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Research Paper

Microbiological Quality and Prevalence of β-Lactam Antibiotic Resistance Genes in Oysters (*Crassostrea rhizophorae*)

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ABSTRACT

The microbiological quality of oysters reflects the microbiological quality of their habitats because they are filter feeders. The objective of this study was to assess the bacterial composition of the edible oyster *Crassostrea rhizophorae* in urban and preserved estuaries. Particularly, we assessed the presence of pathogenic bacteria, investigated antibiotic susceptibility in bacterial isolates, and quantified β-lactam antibiotic resistance genes (bla_{TEM}, bla_{SHV}, and bla_{KPC}) via quantitative PCR of oyster DNA. Our results detected total coliforms, *Escherichia coli,* and enterobacteria in the oysters from urban estuaries, which is indicative of poor water quality. In addition, our detection of the *eaeA* and *stxA2* virulence genes in 16.7% of *E. coli* isolates from oysters from this region suggests the presence of multiantibiotic-resistant enteropathogenic and enterohemorrhagic *E. coli* strains. During periods of low precipitation, increased contamination by *E. coli* (in winter) and *Vibrio parahaemolyticus* (in autumn) was observed. In contrast, cultivated oysters inhabiting monitored farms in preserved areas had low levels of bacterial contamination, emphasizing that oyster culture monitoring enhances food quality and makes oysters fit for human consumption. Distinct antibiotic resistance profiles were observed in bacteria isolated from oysters collected from different areas, including resistance to β-lactam antibiotics. The presence of the bla_{TEM} gene in 91.3% of oyster samples indicated that microorganisms in estuarine water conferred the capability to produce β-lactamase. To our knowledge, this is the first study to directly quantify and detect β-lactam antibiotic resistance genes in oysters. We believe our study provides baseline data for bacterial dynamics in estuarine oysters; such knowledge contributes to developing risk assessments to determine the associated hazards and consequences of consuming oysters from aquatic environments containing pathogenic bacteria that may possess antibiotic resistance genes.

Key words: bla_{TEM}; Microbiological quality control; Oyster culture; Quantitative PCR

Environmental pollution caused by waste that contains antibiotics and pathogenic microorganisms promotes the selection of resistant bacteria in aquatic environments, leading to the dissemination of resistance mechanisms in the local bacterial community *(14, 18)*. Oysters act as bioindicators for environmental contamination because they can filter 19 to 50 liters of water per hour with low selective capacity. They can accumulate pathogenic microorganisms and antibiotic resistance genes, thus becoming a public health hazard when consumed raw *(30)*.

Studies have reported that the presence of antibiotic resistance genes in different environments is indicative of contamination *(12, 13, 17, 20, 31)*. Among antibiotic resistance genes are those that encode the β-lactamase enzyme. This enzyme promotes the hydrolysis of the β-lactam ring, which mechanistically contributes to the action of β-lactam antibiotics. Over 450 β-lactamases have been described in the literature *(3, 6),* and extended-spectrum β-lactamases, encoded primarily by the bla_{TEM} and bla_{SHV} genes, are of considerable importance. Another important group is the carbapenemases, which are encoded by the bla_{KPC} gene *(16)*. These two groups of β-lactamases are medically relevant because they are capable of hydrolyzing several classes of β-lactam antibiotics. Particularly, extended-spectrum β-lactamases degrade third- and fourth-generation cephalosporins and monobactams, and carbapenemases can not only degrade these β-lactam antibiotic classes but also carbapenems *(10)*.

Because of the social and economic value of oysters, it is essential to control their microbiological quality to ensure safe human consumption. Thus, microbiological rapid tests are an important tool for assessing microbiological quality and rapidly identifying and quantifying microorganisms. In this study, we evaluated the microbiological quality, detection of *Escherichia coli* virulence genes, and quantification of β-lactam antibiotic resistance genes (bla_{TEM}, bla_{SHV}, and bla_{KPC}) in *Crassostrea rhizophorae* oysters

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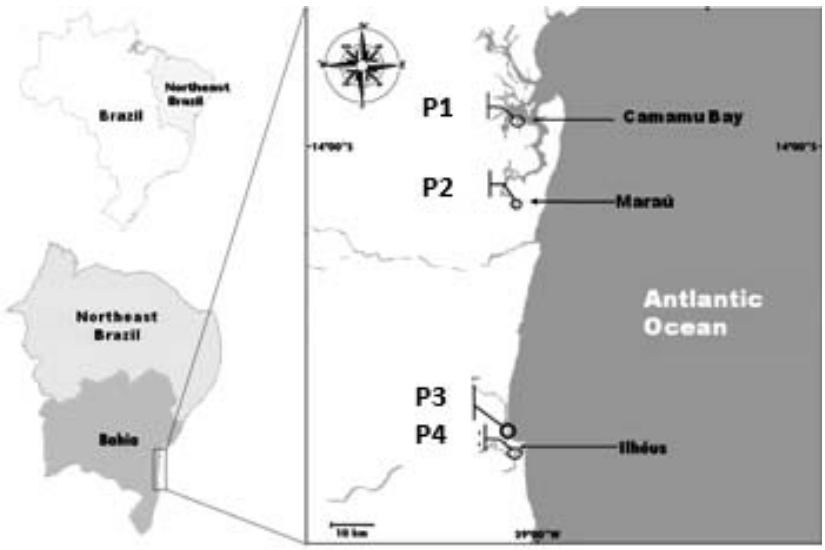

FIGURE 1. *Area map of the study indicating the points of oyster collections. Camamu Bay (P1) and Maraú River estuary (P2) areas were long-line oyster farms, and the locales in Ilhéus (P3 and P4) were urban estuaries.*

Rainfall (mm)

PLACE	Jun-Jul 2013	Aug-Sep 2013	Oct-Nov 2013	Dec 2013 Jan 2014	Feb-Mar 2014	Apr-May 2014
Camamu [P1]	189 (±9,97)	207 (±24,3)	298 (±258,8)	156 (±75,0)	232 (±22,8)	69 (±17,5)
Maraú [P2]	16 (±7,1)	82 (±25,5)	284 (±175,4)	130 (±89,1)	50 (±9,9)	37 (±23,3)
Ilhéus [P3; P4]	26 (±3,5)	16 (±19,8)	107 (±148,5)	42 (±27,6)	50 (±17,0)	30 (±9,0)

destined for human consumption. Specifically, we assessed oysters from long-line farms that were monitored for quality control and from urban estuarine environments lacking such oversight. This is the first study that directly quantified β-lactam antibiotic resistance genes in oysters.

MATERIALS AND METHODS

Study areas. This study was carried out in four locations on the southern coast of Bahia in northeastern Brazil. Locations P1 (13°40′02″S, 39°09′06″W) and P2 (13°40′02″S, 39°09′06″W) are located in the Camamu Bay in the cities of Camamu and Maraú; P3 (14°50′57″S, 39°03′14″W) and P4 (14°49′63″S, 39°03′49″W) are located in the Cachoeira River estuary in the city of Ilhéus (Fig. 1). Specimens from P1 and P2 came from long-line oyster farming systems that were considered to be preserved environments. Specimens from P3 and P4 came from natural habitats in urban areas, the banks of the estuary, and were manually removed from red mangrove roots (*Rhizophorae mangle*). Precipitation data for the study areas were obtained from the Executive Board of the Cocoa Crop Plan for P1 (personal communication) and from the National Institute of Meteorology *(22)* for P2, P3, and P4 (Fig. 1).

Collection and processing of the samples. Oysters in lots of 40 were collected at each site every 2 months for 1 year, for a total of 23 samples and 920 oysters (240 oysters each for sites P1, P3, and P4; and 200 oysters for P2 because logistics made it impossible to collect a sample in December). All samples were placed in hermetically sealed, insulated boxes and taken to the State University of Santa Cruz Veterinary Hospital's Microbiology Laboratory for immediate processing.

With the aid of a sterile abrasive brush, the oysters were rinsed with running water to remove residue from the valves. The specimens were then placed on paper towels and allowed to completely dry. All oysters were opened with the aid of a sterile knife, and the organic material was collected in a sterile beaker and homogenized using an NT136 tissue homogenizer (Novatécnica, Piracicaba, SP, Brazil). One hundred grams of the homogenate was used for downstream analyses. In addition, 0.1 g was used for the extraction of total DNA from the oysters using the Easy-DNA Kit (Invitrogen Life Technologies, Carlsbad, CA), following the manufacturer's instructions. The absorbance at 260 and 280 nm was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and the extracted DNA was stored at –20°C until it was used for the quantification of antibiotic resistance genes.

Microbiological analyses. We carried out the microbiological analyses using rapid identification kits (Compact Dry, HyServe GmbH & Co. KG, Uffing, Germany) to quantify the following pathogens: *Enterobacteriaceae* (Compact Dry ETB), *Salmonella* spp. (Compact Dry SL), *Staphylococcus aureus* (Compact Dry XSA), *Vibrio parahaemolyticus* and *Vibrio vulnificus* (Compact Dry VP), and total coliforms and *E. coli* (Compact Dry EC).

We added 100 g of homogenized oysters to 100 ml of peptone water (Acumedia, Neogen do Brasil, Indaiatuba, SP, Brazil) and performed serial dilutions from 10^{-1} to 10^{-5}. Then, we poured 1 ml of each dilution onto Compact Dry plates and incubated the plates at 37°C for 18 to 24 h. For the *Salmonella* spp. analysis, we initially preincubated the 1:1 (wt/vol) diluted samples at 37°C for 18 to 24 h before pouring them onto a Compact Dry SL plate. All

TABLE 1. *Primers used in PCR and qPCR assays*[a]

Target gene (GenBank accession no.)	Reference strain	Primers (5′–3′)	Amplicon (bp)	Reference
A. Reference strains and primers used in PCR to confirm pathogens in oysters				
Salmonella-specific fragment (AE006468.1)	*Salmonella* Enteritidis PT4 (IOC)	F (ST15): GGTAGAAATTCCCAGCGGGTACTG R (ST11): AGCCAACCATTGCTAAATTGGCGCA	429	*1*
phoA (FJ546461)	*E. coli* INCQS 0033	F: GGTAACGTTTCTACCGCAGAGTTG R: CAGGGTTGGTACACTGTCATTACG	468	*35*
nuc (NC_002758.2)	*S. aureus* ATCC 6538	F: GCGATTGATGGTGATACGGTT R: CAAGCCTTGACGAACTAAAGC	276	*8*
tlh (AB012596.1)	*V. parahaemolyticus* INCQS 00081 (ATCC 17802)	F: AAAGCGGATTATGCAGAAGCACTG R: GCTACTTTCTAGCATTTTCTCTGC	450	*4*
*vvhA*e B (AB124802.1)	*V. vulnificus* INCQS 00630 (ATCC 27562)	F: CTCACTGGGGCAGTGGCT R: CCAGCCGTTAACCGAACCA	383	*38*
B. Primers used in qPCR assay to quantify β-lactam resistance genes				
bla_{TEM}		F: TTCCTGTTTTTGCTCACCCAG R: CTCAAGGATCTTACCGCTGTTG	112	*5*
bla_{SHV}		F: CCATTACCATGAGCGATAACA R: ACCACAATGCGCTCTGC	402	*2*
bla_{KPC}		F: TCTGGACCGCTGGGAGCTGG R: TGCCCGTTGACGCCCAATCC	399	*12*

[a] F, forward primer; R, reverse primer.

analyses were performed in duplicate and following the manufacturer's instructions.

After incubation, we selected three to five colonies of each pathogen and inoculated them separately onto 800 μl of an enrichment medium specific for each pathogen; the plates were then incubated at 37°C for 18 to 24 h. To specify, the *V. vulnificus* samples were inoculated onto nutrient broth (Acumedia) containing 0.5% NaCl, and the *V. parahaemolyticus* samples were inoculated onto nutrient broth containing 3% NaCl;. the *S. aureus* sample strains were cultured onto brain heart infusion media (Acumedia); and the *Salmonella* spp. and *E. coli* samples were cultured onto tryptic soy broth (Acumedia). After incubation, we stored the isolated bacteria at −20°C in the pathogen-specific medium containing 20% glycerol, with the exception of *V. vulnificus*, which was stored at room temperature (between 23 to 25°C).

We confirmed the species of the isolates via PCR and later used them for antibiogram analyses to discern antibiotic sensitivity.

PCR analyses for pathogen species confirmation and virulence gene detection. The positive results we obtained from the Compact Dry method were confirmed using PCR. The primers used to amplify each microorganism are listed in Table 1. Each 25-μl PCR reaction contained 1× PCR buffer (Invitrogen), 1.25 mM $MgCl_2$ (Invitrogen), 0.2 mM deoxynucleotide triphosphate (dNTP) mix (Invitrogen), 10 pmol of each primer (Invitrogen), 1.25 U of *Taq* DNA polymerase (Invitrogen), and one suspected colony of each pathogen. The reaction volume was filled to 25 μl with sterile nuclease-free water. As positive controls, reference strains were used (Table 1). As negative controls, some colonies were omitted from the PCR reaction. The PCR amplification cycles consisted of 5 min at 94°C for initial denaturation; then 35 cycles for 30 s at 94°C, for 30 s at 58°C, and for 1 min at 72°C; and a final extension step for 10 min at 72°C. The PCR products were electrophoresed in a 1% agarose gel stained with SYBR Green (Invitrogen) and examined under UV light. The *E. coli* isolates were sent to the Oswaldo Cruz Institute (Rio de Janeiro, RJ, Brazil) for characterization of the *eagg*, *eaeA*, *stx1*, *stx2*, *ial*, STa, STb, and LT virulence genes.

Antibiotic susceptibility tests. We analyzed each pathogen's susceptibility to several antibiotics using the disk diffusion method in Mueller-Hinton agar (Acumedia) plus the following antibiotics (all purchased from Laborclin, Pinhais (PR), Brazil), as per the recommendations of the Clinical and Laboratory Standards Institute *(15)*: amikacin (30 μg), gentamicin (10 μg), imipenem (10 μg), cefuroxime (30 μg), cefoxitin (30 μg), cefepime (5 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), trimethorprim-sulfamethoxazole (1.25 and 23.75 μg), ampicillin-sulbactan (10 and 10 μg), amoxicillin-clavulanate (20 and 10 μg), ampicillin (10 μg), nalidixic acid (30 μg), doxycycline (30 μg), and tetracycline (5 μg). As a control for the quality and reliability of the results obtained, we evaluated the *E. coli* strain ATCC 25922 under the same culture and incubation conditions in accordance with the Clinical and Laboratory Standards Institute guidelines. We measured the inhibition halo of each antibiotic disc with the aid of a digital caliper and compared the measurement with reference values determined by the Clinical and Laboratory Standards Institute to determine whether microorganisms were sensitive, resistant, or intermediate in response to the tested antibiotics.

Absolute quantification of antibiotic resistance genes in oysters. We performed quantitative PCR (qPCR) to quantify the bla_{TEM}, bla_{SHV}, and bla_{KPC} genes in the total DNA extracted from oysters. First, we generated standard qPCR curves from clones containing each specific gene in a plasmid vector. The clones were produced using the TA Cloning Kit (Invitrogen) in accordance with the manufacturer's instructions. The vector used was pCR 2.1, and the competent cell used was *E. coli* TOP10.

TABLE 2. *Microbiological analysis of the oyster* C. rhizophorae *obtained from the southern coast of Bahia, Brazil* [a]

Microorganism	Collection period						Mean/location
	June–July 2013	Aug.–Sep. 2013	Oct.–Nov. 2013	Dec. 2013–Jan. 2014	Feb.–Mar. 2014	Apr.–May 2014	
Total coliforms							
P1	3.40	3.06	3.90	3.71	3.71	3.54	3.55
P2	2.89	4.20	2.78	NC	2.30	2.40	2.91
P3	3.24	3.04	2.88	3.27	2.35	2.42	2.87
P4	6.59[b]	4.66	3.55	4.31	3.80	4.38	4.55
Mean per period	4.03	3.74	3.28	3.76	3.04	3.19	
E. coli							
P1	1.42	2.27	0.00	0.00	0.00	2.12	0.97
P2	0.00	1.22	0.00	NC	0.00	0.00	0.24
P3	3.24	2.86[c]	2.65	0.00	0.00	2.24	1.83
P4	5.59[b]	3.16	2.40[d]	2.34	2.74	4.38	3.44
Mean per period	2.56	2.38	1.26	0.78	0.69	2.19	
Enterobacteriaceae							
P1	3.40	3.06	3.90	3.71	3.71	3.54	3.55
P2	3.26	4.24	3.53	NC	4.06	3.28	3.67
P3	5.01	4.53	4.59	4.59	3.59	4.59	4.48
P4	6.62[b]	4.59	3.40	4.21	3.76	3.66	4.37
Mean per period	4.57	4.11	3.86	4.17	3.78	3.77	
V. parahaemolyticus							
P1	0.00	0.00	0.00	0.00	0.00	3.57	0.60
P2	0.00	0.00	0.00	NC	0.00	0.00	0.00
P3	0.00	0.00	0.00	0.00	0.00	2.21	0.37
P4	0.00	0.00	0.00	0.00	0.00	3.03	0.51
Mean per period	0.00	0.00	0.00	0.00	0.00	2.90	

[a] $n = 920$. Mean of log of experimental duplicates in three dilutions (10^{-1}, 10^{-2}, and 10^{-3}). All samples were negative for *Salmonella* spp., *S. aureus*, and *V. vulnificus*. NC, not collected.
[b] Significant difference ($P < 0.0001$) as determined by the Tukey's test between locations and sampling period.
[c] Presence of *eaeA* virulence gene.
[d] Presence of *stxA2* virulence gene.

We carried out an initial 100-μl PCR reaction with the plasmid DNA extracted from each clone. The reaction contained 1× PCR buffer, 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, a 0.8 μM solution of each pair of primers (Table 1), 2 U of *Taq* DNA polymerase, and 100 ng of DNA. We performed the PCR reactions under the following conditions: initial denaturation for 5 min at 95°C; then 35 cycles for 60 s at 95°C, for 60 s at the annealing temperature corresponding to each pair of primers used (bla_{TEM}, 56.1°C; bla_{SHV}, 60°C; and bla_{KPC}, 60°C), and for 45 s at 72°C; and a final extension for 10 min at 72°C. The PCR product was purified using the PureLink PCR Purification Kit (Invitrogen), subjected to electrophoresis in 1% agarose gel, and quantified using a NanoDrop 2000 spectrophotometer. We calculated the number of copies per microliter using the following formula: Number of copies per μl = [DNA concentration (in ng) × (6.022×10^{23})]/[Fragment size × (1×10^9) × 650]. We then serially diluted the purified PCR product in ultra-pure sterile water and used the dilutions corresponding to 10^6 to 10^1 gene copies per μl of DNA for the standard qPCR curves.

We set up 20-μl qPCR reactions using the SYBR Green PCR Master Mix Kit (Invitrogen). Each reaction contained 100 ng of DNA, 5 μM solutions of the forward and reverse primers, and 1× SYBR Green PCR Master Mix. We used ultra-pure sterile water as a negative control. We performed qPCR amplification in the Applied Biosystems 7500 Fast Real-Time PCR Systems platform (Life Technologies, Foster City, CA) under the following conditions: for 10 min at 95°C, followed by 40 cycles for 15 s at 95°C and for 1 min at 60°C. The assays were done in triplicate and compared with the standard curves.

Statistical analysis. We calculated the average number of bacteria and the standard deviation, and performed a one-way analysis of variance (ANOVA) with a Tukey's test to determine the statistical significance of the variation in the locales and collection times of the study. We considered $P \leq 0.05$ to be statistically significant. The data were analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA).

RESULTS

Microbiological analyses and virulence genes detection. All the samples were negative for *Salmonella* spp., *S. aureus*, and *V. vulnificus* (Table 2). The samples that showed the greatest microbial contamination came from the urban areas, P3 and P4, and were collected from June to July. We observed a significant difference ($P < 0.0001$) regarding total coliforms of *E. coli* and *Enterobacteriaceae* between P4 and the other locations and periods (Table 2). A total of 26 *E. coli* isolates were recovered (P1, 7 isolates; P2,

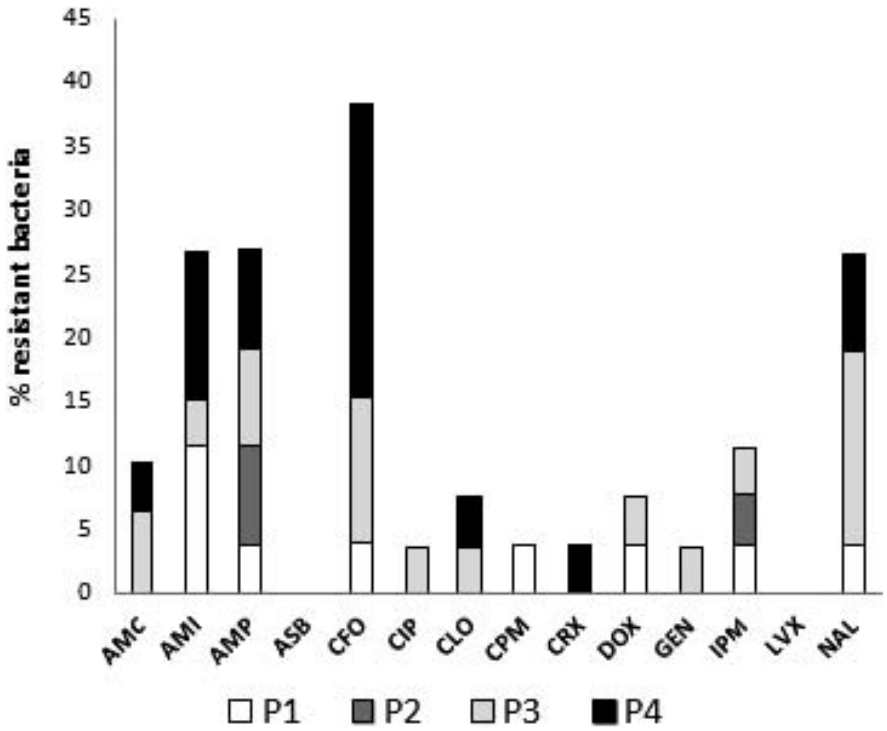

FIGURE 2. *Antibiotic resistance profile in* E. coli *isolated from oysters. Cultivated (P1 and P2) and native oysters were collected (P3 and P4), and* E. coli *isolates were assessed for their sensitivity to 14 antibiotics, according to CLSI (15). AMC, amoxicillin-clavulanate; AMI, amikacin; AMP, ampicillin; ASB, ampicillin-sulbactan; CFO, cefoxitin; CIP, ciprofloxacin; CLO, chloramphenicol; CPM, cefepime; CRX, cefuroxime; DOX, doxycycline; GEN, gentamicin; IPM, imipenem; LVX. levofloxacin; NAL, nalidixic acid.*

1 isolate; P3, 9 isolates; and P4, 9 isolates), and they were tested for the presence of virulence genes. Of these 26 isolates, *eaeA* was detected in three *E. coli* strains from the same oyster sample, collected at P4 in August, and *stxA2* was detected in one *E. coli* strain from the sample collected at P4 in October (Table 2). Thus, a prevalence of 16.7% was found for both genes at location P4. We observed the presence of *V. parahaemolyticus* only at P1, P3, and P4 for the months of April and May, with an average of 2.9 log CFU/g and showing no significant differences among the locations (Table 2).

Antibiotic susceptibility profiles. The antibiotics to which the isolates showed the greatest resistance were amikacin in isolates from P1 (11.6% of the isolates), ampicillin in isolates from P2 (7.6% of the isolates), nalidixic acid in isolates from P3 (15.4% of the isolates), and cefuroxime and cefoxitin in isolates from P4 (26.9 and 23.4% of the isolates, respectively). Levofloxacin was the antibiotic to which 96.2% of the *E. coli* isolates showed sensitivity (Fig. 2). Six samples of *V. parahaemolyticus* were isolated from P1, P3, and P4, and all were resistant to ampicillin and sensitive to the other antibiotics.

Quantification of the bla_{TEM}, bla_{SHV}, and bla_{KPC} resistance genes in oysters. All the oyster samples were negative for the bla_{SHV} and bla_{KPC} genes. The bla_{TEM} gene was present in all four locations studied (and in 21 of the 23 oyster samples tested, a prevalence of 91.3%), with an average of 3.81 log copies per g of oysters (±3.61 log copies per g), during all periods of the study, except for the negative results obtained in P2 for August to September 2013 and for February to March 2014. An ANOVA revealed significant differences among the averages ($P = 0.0468$); however, we observed no significant differences using the Tukey's test when we compared the locations. Despite this, the samples from urban areas showed a higher average for the presence of the bla_{TEM} gene than those from the monitored culture areas (Fig. 3C).

DISCUSSION

Brazilian legislation, through the National Program for Sanitary Control of Bivalves Molluscs *(27),* sets the acceptable limit of *E. coli* at <230 most probable number (MPN) per 100 g (<2.36 log) for consumption in natura, and at between 230 and 4,600 MPN/100 g (3.36 to 3.66 log) for consumption after depuration. Nevertheless, enumerating only the level of *E. coli* is not enough to ascertain the microbiological quality of oysters. This is evidenced in our work. Between April and May 2014, *V. parahaemolyticus* was detected in P1 and P3, but the level of *E. coli* was below the acceptable limit for consumption in natura. Although we did not use the MPN methodology to quantify the *E. coli* (as recommended by legislation), the Compact Dry EC correlates well ($R^2 = 0.93$) with MPN methodology and is thus a valid test *(25)*.

In the preserved areas, where there is monitored oyster farming, the level of microbial contamination remained low and stable during our study period (Table 2) and also during the previous period, according to research by Silva Neta et al. *(36)*. This finding may be associated with lower population density, less deforestation, and the absence of dam building. In addition, it is likely that the higher precipitation observed in the preserved areas throughout the study period contributed to the lower level of contamination (Fig. 1).

In contrast, oysters from the urbanized areas presented a higher level of contamination. In particular, during June to July 2013 the number of total coliforms and *Enterobacte-*

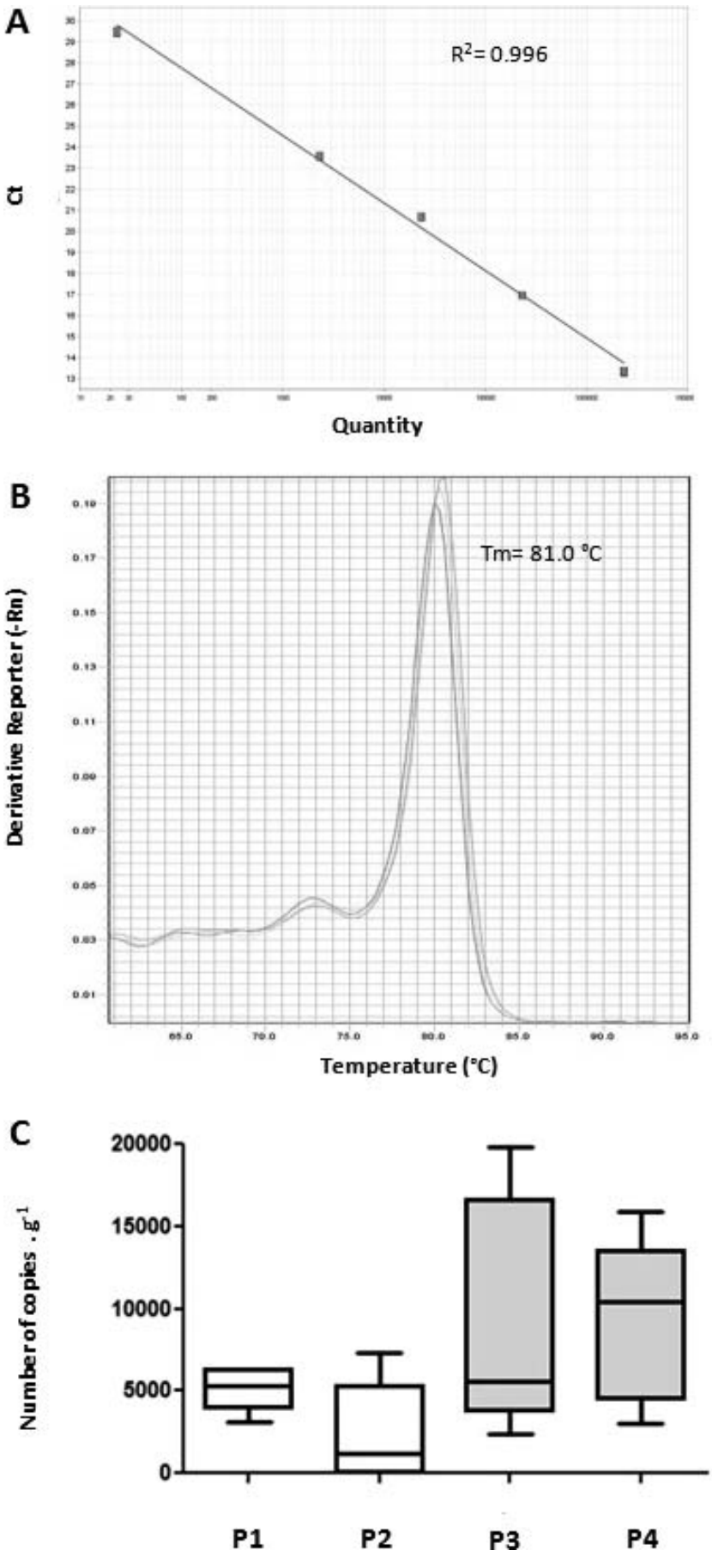

FIGURE 3. *qPCR analysis of the* bla$_{TEM}$ *gene in oysters. (A) Standard curve showing dilutions* 10^6 *to* 10^1 *copies of the* bla$_{TEM}$ *gene per μl* ($R^2 = 0.996$). *(B) Melting curve* ($T_m = 81°C$). *(C) Number of copies of* bla$_{TEM}$ *gene per gram of oyster found in each studied region. Gray bars represent oysters from urban areas; white bars represent oysters from preserved areas.*

riaceae surpassed 10^6 CFU/g, and the level of *E. coli* was over 10^5 CFU/g (Table 2). A high amount of *E. coli* is an indicator of the presence of other disease-spreading agents in the water. Urbanized riverside communities are especially at risk via extractive fishing, including oyster extractions, in these ecosystems, in which microbial contamination is rampant *(34, 36)* due to anthropogenic actions, such as discharges of sewage and industrial waste. Controlled experiments *(9)* have demonstrated that fecal pollution in the water has a dynamic relationship with shellfish-related illnesses because of the ability of shellfish to accumulate and retain enteric pathogens. The accumulation of bacteria by shellfish takes place particularly in the digestive glands, and shellfish organisms proceed to reach microbial equilibrium with the environment based on the bacterial content of the water *(11)*.

In our study, the *eaeA* and *stx2* virulence genes were detected in *E. coli* strains isolated from P4 oysters. This means that oysters from this locale could transmit enteropathogenic and enterohemorrhagic *E. coli,* constituting a serious public health concern. The *eaeA* gene, absent in nonpathogenic *E. coli* strains, is present in all enteropathogenic and enterohemorrhagic *E. coli* strains. This gene is responsible for encoding the intimin protein, which interacts with the Tir receptor present on the surface of intestinal cells, adhering and forming lesions. These injuries then result in the malabsorption of nutrients and fluids *(24)*. The *stx2* gene is present in 97% of enterohemorrhagic *E. coli,* with or without simultaneous presence of *stx1*. Together, these genes are responsible for encoding Shiga toxins. The *stx1* gene encodes toxins that are 99% identical to the Shiga toxin produced by *Shigella,* and the *stx2* gene is 55% identical to *stx1 (21)*. The simultaneous presence of the *eaeA* and *stx2* genes indicates the presence of enterohemorrhagic *E. coli,* which can result in several syndromes, including hemorrhagic colitis and hemolytic uremic syndrome.

According to our study and Silva Neta et al. *(36),* periods of low precipitation, such as winter (from June to August) and autumn (from April to May), are critical for the presence of *E. coli* and *V. parahaemolyticus,* respectively. These seasons have precipitation levels of 23 to 28 mm; in contrast, from November to March, the average precipitation is more than 66 mm, according to the National Institute of Meteorology *(22)*. In particular, Silva Neta et al. *(36)* demonstrated that rainfall is the critical abiotic factor that affects bacterial contamination in oysters in this region because the water temperature and salinity remain constant. As the rainfall decreases, pathogen contamination increases, and as rainfall increases, the retention of microorganisms by oysters decreases. Regarding *V. parahaemolyticus,* current data differ on the prevalence of this pathogen in relation to the period of the year because it has been found both during the coldest period in marine sediments *(33)* and during the summer *(28)*.

Based on our results, during winter and autumn periodical sampling could be done and a simulation and prediction model *(7)* of *E. coli* fluxes could be applied to oyster cultivation areas to understand and predict the impact of the bacteria on water and shellfish quality. From this, a risk period could be determined, which would contribute greatly to shellfish management and consumer protection *(32)*. In addition, during the risk period a depuration process should be implemented to minimize the risk of bacterial contamination.

The antibiogram results demonstrated that most of the *E. coli* strains were sensitive to the antibiotics we tested. Nevertheless, different susceptibility profiles were found in each region of the study (Fig. 2). In particular, we observed resistance to amikacin in P1, to ampicillin in P2, to nalidixic acid in P3, and to cefoxitin and cefuroxime in P4. We detected multiantibiotic resistance in all strains that carried the virulence genes. The *stx2*-positive strains were resistant to ampicillin, amikacin, and cefoxitin, and the *eaeA*-positive strains were resistant to ampicillin, chloramphenicol, cefoxitin, cefuroxime, doxycycline, and nalidixic acid. The presence of these genes in multiresistant strains presents a reasonable public health concern, especially in environments conducive to waterborne and foodborne transmission, such as oyster culture and extraction areas.

The detection of the bla_{TEM} gene in the total DNA samples extracted from oysters (Fig. 3) may explain the resistance to ampicillin, cefoxitin, and cefuroxime. Several extended-spectrum β-lactamases are encoded in plasmids that carry the bla_{TEM} gene *(29),* increasing its environmental dissemination. The high prevalence of the bla_{TEM} gene (91.3%) in this study, including in the preserved areas that did not have any contact with hospital waste, warrants more attention and further investigation because this gene may be present in the environment at a stable level. Marine and freshwater environments are natural reservoirs of resistant bacteria because these environments facilitate horizontal gene transfer through conjugation between bacteria present in biofilms and through lysogenic bacteriophages *(14)*. Studies (R.P.R., unpublished data) have found the bla_{TEM} gene in mangrove and cave sediments in Bahia, Brazil, suggesting that the bacteria harboring this gene are adapted to these environments. The search for resistance genes is not part of the official methods for quality control in food. Nevertheless, this search is as important as the search for microorganisms relevant to public health because horizontal gene transfer may also occur through nonpathogenic bacteria, thereby disseminating resistance genes to different species. Research on depuration processes should be conducted to evaluate the capability of diminishing the microbial carriers of the bla_{TEM} gene.

For amikacin, enzymatic modification is the most common mechanism of aminoglycoside resistance. The genes responsible for this resistance are usually found on plasmids or transposons that also increase its dissemination. This kind of mechanism may lead to a high level of resistance to these antibiotic agents. As for quinolone resistance, we believe that it was mediated by chromosomal genes only, occurring in a gradual and cumulative manner. However, plasmid genes may also confer low levels of resistance to nalidixic acid and ciprofloxacin, although less frequently *(23, 26)*.

Regarding our results on the susceptibility of *V. parahaemolyticus* to antibiotics, 100% of the isolates from both the preserved and urban areas were resistant to ampicillin. The dispersion of antibiotic-resistant microor-

ganisms in aquatic ecosystems has been a grave public health concern because hydric transmission has a significant epidemiological impact *(37)*.

According to the Food and Agriculture Organization of the United Nations *(19),* Brazil is 11th in the world regarding the inland water fish industry, producing 235,527 tons in 2014. In addition, Brazil is 14th in the production of farmed species, with 562,500 tons of total aquaculture production, almost 20% of all fish production in northeastern Brazil. The goal of the Brazilian government is to increase fish production, specifically to produce 2 million tons by 2020. However, this goal may be significantly affected by pollution, environmental degradation, and overfishing *(19)*. Regarding mollusks, Brazil produces 22,100 tons *(19),* and 90% of this production is concentrated in Santa Catarina State in southern Brazil, all of which is destined for domestic trade. Currently, the large-scale production of bivalve mollusks (mainly oysters) in northeastern Brazil is an economically viable option to increase national production, and this must be encouraged by governmental actions. The oyster cultivation systems contribute to environmental and family sustainability, tend to reduce the extraction of oysters from natural banks, and provide quality products because oysters are typically consumed raw in Brazil. Moreover, to access international trade, a systematic quality control program must be employed, which is possible only via monitored cultivation. To achieve successful quality management systems for oyster cultivation, data must be collected periodically in different geographical regions and in different seasons to provide baseline data that can be used to inform and develop risk assessment models for foodborne pathogens in oysters.

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