

Research Paper

Enterobacteria identification and detection of diarrheagenic *Escherichia coli* in a Port Complex

Clarissa Frota Macatrão Costa¹, Valério Monteiro Neto², Bruno Rafael de Carvalho Santos³, Bruno Rafael Rabelo Costa¹, Alexandre Azevedo⁴, Josilene Lima Serra⁵, Hermínio Benítez Rabello Mendes⁶, Adenilde Ribeiro Nascimento⁷, Mariana Bonfim Pinto Mendes³, Oliver Kuppinger⁸

¹Biodiversidade e Conservação, Universidade Federal do Maranhão, São Luís, MA, Brazil.

²Departamento de Patologia, Universidade Federal do Maranhão, São Luís, MA, Brazil.

³Laboratório de Geologia Sedimentar, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil.

⁴Ciências Biológicas, Universidade Federal do Maranhão, MA, Brazil.

⁵Instituto Federal do Maranhão, MA, Brazil

⁶Programa Bionorte, Universidade Federal do Amazonas, Manaus, Brazil.

⁷Departamento de Tecnologia Química, Universidade Federal do Maranhão, São Luís, Brazil.

⁸Laboratório de Genética e Biologia Molecular, Departamento de Biologia, Universidade Federal do Maranhão, São Luís, Brasil

Submitted: April 21, 2013; Approved: March 14, 2014.

Abstract

The Port Complex of Maranhão (PCM) is the second largest port complex in Brazil, receiving ships with large volumes of ballast water. To evaluate the microbiological quality of its waters, physicochemical parameters (pH and salinity), the number of coliforms (thermotolerants and totals), and the presence of enterobacterias and diarrheagenic *Escherichia coli* strains were analyzed. In order to identify the presence of *E. coli* virulence genes target regions of the *stx*, *elt*, *est*, *aggR*, CVD432, *ipaH* and *eae* nucleotide sequences were studied. The presence of totals and thermotolerants coliforms were positive. Analyzing the salinity parameter, a significant increase in total coliforms was observed during the rainy season. We identified the species *Escherichia coli*, *Proteus mirabilis*, *Citrobacter freundii*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Klebsiella ozaenae*, *Morganella morganii*, *Enterobacter cloacae* and *Edwardsiella tarda*. Out of the 51 *E. coli* isolated, two were positive for the *elt* gene and one was positive for the CVD432 sequence, features of enterotoxigenic and enteroaggregative strains, respectively. This study reveals that the PCM is contaminated by enterobacteria and diarrheagenic *E. coli* thus providing evidence regarding the risk of these bacteria being carried by ships to other countries, and draws attention to the input of fecal bacteria brought by ships in the port waters of Maranhão.

Key words: microbiological analysis, coliforms, enterobacterias, ballast water, *Escherichia coli*.

Introduction

Ballast water discharge in coastal waters is a great global concern. Ships can act as vehicles for the transportation of pathogenic and toxic species, which occurs primarily when there is sewage discharge near the port or coastal area where water is collected.

To address this issue, the National Health Surveillance Agency (Agência Nacional de Vigilância Sanitária - ANVISA) completed an exploratory study for the identification and characterization of pathogenic agents in ballast water (ANVISA, 2003). This study examined nine Brazilian ports, including the Port of Itaqui, which is part of the

Port Complex of Maranhão, the second largest port complex in Brazil.

To conduct microbiological analyses of water quality, indicator microorganisms, such as the total and thermotolerant coliforms, *E. coli*, *Enterococci* spp., and coliphages, are assayed (Bonilla *et al.*, 2007). Total coliform concentration is often used to measure water quality for recreational purposes and is correlated with the incidence of diseases among bathers (Bordalo, 2003; Noble *et al.*, 2003). Thermotolerant coliforms point to the level of faecal contamination and, therefore, suggest the existence of pathogenic bacteria (Bitton, 2005).

These pathogenic microorganisms include the enterobacteria, which are responsible for 70% of the urinary infections and 50% of the septicaemias that affect humans (ANVISA, 2004). The most common species, *E. coli*, inhabits the gastrointestinal tract of humans and other endothermic animals, and although most of its strains are commensal, a portion of these strains are pathogenic, causing intestinal and extra-intestinal diseases, such as diarrhea and urinary infections (Picard *et al.*, 1999; Albertini, 2009). Previous studies have focused mainly on strains from clinical samples, but notably little is known about the presence of diarrheagenic *E. coli* in marine environments or, more specifically, in port waters, such as Albertini (2009).

The identification of diarrheagenic *E. coli* using biochemical and serological tests is unreliable (Fialho, 2008). Therefore, several PCR tests have been developed to amplify the target regions present in virulence genes and identify strains of enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), and enterohaemorrhagic *E. coli* (EHEC) (Toma *et al.*, 2003; Vidal *et al.*, 2004).

In their study, ANVISA detected all of the microbiological indicators analysed, and although the vessel masters have declared open water exchange of ballast waters, as suggested by the International Maritime Organization (IMO), no exchange was observed in 86% of the ships (ANVISA, 2003).

In light of this problem, the aim of the present study was to evaluate the microbiological quality of the waters of

the Port Complex of Maranhão by quantifying the total and thermotolerant coliforms and identifying the enterobacteria and diarrheagenic *E. coli*. Furthermore, this study intended to determine the effects of salinity and seasonality on the number of total coliforms.

Materials and Methods

Area location and sampling

The Port Complex of Maranhão (PCM) is situated in the Bay of São Marcos, west of São Luís Island, Maranhão, Brazil. We selected three sampling points: Point 1 was near Alumar Port (02°40'42" S/44°21'18" W), Point 2 was close to the Vale and Itaqui Ports (02°33'9" S/44°22'9" W), and Point 3 was in front of Guia Beach (02°31'S/44°20'W). As a negative control, samples were collected in the Bay of São José (Caúra (P1-CN), Tapari (P2-CN), and Ponta Verde (P3-CN) beaches), east of São Luís Island, at a site opposite the port complex (Figure 1). The samples were collected in sterile flasks, 30 cm from the surface during high tide, at a distance of 100 to 200 m from the coast, between September 2008 and December 2009. We selected four months from the rainy season (February to July) and four months from the dry season (August to January) (Table 2). The samples were analyzed on the same day they were collected.

Salinity and pH measurement

To measure the salinity and pH, we used a portable brix refractometer (*model 211, Mingda Co. Ltd.*) and a Quimis pH meter (*model Q 400A*).

Determination of total and thermotolerant coliforms

The multiple tube fermentation method was used according to the methodology described in APHA (2005) beginning with 250-mL flasks and using lactose broth for the presumptive test and brilliant green and EC (*E. coli*) broth for the confirmation tests. The most probable number (MPN) of total and thermotolerant coliforms was calculated using the Hoskins table (APHA, 2005).



Figure 1 - Location of the study area.

Cultivation and bacterial isolation

Coliforms

Aliquots of the positive tubes of brilliant green broth were collected and streaked onto MacConkey (MC) agar. Colonies with different morphotypes were collected and transferred into tubes containing tryptic soy agar (TSA) and incubated at 37 °C for 24 to 48 h for subsequent biochemical identification.

Salmonella

In parallel with coliforms analysis, for each sample point, a second cultivation method, indicated for *Salmonella* research, was performed according to the methods described in APHA (2005), with modifications. The samples were collected in 1000 mL bottles and aseptically filtered through filter papers, which were then placed in vials with 225 mL of buffered peptone water and incubated at 37 °C for 24 h. One millilitre of each sample was aseptically transferred into a tube containing nine mL of tetrathionate broth and incubated at 37 °C for 24 h. The culture was plated on Hektoen selective media. Next, morphologically different colonies were isolated in TSA for subsequent biochemical identification.

Biochemical identification

For biochemical identification, oxidase-negative bacteria were selected, and the colonies were subjected to 20 biochemical tests using the Bactray I and II Systems according to the manufacturer's instructions (Laborclin). These results were analysed using the Bactray Software to identify the strains at a specific level.

Identification of diarrheagenic *E. coli*

For the PCR, the *E. coli* strains were subcultured on nutrient agar plates and inoculated into 1 mL of sterile water. Multiplex PCR was performed as described by Toma *et al.* (2003). *Hot-start* monoplex PCR was performed employing primers that amplified specific sequences of nucleotides in each virulence gene as illustrated in Table 1. The PCR reactions, at a final volume of 50 µL each, contained 1x PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 25 pmol of each primer and 2.5 µL of suspension from each strain. The reaction mixture (45 µL), covered with mineral oil was heated to 95 °C before adding the Taq-Polymerase (2.0 U in 5 µL of water with 1x PCR buffer). The thermocycling conditions included 30 cycles at 95 °C for 40 s, 40 s at the annealing temperature of each specific primer (Table 1), 72 °C for 40 s for the extension, with an initial denaturation at 95 °C for five minutes and a final extension at 72 °C for ten minutes.

The 1381-7 (*ipaH*), EDL 933 (*stx*), 042 (*aggR* and CVD432), 0691-3 (*elt*), 0361-1 (*est*) and E2348-6 (*eae*) reference strains, and the C-8 and C-15 (EAEC), and STEC (*stx*) strains were used as positive controls of *E. coli* virulence genes. They were provided by the Laboratory of Parasitic Biology of UNICEUMA and were sub cultured, grown and suspended in the same way as the strains of the study. For negative controls, sterile water was used. The PCR products were separated in a 2.0% agarose gel.

Statistical analysis

The relationship between the salinity and the number of total coliforms of port water samples was analysed using a simple linear regression. Using the paired t-test, the mean MPN values of the coliforms collected during the dry sea-

Table 1 - Description of the primers, markers used, amplicon sizes and annealing temperature.

Designation	Sequence (5'-3')	Gene	Size of the amplicon (bp)	Reference	Annealing temperature
SK1	CCCGAATTCGGCACAAGCATAAGC	<i>eae</i>	881	Toma <i>et al.</i> 2003	65 °C
SK2	CCCGGATCCGTCTCGCCAGTATTCG				
VTcom-u	GAGCGAAATAATTTATATGTG	<i>Stx</i>	518	Toma <i>et al.</i> 2003	50 °C
VTcom-d	TGATGATGGCAATTCAGTAT				
AL65	TTAATAGCACCCGGTACAAGCAGG	<i>Est</i>	147	Toma <i>et al.</i> 2003	56 °C
AL 125	CCTGACTCTTCAAAAAGAGAAAATTAC				
LTL	TCTCTATGTGCATACGGAGC	<i>Elt</i>	322	Toma <i>et al.</i> 2003	54 °C
LTR	CCATACTGATTGCCGCAAT				
IpaIII	GTTCCCTTGACCGCCTTTCCGATACCGTC	<i>ipaH</i>	619	Toma <i>et al.</i> 2003	65 °C
IpaIV	GCCGGTCAGCCACCCTCTGAGAGTAC				
aggRkas1	GTATACACAAAAGAAGGAAGC	<i>aggR</i>	254	Toma <i>et al.</i> 2003	52 °C
aggRkas2	ACAGAATCGTCAGCATCAGC				
Eaggfp	AGACTCTGGCGAAAGACTGTATC	CVD432	194	Toma <i>et al.</i> 2003	62 °C
Eaggbf	ATGGCTGTCTGTAATAGATGAGAAC				

son and those collected during the rainy season were compared. The statistical software used was Statistica 7.0.

Results

Microbiological quality

The levels of thermotolerant coliforms in the water samples from the Port Complex of Maranhão were within the quality standards recommended by CONAMA Resolution 274/2000 (CONAMA, 2001), with the exception of one sample (P1, June 2009), which showed values above the limit established by the Brazilian legislation (1000 MNP/100 mL) for bathing waters. The mean value of the total coliforms was approximately four times higher during the rainy season than during the dry season, with mean values of 1287 and 370 MNP/100 mL, respectively (Table 2). This difference was statistically significant ($p < 0.05$), as illustrated in Figure 2, which also reveals a greater variation in the number of total coliforms during the rainy season.

Samples from the Bay of São José (negative control) revealed no thermotolerant coliforms. In addition, no total coliforms were detected in Caúra (P1-CN), although total coliform values of 100 and 300 MNP/100 mL were observed in Tapari (P2-CN) and Ponta Negra (P3-CN), respectively.

Salinity and pH

The salinity of the Port Complex samples ranged from 14 to 35, with more than 70% of the samples with salinity less than or equal to 30; these values characterise the port waters as brackish (CONAMA, 2005). Regression analysis (Figure 3) revealed a moderately negative correlation ($r = -0.63$) between the total coliforms and the salinity. In contrast, the samples from the Bay of São José had the highest values of salinity, at 35, characterizing the water as seawater.

The pH analysis showed variations between 6.76 and 8.21 for the port waters, which are within the range recommended by CONAMA Resolution 357/2005 for brackish

waters (CONAMA, 2005). The pH analysis of the water samples from the Bay of São José showed values of 8.21.

Characterization of enterobacteria

Among the colonies of bacteria that were isolated, 105 colonies were identified, of which 98 were from the Port Complex and 7 were from the Bay of São José. The most frequently encountered species in the Port Complex of Maranhão was *E. coli*, which represented 51/98 (52.04%) of the isolates and was present in all of the samples collected, followed by *P. mirabilis*, with 16/98 (16.32%) of the isolates. Other enterobacteria identified included *C. freundii*, *P. vulgaris*, *K. pneumoniae*, *K. ozaenae*, *M. morgani*, *E. cloacae*, and *E. tarda*, representing 12, 6, 5, 4, 2, 1, and 1 of the 98 isolates (12.24%, 6.12%, 5.10%, 4.08%, 2.04%, 1.02%, and 1.02%), respectively. The species *E. coli*, *P. mirabilis*, *C. freundii*, *P. vulgaris*, *K. pneumoniae*, and *K. ozaenae* were found at the 3 sampling points (Table 3). Bacteria from the genus *Salmonella* were not detected.

In the samples from the Bay of São José, *K. pneumoniae*, *E. cloacae*, and *P. mirabilis* were identified.

Identification of diarrheagenic *E. coli*

The multiplex PCR of the identification of the virulence genes was efficient in the amplification of target sequences as well as nonspecific fragments. However, performing PCR monoplex with the same primers, but modifying the thermocycling conditions (Table 1, Figure 4) exclusively amplified the specific fragments of the virulence genes.

PCR for the identification of the virulence genes

Out of the 98 colonies isolated from the samples of the port waters, 51 were specimens of *E. coli*. Three *E. coli* strains, representing 5.88% of the strains, tested positive for virulence genes. The target DNA region of the heat-labile toxin gene (*elt*), characteristic of enterotoxigenic strains, was amplified in two strains (3.92%). The CVD432 fragment was amplified in one other strain (representing 1.96% of col-

Table 2 - Most probable number of total and fecal coliforms per sample (NMP/100 mL) of port water samples.

Collections	P 1 (ALUMAR Port)		P 2 (Vale and Itaqui Ports)		P 3 (Guia Beach)	
	Totals	Thermotolerants	Totals	Thermotolerants	Totals	Thermotolerants
September-08	230	230	1500	930	230	36
October-08	460	-	91	-	23	-
April-09	460	93	1100	210	75	43
May-09	1100	24	2400	290	2400	240
June-09	2400	1100	2400	44	2400	44
July-09	240	23	240	150	240	240
October-09	93	93	460	460	43	15
December-09	23	23	1100	210	290	35

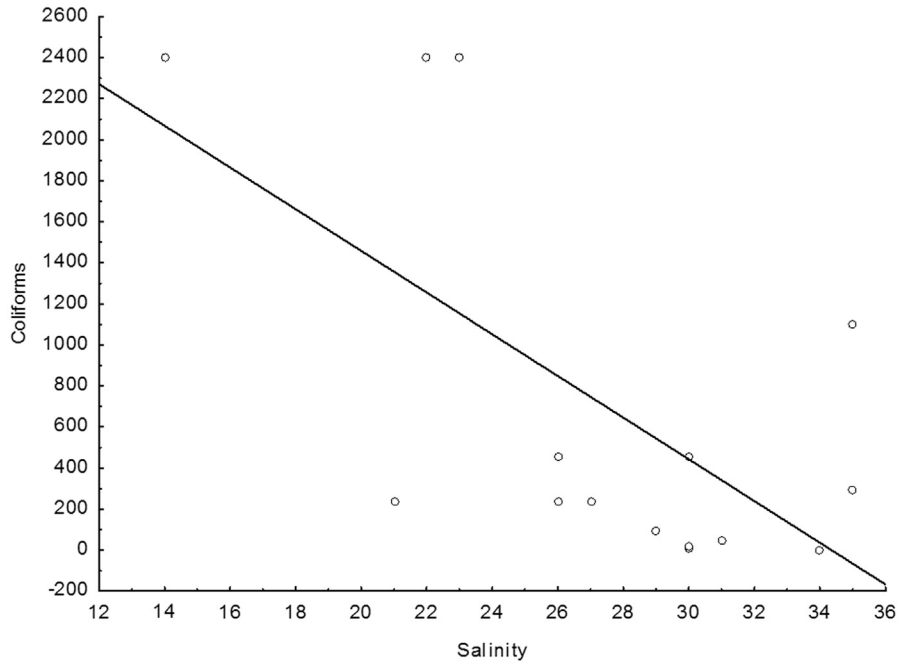


Figure 2 - Mean, standard deviation and standard error of the mean values of coliforms in dry and wet seasons ($t = 2.523, p = 0.02, = 11 \text{ Gl}$).

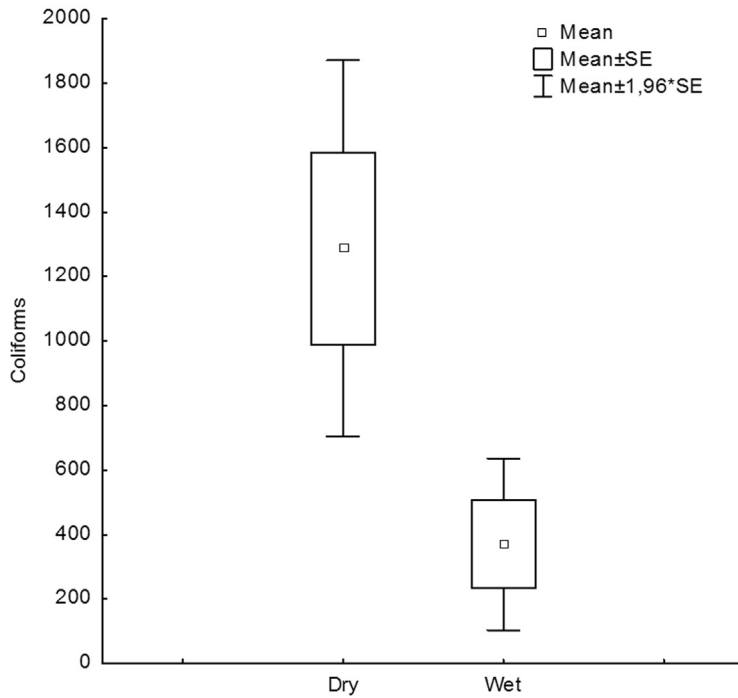


Figure 3 - Regression between total coliform samples from the Port Complex and salinity (Salinity:Coliforms: $r^2 = 0.4033; r = -0.6350; p = 0.0110; y = 3490.439 - 101.5888 * x$).

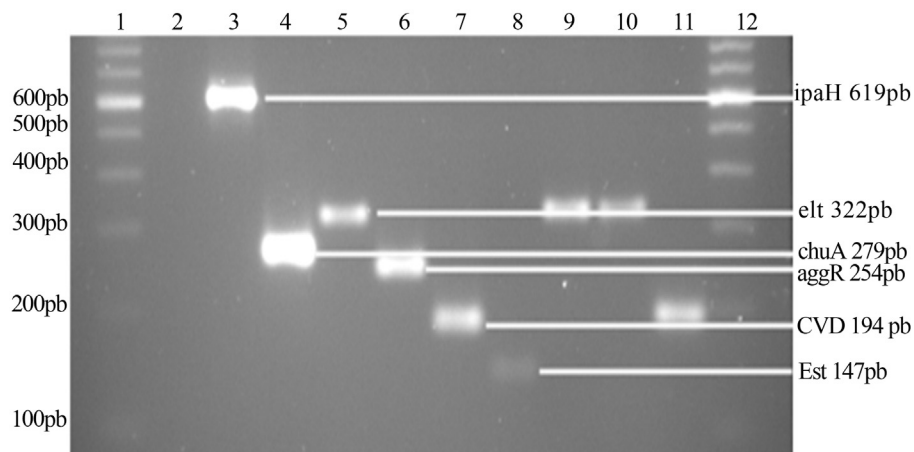
onies), characterized as an EAEC strain. None of the *E. coli* strains amplified the *est, eae, ipaH, aggR* and *stx* target DNA regions present in the enterotoxigenic strains, which produces heat-stable protein (ETEC), in the enteropathogenic (EPEC), enteroinvasive (EIEC), enteroaggregative (EAEC) and enterohaemorrhagic (EHEC) strains.

Discussion

This study is one of the first on the analysis of enterobacterias and pathogenic strains in port waters. The number of supply ships in the Port Complex of Maranhão have grown significantly in recent years. The subsequent

Table 3 - Enterobacteria species identified in samples.

Collections	P 1 (ALUMAR Port)	P 2 (Vale and Itaquí Ports)	P 3 (Guia Beach)
September 08	<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i>
	<i>C. freundii</i>	<i>K. ozaenae</i>	<i>C. freundii</i>
		<i>P. mirabilis</i>	<i>K. ozaenae</i>
			<i>P. mirabilis</i>
October 08	<i>P. mirabilis</i>	<i>E. coli</i>	<i>E. coli</i>
	<i>C. freundii</i>		
November 08	<i>E. coli</i>	<i>C. freundii</i>	-
	<i>E. tarda</i>	<i>P. mirabilis</i>	
April 09	<i>P. mirabilis</i>	<i>P. vulgaris</i>	<i>E. coli</i>
May 09	<i>E. coli</i>	<i>P. vulgaris</i>	<i>E. coli</i>
	<i>P. mirabilis</i>	<i>K. pneumoniae</i>	<i>E. cloacae</i>
	<i>P. vulgaris</i>		
June 09	<i>P. mirabilis</i>	<i>E. coli</i>	
		<i>M. morgani</i>	<i>E. coli</i>
		<i>P. mirabilis</i>	
		<i>K. pneumoniae</i>	
July 09	<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i>
	<i>P. mirabilis</i>	<i>M. morgani</i>	
October 09	<i>E. coli</i>	<i>P. mirabilis</i>	<i>E. coli</i>
	<i>K. ozaenae</i>	<i>K. pneumoniae</i>	<i>K. pneumoniae</i>
	<i>P. mirabilis</i>		
December 09	<i>E. coli</i>	<i>E. coli</i>	
	<i>K. pneumoniae</i>	<i>C. freundii</i>	<i>E. coli</i>

**Figure 4** - DNA fragments amplified by PCR for the ipaH, elt, aggR, CVD 432 and est virulence genes. Ladder (1). Positive controls: ipaH (3), elt (5), aggR (6), CVD 432 (7), est (8). Positive strains of this study: 2-9c (7) and 8-9c (8), positives for the elt gene; 5-6c (9) strain, positive for CVD 432. Ladder (10).

increase in ballast water dumped by ships increases the plausibility that pathogenic enterobacterias and *E. coli* strains have thereby been dumped in the port water and/or are captured and transported by ships to other countries.

The levels of coliforms were within the limits recommended by CONAMA Resolution 274/2000 in 98% of the samples (CONAMA, 2001); however, these values confirmed the biological contamination in the Maranhense

Port Canal that had already been observed in a previous study (ANVISA, 2003).

Although these water samples exhibited levels of coliforms that were below the legal limit, the risks of the microbiological contamination observed in the port canal should not be downplayed, as the sediment and the animals present there can concentrate microorganisms at much higher levels than those found in the water samples, thereby acting as a reservoir for coliforms (La-Liberte and Grimes, 1982).

In that context, studies have indicated that much higher concentrations of coliforms are present in sand (Vieira *et al.*, 2003; Bonilla *et al.*, 2007) and oysters (Fernandez-Delgado *et al.*, 2007) in comparison to the seawater at the same beach. This increased concentration increases the risk of epidemic outbreaks that would greatly compromise the local economy and tourism.

Remarkably, a sample with levels of thermotolerant coliforms above the limit was collected in June 2009 following floods that affected the state of Maranhão. It is noteworthy that this sample was collected in Alumar Port (P 1) near the Cachorros River, where enterotoxigenic *E. coli* were also identified.

The fact that levels of thermotolerant coliforms exceeded the limits established by the legislation were not found in the Vale and Itaqui Ports (P 2) and at Guia Beach (P 3) in June 2009 may be related to the less isolated nature of these locations and the fact that they are more subject to tidal action.

There was also a significant increase in the number of total coliforms in samples collected during the rainy season, possibly because the higher volume of water leads to an increase in the number of nutrients and a decrease in the salinity, favouring the survival of enterobacteria. The superficial drainage of rainwater has also been reported as a main factor responsible for deteriorating the microbiological quality of the water due to the washing off of human and animal excrements, particularly where there is no sewage treatment (Amaral *et al.*, 2003). Other studies have also shown an increase of bacteria in the water during the rainy season (Vieira *et al.*, 2003; Da Silva *et al.*, 2008). However, an improvement in the microbiological quality of the water was observed in relation to an increase in the salinity, similar to results that have been observed at urban beaches in Portugal (Bordalo, 2003).

The increased level of coliforms during the rainy season (February to July) suggests that the contamination of the waters of the Port Complex of Maranhão may originate mainly in sewers and storm drains that are discharged into the rivers that empty nearby.

Although seawater affects the survival of enterobacteria, the existence of latent forms of these pathogens, which remain viable and potentially virulent for extended periods in seawater (Grimes *et al.*, 1986), has been reported previously.

The ability of *E. coli* to become dormant in salt water (Xu *et al.*, 1982) facilitates their survival in the ballast tanks of ships. Since the transfer of genes between organisms is known to occur in natural environments (Bitton, 2005), the possibility exists that the virulence genes of diarrheagenic present in the port waters may be transferred to other bacteria.

The presence of enterobacterias and *E. coli* strains with virulence genes revealed the existence of pathogenic potential in water samples from the PCM. This fact suggests further studies in the rivers of the western region of São Luis Isle and in the ballast water of docked ships are needed in order to better understand the origin of this contamination. The microbiological analysis of edible marine organisms, such as oysters, in the coastal areas near the port complex is perhaps the best monitoring tool to prevent possible outbreaks of gastroenteritis caused by the ingestion of shellfish contaminated with pathogenic *E. coli*. The presence of *E. coli* with virulence genes in the PCM makes it possible that these bacteria are carried to other countries via the ballast water of ships, transferring virulence genes to the microbiota of their ports and, thereby, may cause epidemiological outbreaks in these places.

References

- Amaral LAA, Nader Filho A, Rossi Junior OD, Ferreira, FLA, Barros, LSS (2003) Água de consumo humano como fator de risco à saúde em propriedades rurais. *Rev Saúde Pública* 37(4):510-4.
- Albertini LS (2009) Ecology, virulence-associated factors and diversity of *Escherichia coli* isolated from samples of ballast water, water from port regions and bivalve shellfishes in Brazil. PhD Thesis, Universidade de São Paulo, São Paulo.
- Agência Nacional de Vigilância Sanitária (2004) Detecção e identificação de bactérias de importância médicas. Available at: http://www.anvisa.gov.br/servicos/auda/microbiologia/mod_5_2004.pdf.
- Agência Nacional de Vigilância Sanitária (2003) Brasil - Água de lastro, http://www.anvisa.gov.br/divulga/public/paf/agua_lastro3.pdf
- American Public Health Association (2005) Standard methods for examination of water and wastewater, American Public Health Association, Washington, 21th ed.
- Bitton G (2005) Microbial indicators of fecal contamination. In: Wiley-Liss (ed) *Wastewater Microbiology*. 3rd ed. Florida, pp 153-172.
- Bonilla TD, Nowosielski K, Cuvelier M, Hartz A, Green M, Esiobu N, McCorquodale DS, Fleisher JM, Rogerson A (2007) Prevalence and distribution of fecal indicator organisms in South Florida beach sand and preliminary assessment of health effects associated with beach sand exposure. *Mar Pollut Bull* 54:1472-1482.
- Bordalo AA (2003) Microbiological water quality in urban coastal beaches: the influence of water dynamics and optimization of the sampling strategy. *Water Res* 37:3233-3241.

- Conama - Conselho Nacional de Meio Ambiente (Brasil) Resolucao nº 274, de 29 de novembro de 2000. Dispõe sobre a qualidade das águas de balneabilidade e alerta o disposto na Resolução 020, de 18 de junho de 1986. Diário Oficial da República Federativa do Brasil, Brasília, 8 jan. 2001.
- Conama - Conselho Nacional de Meio Ambiente (Brasil) Resolucao nº 357, de 17 de março de 2005. Dispõe sobre a classificação de corpos de água e diretrizes ambientais para o seu enquadramento, bem como estabelece as condições e padrões de lançamento de efluentes, e dá outras providências. Diário oficial da República Federativa do Brasil, Brasília, 18 mar. 2005.
- Fernandez-Delgado M, Contreras M, García-Amado A, Gueneau P, Suárez P (2007) Occurrence of *P. mirabilis* associated with two species of venezuelan oysters. *Rev Inst Med Trop São Paulo* 49(6):355-359.
- Fialho OB (2008) Identificação de estirpes de *E. coli* diarréogênicas por PCR- multiplex, Curitiba, M.Sc. Dissertation, Universidade Federal do Paraná, 106 p.
- Grimes DJ, Atwell RW, Brayton PR, Palmer LM, Rollins DM, Roszak DB, Singleton FL, Tamplin ML, Colwell RR (1986) The fate of enteric pathogenic bacteria in estuarine and marine environments. *Microbiol Sci* 3:324-329.
- La-Liberte P, Grimes DJ (1982). Survival of *E. coli* in lake bottom sediment. *Appl Environ Microbiol* 43:623-628.
- Noble RT, Moore DF, Leecaster MK, McGee CD, Weisberg SB (2003) Comparison of total coliform, fecal coliform, and enterococcus bacterial indicator response for ocean recreational water quality testing. *Water Res* 37:1637-1643.
- Picard B, Garcia JS, Gouriou S, Duriez P, Brahim N, Bingen E, Elion J, Denamur E (1999) The link between phylogeny and virulence in *E. coli* extraintestinal infection. *Infect Immun* 67:546-553.
- Da Silva VC, Nascimento AR, Mourão APC, Coimbra Neto SV, Costa FN (2008) Contaminação por *Enterococcus* da água das praias do município de São Luís, Estado do Maranhão. *Acta Scient Tech* 30(2):187-192.
- Toma C, Lu, Y, Higa N, Nakasone N, Chinem I, Baschkier A, Rivas M, Iwanaga M 2003. Multiplex PCR assay for identification of human diarrheagenic *E. coli*. *J Clin Microbiol*, 41:2669-2671.
- Vieira RHSF, Do Nascimento SCO, De Menezes FGR, Do Nascimento SMM, De Lucena LHL (2003) Influência das águas das galerias pluviais como fator de poluição costeira, Fortaleza, Ceará. *Arquivos de Ciências do Mar* 36:123-127.
- Vidal R, Vidal M, Lagos R, Levine M, Prado V (2004) Multiplex PCR for diagnosis of enteric infections associated with diarrheagenic *E. coli*. *J Clin Microbiol* 42:1787-1789.
- Xu, HS, Roberts N, Singleton FL, Atwell RW, Grimes DL, Colwell RR (1982) Survival and Viability of Nonculturable *E. coli* and *Vibrio Cholerae* in the Estuarine and Marine Environment. *Microb Ecol* 8:313-323.