials tested. A similarity dendrogram was built using the neighbor joining UPGMA method with the BioDiversity Professional Beta (McAleece *et al.*, 1997) software package and Bray-Curtis analysis as similarity matrix.

RESULTS AND DISCUSSION

In Beach 1, bathing suitability was observed in 60% of the weeks, followed by Beachs 2 and 3 which were safe in 40% and 20% of the weeks, respectively. The combined analysis of Beaches 1 and 2 showed that they were suitable during 4 weeks (4th, 6th, 7th and 10th) (Table I). During the last week of the experiment, all three beaches were suitable for bathing. Therefore, water quality in Beach 1 was considered excellent or very good in 30% of the weeks. Water quality in Beach 2 was excellent in 20% of the weeks and very good or satisfactory in 10% of the weeks.

Resolution 274 (BRASIL, 2000) of the Brazilian National Environmental Council (CONAMA) establishes the suitability of marine and freshwater bathing sites for contact recreation, and grades the water at any given site as satisfactory when no more than 400 enterococci/100 mL are found in 80% of the analyzed samples during the five previous weeks. Water is graded as Excellent, Very Good, Satisfactory and Unsuitable, being the first three considered Suitable.

Table I - Most Probable Number (MPN) of Enterococcus per 100 mL in water samples of three beaches (Fortaleza, Ceará), classified according to the criteria of bathing suitability established by CONAMA´s Resolution n° 274, 2000.

Weeks	Samples	Source					
		Beach 1		Beach 2		Beach 3	
		NMP/100	CL	NMP/100	CL	NMP/100	CL
01	01	11,000		200		930	
	02	130		45		490	
	03	230	U	45	U	9,400	U
	04	>160,000		680		43,000	
	05	20		230		45	
02	01	130		45		490	
	02	230		45		9,400	
	03	>160,000	U	680	U	43,000	U
	04	20		230		45	
	05	< 1.8		45		170	
03	01	230		45		9,400	
	02	>160,000		680		43,000	
	03	20	U	230	U	45	U
	04	< 1.8		45		170	
	05	< 1.8		< 1.8		45	
04	01	>160,000		68		43,000	
	02	20		230		45	
	03	< 1.8	S	45	S	170	U
	04	< 1.8		< 1.8		45	
	05	20		< 1.8		170	
05	01	20		230		45	
	02	< 1.8		45		170	
	03	< 1.8	S	< 1.8	U	45	U
	04	20		< 1.8		170	
	05	< 1.8		130		45	
06	01	< 1.8		45		170	
	02	< 1.8		< 1.8		45	
	03	20	S	< 1.8	S	170	U
	04	< 1.8		130		45	
	05	45		20		230	
07	01	< 1.8		< 1.8		45	
	02	20		< 1.8		170	
	03	< 1.8	S	130	S	45	U
	04	45		20		230	
	05	45		20		< 1.8	
08	01	20		< 1.8		170	
	02	< 1.8		130		45	
	03	45	U	20	U	230	U
	04	45		20		< 1.8	
	05	790		110		68	
09	01	< 1.8		130		45	
	02	45		20		230	
	03	45	S	20	U	< 1.8	S
	04	790		110		68	
	05	20		< 1.8		18	
10	01	45		20		230	
	02	45		20		< 1.8	
	03	790	S	110	S	68	S
	04	20		< 1.8		18	
	05	45		20		20	

CL = classification, S = suitable, U = unsuitable.

The results indicate that during some weeks the beaches were not suitable for bathing. This condition can be due to intense rainfall at the beginning of the

sampling, and also to the sewage discharged into culverts and berths which might carry fecal bacteria into the sea (Vieira *et al*., 2007). In the 8th week, 80% of the samples collected in Beach 1 were within the allowed concentration of enterococci. Nevertheless, the location was classified as unsuitable for bathing because, according to CONAMA´s Resolution, the value obtained in the last sample must not be higher than 400 enterococci/100 mL (Table I). The water in Beach 3 has been reported as unsuitable for primary contact recreation. The Maceió Creek´s water is an open sewage with foul smell and its direct drainage into the beach might be responsible for the observed water contamination (Vieira *et al*., 2003).

Of the 110 probable *Enterococcus* strains, 52 (63.4%) were identified as *E. faecalis* and 30 (36.6%) as *E. faecium* (Figure 1), which amounts to 82 isolates. In Beach 1, a total of 31 isolates (37.8%) were found, followed by Beach 2 with 26 isolates (31.7%) and by Beach 3 with 25 isolates (30.5%). The independent analysis of the beaches showed that the presence of *E. faecalis* was 32.7%, 34.6% and 32.7% in Beaches 1, 2 and 3, respectively. For *Enterococcus faecium,* the presence of isolates in the same locations was 46.7%, 26.7% and 26.7%, respectively.

Despite the high number of species known today, 85-95% of the enterococci infections are caused by *E. faecalis* and 5-10% by *E. faecium* (Bender *et al*., 2009). The predominance of *E. faecalis* isolates when compared to *E. faecium* is in agreement with the findings of Bonilla *et al*. (2006), according to which *E. faecium,* commonly found in the gastrointestinal tract and human feces, might be easily inactivated by environmental factors such as temperature or culture medium. These microorganisms have been found to be the main cause of infections in humans and present natural resistance to several antimicrobials (Tavares, 2000).

Beach 3 was the location with the highest MPN/100 mL and therefore a higher number of isolates was expected (Figure 1). The high concentration of thermo tolerant coliforms in the water (Evangelista-Barreto *et al*., 2007) may have influenced the enterococci MPN results. The presence of these bacteria at Beaches 1 and 2 can be due to their longer survival time when compared to *E. coli* and to thermotolerant coliforms (Dufour, 1994). Another aspect that should be taken into account is the fact that samples were always collected at the same time of the day, independently of the tide. At high tide, the sea water washes the sand and might thus have contributed to enhance the isolation of microorganisms.

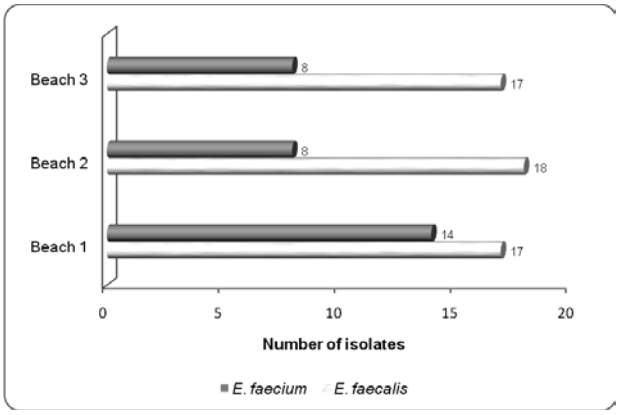

Figure 1 - Number of *Enterococcus* isolates from water samples collected at Beaches 1, 2 and 3, Fortaleza, Ceará, Brazil.

In relation to the susceptibility profile to antimicrobials, 52 strains were tested: 19 (36.5%) for *E. faecium* and 36 (63.5%) for *E. faecalis*. Ninety-eight percent of the selected strains were resistant, although all strains presented sensibility to ampicillin and imipinem. High resistance percentage was observed for nalidixic acid (94.3%), tetracycline (26.4%), amicacin (26.0%), erythromycin (17.0%), gentamicin (9.4%), penicillin (3.8%) and cefalotin (01.9%) (Table 2). The strains showed intermediate sensibility to all the antibiotics tested, excepting the betalactamic group (penicillin, ampicillin and imipinem) and were therefore considered resistant (Table II).

Assessing the susceptibility profile of the genus *Enterococcus* is of importance because these microorganisms have been increasingly involved in opportunistic infections and multiresistant lineages have been isolated (Poeta *et al*., 2005). The most relevant resistance phenotypes are those related to aminoglycosides (streptomycin and gentamicin), betalactamics (amoxicillin and ampicillin) and glycopeptides (teicoplanin and vancomycin) (D'Azevedo *et al*., 2004).

Only one strain showed susceptibility to all antibiotics tested. One *E. faecium* strain presented intermediate resistance to vancomycin. This result is of concern considering that vancomycin is one of the few, if not the only, antibiotic effective for the treatment of *E. faecium* infections (Poeta *et al*., 2005). The high ampicillin susceptibility observed in our research has also been reported by other authors (*e.g*., Bender *et al*., 2009).

Most of the isolates were resistant to nalidixic acid (Table II). The resistance mechanism to quinolones is cromossomic and results from clone diffusion from mutagenic samples (Lima *et al*., 2006) that reach the environment through sewage and

Table II - Antimicrobial susceptibility profile according to disc diffusion method among 52 enterococcal isolates in three beaches in the city of Fortaleza, Brazil.

Antimicrobial agents	*E. faecalis* (33)			*E. faecium* (19)		
	Susceptible N (%)	Intermediate N (%)	Resistant N (%)	Susceptible N (%)	Intermediate N (%)	Resistant N (%)
Aminoglycosides						
- Gentamicin	32 (88.9)	01 (02.8)	3 (08.3)	13 (76.5)	02 (11.8)	02 (11.8)
- Amikacin	19 (52.8)	07 (19.4)	10 (27.8)	09 (52.9)	04 (23.5)	04 (23.5)
Betalactamics						
- Penicillin	34 (94.4)	0	2 (05.5)	17 (100)	0	0
- Ampicillin	36 (100)	0	0	17 (100)	0	0
- Cefhalotin	33 (91.7)	03 (08.3)	0	16 (94.1)	0	01 (05.9)
- Ceftriaxon	22 (61.1)	14 (38.9)	0	11 (64.7)	06 (35.3)	0
- Imipenem	36 (100)	0	0	17 (100)	0	0
Tetraciclina						
- Tetracycline	24 (66.7)	01 (02.8)	11 (30.5)	13 (76.5)	01 (05.9)	03 (17.6)
Macrolide						
- Erythromycin	17 (47.2)	13 (36.1)	6 (16.7)	07 (41.2)	07 (41.2)	03 (17.6)
Glycopeptides						
- Vancomycin	36 (100)	0	0	16 (94.1)	01 (05.9)	0
Quinolone						
- Nalidixic acid	01 (02.8)	0	35 (97.2)	01 (05.9)	01 (05.9)	15 (88.2)

N = number of isolates

through animal and human feces. However, Cabello et al. (2006) reported plasmid mediated antimicrobial resistance to quinolones between bacteria from aquatic and terrestrial environments. The observed resistance to macrolides and tetracyclin (Table II) is of importance, for these are alternative antibiotics to treat *Enterococcu*s infections, which make them unsuitable for empirical antimicrobial therapy (Bender *et al*., 2009). According to Oliveira & Pinhata (2008), high transference of plasmid resistance mainly to tetracycline has been reported in bacteria isolated from marine sediments.

The high susceptibility of the isolates to aminoglycosides is a genus-specific property. These antimicrobials show a sinergic effect when associated to betalactamics and glycopeptides. Notwithstanding, in the last years enterococci lineages with high resistance to aminoglicosides have been reported. That was also the case in the present study, where high resistance to amicacin was observed (Table II). The resistance results in the loss of synergism with betalactamics and glycopeptides and the consequent loss of bactericidal effect (Saraiva *et al*., 1997).

Considering that water bodies contain a great diversity of bacteria species, the study of autochthonous bacteria antimicrobial resistance in terms of the maintenance and transference of resistance genes to other bacteria, including pathogenic ones, improves our understanding of the environmental dissemination of resistant microorganisms (Miranda & Zemelman, 2002). Domestic sewage draining into the sea or recreational areas contributes to establishing routes for microorganisms carrying resistance genes.

Multiple antibiotic resistance (MAR) values are shown in Table III. From the 52 strains tested, 46 presented values equal to or higher than 0.18, which indicates resistance to one or two antibiotics (Table III). Multiple resistance profile was observed in 94.7% of the strains isolated from Beach 2, followed by Beaches 1 with (88.8%) and 3 (80%). Isolates with MAR values of 0.54 were found in Beaches 1 and 2.

Table III - Number of isolates with multiple antibiotic resistance (MAR) in three beaches of Fortaleza, Brazil.

MAR	Beach 1	Beach 2	Beach 3	Total
0.18	2	6	6	14
0.27	6	7	3	16
0.36	5	1	2	8
0.45	2	2	1	5
0.54	1	2	0	3
Total	16	18	15	46

Lineages with MAR varying from 0.18 (resistance to two antibiotics) to 0.54 (resistance to six antibiotics) in all but Beach 3 which presented a maximum MAR of 0.45 (resistance to five antibiotics) (Table III). The presence of multiresistant *E. faecalis* and *E. faecium* in the aquatic environment has ecological and public health implications that justify further studies, mainly with respect

to resistance determinants in different species (Miranda & Zemelman, 2002). Isolates with multiple resistance had MAR values of 0.27 (resistance to three antibiotics) and were predominant in Beach 2. Most of the strains had MAR values of 0.27 (16 strains) and 0.18 (14 strains) (Table III). Multiple resistance to various antibiotics due to acquired resistance has been observed in *E. faecium* which is presently considered a serious nosocomial pathogen worldwide (Iversen *et al*., 2002).

In terms of the susceptibility profile, the similarity coefficient for the nine antimicrobials tested revealed that *E. faecalis* and *E. faecium* lineages formed three major groups. The highest similarity values (about 60%) were obtained in groups A and B, and the lowest in group C (Figure 2). The response to the antimicrobials was not influenced by the isolated species of *E. faecium* and *E. faecalis* or by the location where the strains were isolated. Other factors such as untreated sewage discharged into marine environments through rivers, soil or water modifies the microbial environment causing changes that are still not well understood. The horizontal transference of resistance plasmids among or within species is another crucial factor for the appearance of populations of resistant bacteria in the aquatic environment.

CONCLUSION

The low bathing suitability of the beaches under study due to the presence of fecal bacteria may have deleterious bearing on human health. Besides being a potential source of pathogenic agents, the contaminated water also contributes to the spread of multiresistant lineages and to the increase of antimicrobial resistance in autochthonous microorganisms.

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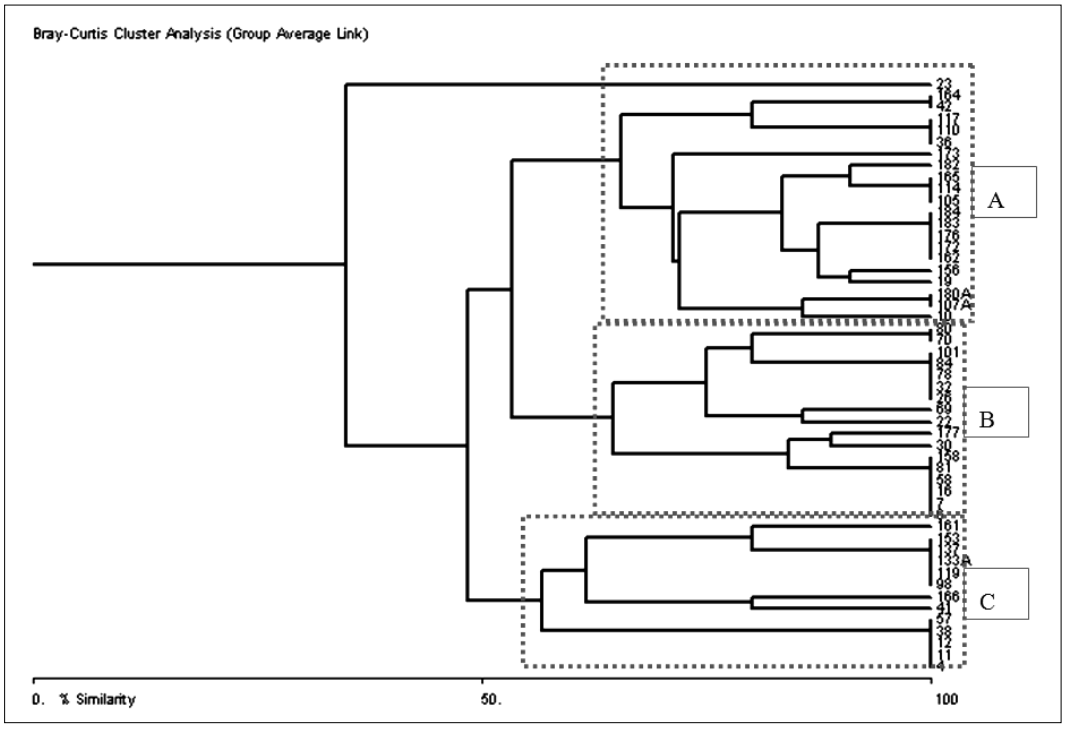

Figure 2 - Dendrogram based on the susceptibility profiles to antimicrobials of *E. faecalis* and *E. faecium* lineages isolated from water samples collected at three different beaches along the waterfront of the city of Fortaleza, Brazil.

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