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GENES OF VIRULENCE AND ANTIMICROBIAL RESISTANCE IN *Vibrio parahaemolyticus* PREVALENT IN AREAS OF OSTREICULTURE

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ABSTRACT

This study aimed to quantify *Vibrio* spp. and evaluate the profile of antimicrobial susceptibility and virulence factors in *Vibrio parahaemolyticus* strains in the samples of oysters collected from two estuaries of the Baixo Sul, Bahia. The samples were collected between June 2015 and January 2016 from natural banks (E1) (oyster) and from cultivation areas (E2) (water and oyster). For the quantification of *Vibrio* spp., the most probable number (MPN) was determined and the characterization of *V. parahaemolyticus* marker (species-specific) gene *tl* was performed. Pathogenicity was observed with the Kanagawa test, and the presence of the *tdh*, *trh* and *ure* genes were tested. The antimicrobial sensitivity test included disc diffusion method with 15 antimicrobial discs, β-lactamase enzyme production, and the presence of *bla*TEM, *bla*SHV, and *bla*CTX-M. The maximum density of *Vibrio* spp. in the samples from cultivation was 4.70 log MPN g^{-1} and extractivism was 6.10 log MPN g^{-1}, with the temperature being the most influencing variable on the presence of microorganisms in the cultivation area (E2). The *tl* gene was detected in 71% of the isolates, without the presence of *tdh*, *trh* and *ure* genes. All strains of *V. parahaemolyticus* were Kanagawa negative. High antimicrobial resistance was observed in the β-lactam antibiotics (cephalothin - 72%, ampicillin - 60%) and aminoglycosides (amikacin - 64%), with multi-resistance in 88% of the strains of *V. parahaemolyticus*, of which 68% of the resistance was mediated by plasmids. Phenotypically, no production of β-lactamase enzymes was observed, but the presence of *bla*TEM genes was observed. The multiresistant character of plasmids reported in *V. parahaemolyticus* strains increases the concern about native bacteria in the marine environment since it can potentially compromise the control of this bacterium infection in bivalve mollusks.

Key words: estuaries; antimicrobials; anthropogenic action; oysters.

GENES DE VIRULENCIA E RESISTÊNCIA ANTIMICROBIANA DE *Vibrio parahaemolyticus* EM ÁREAS DE OSTREICULTURA

RESUMO

Este estudo teve como objetivo quantificar *Vibrio* spp. e avaliar o perfil de suscetibilidade antimicrobiana e fatores de virulência em cepas de *Vibrio parahaemolyticus* em amostras de ostras em dois estuários do Baixo Sul da Bahia. As coletas foram realizadas no período de junho de 2015 a janeiro de 2016, com coletas de ostras provenientes de bancos naturais (E1) e coletas de água e ostras provenientes de cultivo (E2). Para a quantificação de *Vibrio* spp. usou-se o número mais provável (NMP) e para a caracterização de *V. parahaemolyticus* o marcador (espécie-específica) gene *tl*. A patogenicidade foi observada com o teste de Kanagawa e presença dos genes *tdh, trh* e *ure*. Para o perfil de suscetibilidade antimicrobiana foram usados 15 antimicrobianos (método disco difusão), produção de enzimas β-lactamases e presença dos genes *bla*TEM, *bla*SHV e *bla*CTX-M. A densidade máxima de *Vibrio* spp. em ostras de cultivo foi de log 4,70 NMP g^{-1} e de extrativismo log 6,10 NMP g^{-1}, sendo a temperatura a variável que mais influenciou na presença dos microrganismos na área de cultivo (E2). O gene *tl* foi encontrado em 71% dos isolados, sem a presença dos genes *tdh, trh* e *ure*. Todas as cepas de *V. parahaemolyticus* foram Kanagawa negativo. Elevada resistência antimicrobiana foi observada aos antimicrobianos betalactâmicos (cefalotina - 72% e ampicilina - 60%) e aminoglicosídeos (amicacina - 64%), com multirresistência em 88% das cepas de *V. parahaemolyticus*, e dessas, em 68% a resistência era mediada por plasmídeos. Fenotipicamente não foi observada produção de enzimas β-lactamases, embora tenha sido observado a presença de genes *bla*TEM. O caráter de multirresistência sobretudo por plasmídeos em cepas de *V. parahaemolyticus* aumenta a preocupação de bactérias autóctones do meio marinho, pois compromete o controle de infecção por esta bactéria em moluscos bivalves.

Palavras-chave: estuários; antimicrobianos; ação antrópica; ostras.

INTRODUCTION

The Baixo Sul da Bahia occupies an area of 6.451 km^2, corresponding to about 1.14% of the entire state. It comprises of 11 municipalities and 7 coastal areas (Cairu, Taperoá, Camamú, Igrapiúna, Ituberá, Nilo Peçanha, and Valença). It is a region of great environmental and landscape diversity, cut off by islands, estuaries, and deep bays (FISCHER *et al*., 2007); the extension of mangroves (120,000 hectares area) is significant, as >20% of the local population survives directly or indirectly from artisanal fishing (IBAM, 2010).

In the Northeast region of Brazil, oyster breeding has gradually been consolidated with the cultivation of the native species *Crassostrea rhizophorae*. Oysters are filtering organisms, with a water filtration capacity of 19-50 L h^{-1}, and, through a low-selective capacity, they can accumulate biological pollutants such as bacteria in their tissues (ORBAN *et al*., 2007; BALLESTEROS *et al*., 2016). These pollutants when ingested in natura or poorly cooked, which is a common occurrence in the coastal regions, can cause significant food disease outbreaks, mainly through *Vibrio* strains (YU *et al*., 2016). The genus *Vibrio* includes native species of marine environments, estuaries, and coastal waters (NASCIMENTO *et al*., 2011).

The pathogenesis of *V. parahaemolyticus* can mainly be attributed to the presence of two virulence factors, the direct thermostable hemolysin (TDH) and the related thermostable hemolysin (TRH). The virulence factor TDH is considered as an important enterotoxin and has been identified as the main virulence factor for the species (XIE *et al*., 2017). Thus, phenotypical tests for the purpose of rapid identification and effective monitoring of oyster culture or extractive environments are essential to avoid and control the outbreaks of food diseases.

In the outbreaks of alimentary gastroenteritis involving *V. parahaemolyticus,* the treatment is conducted with chemotherapeutics. However, the transmission *Vibrio* strains resistant to the various drugs have increased in recent years (MANJUSHA and SARITA, 2013; LOU *et al*., 2016), because the aquatic environment is continuously receiving human and veterinary antimicrobial residues via domestic and hospitals sewage (BOUKI *et al*., 2013). Bacteria produce and secrete antibiotics into the environment for signaling and regulatory purposes as well as to protect themselves from antimicrobial toxicity through the acquisition and expression of resistance genes in their respective microbial communities (KANG *et al*., 2017).

Considering the epidemiological importance of *Vibrio*, as well as the increase in antimicrobial-resistant bacteria in the food of marine origin due to the increase in anthropic action in estuarine systems, where the aquaculture activities or extraction of bivalve mollusks are carried out, this study aimed to quantify *Vibrio* spp. and evaluate the profile of antimicrobial susceptibility and virulence factors in *V. parahaemolyticus* strains in water samples, cultured oysters (*C. rhizophorae*), and oysters extractivism, in two estuaries of the Southern Bahia Lowlands region, Brazil.

METHODS

Study area

The estuary of Taperoá (E1), which houses a natural oyster bank, is located in the municipality of Taperoá, Baixo Sul da Bahia, and is bathed by the Almas river (13º32'17"S 39º05'55"W). The estuary (E2) that bathes the locality of Torrinhas receives several arms of rivers, of which the river Una (13°33'54.84"S 39°0'29.99"W) is the main one. In this area, oyster cultivation is conducted in a flashlight system (Figure 1). Cultivation in lanterns is recommended for deep waters in the absence of strong currents, as the lanterns stand vertically (Figure 2). The cultivation practice consists of collecting oyster seeds directly from nature and fattening them in lanterns through the filtration of organic matter suspended in the water (SILVA and SILVA, 2007). The extraction areas E1 and E2 are approximately 6-km apart (Figure 1).

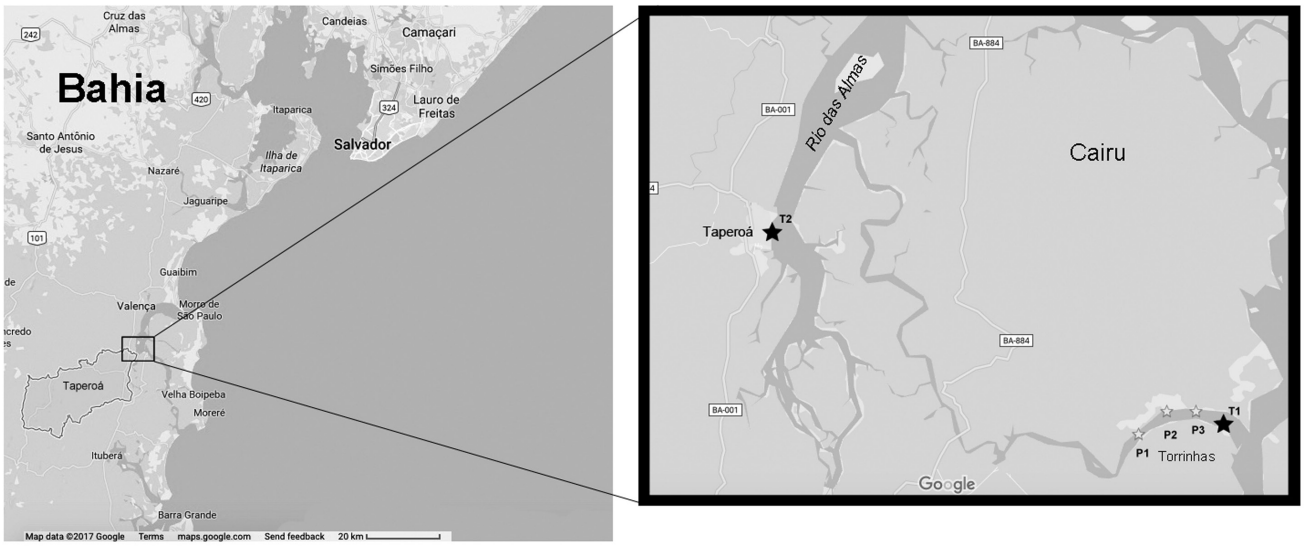


Figure 1. Map of the oyster and water collection points (P1, P2 and P3) in the E1 and E2 estuaries, Baixo Sul da Bahia.

Figure 2. Cultivation of oysters in a lantern system (E1) the locality of Torrinhas, Baixo Sul, Bahia.

Collection and microbiological analysis

- Natural banks (E1)

Six oyster samples were collected from natural banks (E1-13°32'43.8"S 39°05'09.1"W) (Figure 1) between June 2015 and January 2016, totaling 720 oysters. The oysters were taken from the women engages in culturing oyster due to the difficulties in a direct access to the place.

- Area of cultivation (E2)

Oyster collections in the E2 area were conducted on the same day as that of sample collection from the E1 area. Oyster collection was done randomly. At each harvest, about 36 oysters were taken from the two lanterns, totaling 216 oysters. The water samples collected for each point (point P1 [13°34'39.576"S 39°1'22.800"W] after cultivation (peck-crop direction), point P2 [13°34'23.196"S 39°0'41.652"W] in cultivation, and point P3 [13°34'23.664"S 39°1'2.640"W], prior to cultivation (Figure 1) consist of a composite sample (1 L of water at five equidistant points), homogenates (5 L), and a 1-L aliquot was withdrawn for analysis. In each collection, the parameters of temperature, pH, and salinity were determined by means of a HORIBA multiparameter probe.

All the collected materials were conditioned in a thermal box containing ice and sent for immediate analysis. The interval between collection and analysis was less than 4 h. The oysters were washed under running water, dried on a paper towel, and opened under aseptic conditions for the withdrawal of the liquid and intervalvar muscle.

For the quantification of *Vibrio* spp., 50 g of oysters and 50 mL of water were aseptically transferred to an Erlenmeyer flask containing 450 mL of phosphate-buffered saline (PBS), with decimal dilutions up to 10^{-4}. Aliquots of 1 mL were transferred to a series of 5 tubes (oysters and water) containing alkaline peptone water (APA) + 3% NaCl, incubated at 37 °C for 18-24 h. Each microbial growth tube was seeded on citrate bile sucrose (TCBS) thiosulfate agar plates and incubated at 37 °C for 18-24 h. From each TCBS agar plate, 5 colonies characteristic of *V. parahaemolyticus* (sucrose negative, round and opaque blue-green) were selected and transferred to 3% NaCl Tryptone Soya Agar (TSA), incubated at 37 °C for 24 h, and submitted to Gram-staining for the verification of the morpho-tinctorial characteristics. The microbial density of *Vibrio* spp. was determined from the positive APA tubes [sucrose negative strains and confirmed according to the identification code of NOGUEROLA and BLANCH (2008)] in Most Probable Number (MPN) per gram or milliliter (SILVA *et al*., 2010).

Pheno-genotypic characterization and molecular identification of *V. parahaemolyticus*

A total of 78 strains were analyzed with specific molecular markers for the identification of *Vibrio tl* species. From the positive results, virulence tests were performed on Petri dishes containing Wagatsuma® agar incubated at 37 °C for 18 h. The colonies with transparent halos around them were considered as positive (SILVA *et al*., 2010). These strains were submitted to the tests for genes of virulence and pathogenicity.

Total DNA extraction was performed using the Wizard Genomic DNA Purification Kit (PROMEGA®), following the manufacturer's recommendations. DNA integrity and amount were verified using 0.8% agarose gel electrophoresis and Qubit® 2.0 Fluorometer (Invitrogen), respectively. Amplifications were performed with 60 ng of DNA; 1x Taq DNA polymerase buffer enzyme; 3.7 mM MgCl2; 0.6 pmol μL^{-1} dNTPs; 0.4 pmol μL^{-1} of each *primer;* 1 U of Taq DNA polymerase, and the final volume was adjusted to 50 µL with ultrapure water. The amplification cycles of each primer were performed on a Veriti Thermal Cycler PCR (Appplied Biosystems) according to the manufacturer's recommendations for each pair of primer (markers): Tl gene, *primers:* F5'-AAAGCGGATTATGCAGAAGCACTG-3' and R5'-GCTACTTTCTAG CATTTTCTCTGC-3' (BEJ *et al*., 1999), as the indicator of pathogenicity; the *primers tdh*: F5'-GTAAAGGTCTCTGACTTTTGGAC-3' and R5'-TGGAATAGAACCTTCATCTTCACC-3' were used; *primers* to amplify the *trh*: F5'-TTGGCTTCGATATTTTCAGTATCT-3' and R5'-CATAACAAACATATGCCCATTTCCG-3' gene region (BEJ *et al*., 1999); and the *primers* for amplifying regions of the *ure*: F5'-CTTGTCATCGGGTGTCACTA-3' and R5'-GATGTTAGGTTCACCTACTGACT-3' (CABURLOTTO *et al*., 2009). The PCR products were subjected to 2% agarose gel electrophoresis, stained with ethidium bromide, and documented in the L-pix digital photo documentation system (Locus Biotecnologia) to observe the presence and absence of marker bands and their respective controls.

The strains positive for the phenogenotypic tests were submitted to molecular identification from the 16S rDNA region with

the primers: 8FN:5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R':5'-TACGGYTACCTTGTTACGACTT-3' as described by WEISBURG *et al*. (1991). The amplicons were purified using the ILLUSTRA® GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences) for further nucleotide identification using the ABI-*Prism* 3500 *Genetic Analyzer* (Applied Biosystems) automated sequencer. Sequence editing and assembly was performed with the Sequencher 4.1.4 program (Gene Code Corporation). The BLAST program was used to compare the sequences of each isolate with those found in the public databases NCBI (2018) and ENSEMBL BACTERIA (2018).

Antimicrobial susceptibility

Antimicrobial susceptibility was evaluated by the plate diffusion method using 15 commercial antimicrobials of different classes: aminoglycosides (30 µg amikacin and 10 µg gentamicin); β-lactams (ampicillin 10 µg, aztreonam 30 µg, cephalothin 30 µg, cefoxitin 30 µg, ceftazidime 30 µg, ceftriaxone 30 µg and imipenem 10 µg); phenicols (chloramphenicol 30 µg); nitrofuranols (nitrofurantoin 300 µg); quinolones (30 µg nalidixic acid and 5 µg ciprofloxacin); sulfonamides (25 µg sulfazothrin); and tetracyclines (tetracycline 30 µg) (CLSI, 2010).

For the calculation of the index of multiple antimicrobial resistance (MAR index), the ratio between the numbers of antimicrobials to which the isolate was resistant to the total number of antimicrobials tested was performed. Index >0.13 was characterized as a multidrug-resistant strain (KRUMPERMAN, 1983).

Plasmid-R-mediated resistance was evaluated for the strains showing an antimicrobial resistance profile. The strains were picked in nutrient broth at 37 °C for 24 h. Subsequently, 200 µL aliquots were transferred to tubes containing Luria Bertani (LB) 3% NaCl (control) and 3% LB broth NaCl + acridine orange (AO) at the concentration of 100 µg mL^{-1} as a curing agent. After incubation at 37 °C for 24 h, the cultures were again submitted to the antibiogram (MOLINA-AJA *et al*., 2002).

The phenotypic screening for Extended Spectrum Betalactamases (ESBL) producers was conducted using the disk approximation method in compliance with the standards of the CLSI (2009). A disc containing a third generation cephalosporin and another, 20 mm apart, containing the beta-lactamase inhibitor (amoxicillin + clavulanic acid) was used. The positivity of test was verified by the appearance of a "ghost zone" or widening of inhibition halo of cephalosporin.

In the molecular characterization of genes responsible for beta-lactamase enzymes, the presence of *bla*TEM genes (including TEM-1 and TEM-2 variants) (F5'-CATTTCCGTGTCGCCCTTATTC-3' and R5'-CGTTCATCCATAGTTGCCTGAC-3'), the *bla*SHV genes (including the SHV-1 variants): (F5'-AGCCGCTTGAGCAAATTAAAC-3' and R5'-ATCCCGCAGATAAATCACCAC-3') and the *bla*CTX-M gene (including CTX-M group 1 variants such as CTX-M-1, CTX-M-3 and CTX-M-15) (F5'-TTAGGAARTGTGCCGCTGYA-3' and F5'-CGATATCGTTGGTGGTRCCAT-3') (ELLINGTON *et al*., 2006) were explored.

Statistical analysis

The values of the MPN g^{-1} or mL^{-1} variables of the oyster and water samples were transformed into Log (x + 1). The averages values obtained were submitted to the Bartlett test for homoscedasticity and Shapiro-Wilk test for normality. To perform the tests and confection of the Boxplot graphics and contour area, the software R 3.4.4 (R CORE TEAM, 2016) was used.

RESULTS

Microbiological analysis of *Vibrio* spp.

The variation in the amount of *Vibrio* spp. (log MPN mL^{-1} and log MPN g^{-1}) in the samples of oysters and water was verified during the collection period, with oysters exhibiting greater variation in their bacterial microbiota (Figure 3A).

For the environmental parameters of the water, the average temperature at the collection points (P1, P2, and P3) was 27 °C, pH 7.5, and a salinity of 20 ppm, showing interactions between the

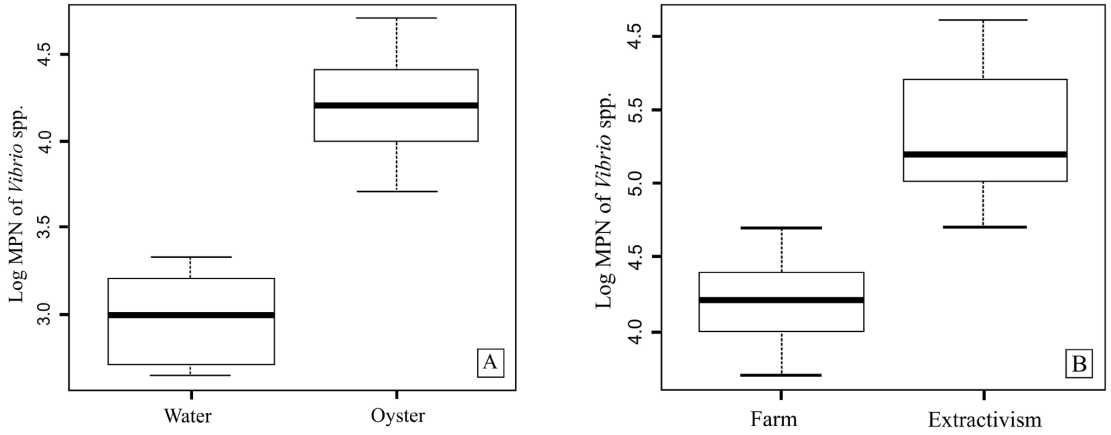


Figure 3. *Boxplot* of the density of *Vibrio* spp. in water samples (log MPN mL^{-1}) and oysters (log MPN g^{-1}) from the farming area (A), and cultivation oysters and extractivism oysters samples (B) in two estuaries of the Baixo Sul, Bahia.

quantification of *Vibrio* spp. and the physicochemical parameters, that is, the highest concentration of *Vibrio* spp. occurred at 29 °C with a salinity of 19 ppm (Figure 4A) and a pH of 7.1 and 8.0 (Figure 4B).

The estimation of the density variation of *Vibrio* spp. among the oyster samples (cultivation × extractivism) verified that the variation of *Vibrio* spp. (E2) was lower than that in extractive oysters (E1) (Figure 3B).

Of the 78 isolates of *Vibrio* spp., 71% (55) presented the *tl* gene, and amplification of 16S rDNA sequences identified it as *V. parahaemolyticus*. All strains of *V. parahaemolyticus* were negative for the Kanagawa test (β-hemolysis), with the absence of the *tdh*, *trh,* and *ure* genes.

Antimicrobial susceptibility

As shown in Figure 5, a high antimicrobial resistance for the antimicrobials amikacin (aminoglycoside), cephalothin, ampicillin and aztreonam (β-lactam) was observed. Moreover, 100% efficiency was observed for β-lactam cefoxitin in 68% of *V. parahaemolyticus* strains. For other antimicrobials belonging to these two families, a high intermediate resistance was observed, mainly to aztreonam (68%) and imipenem (52%).

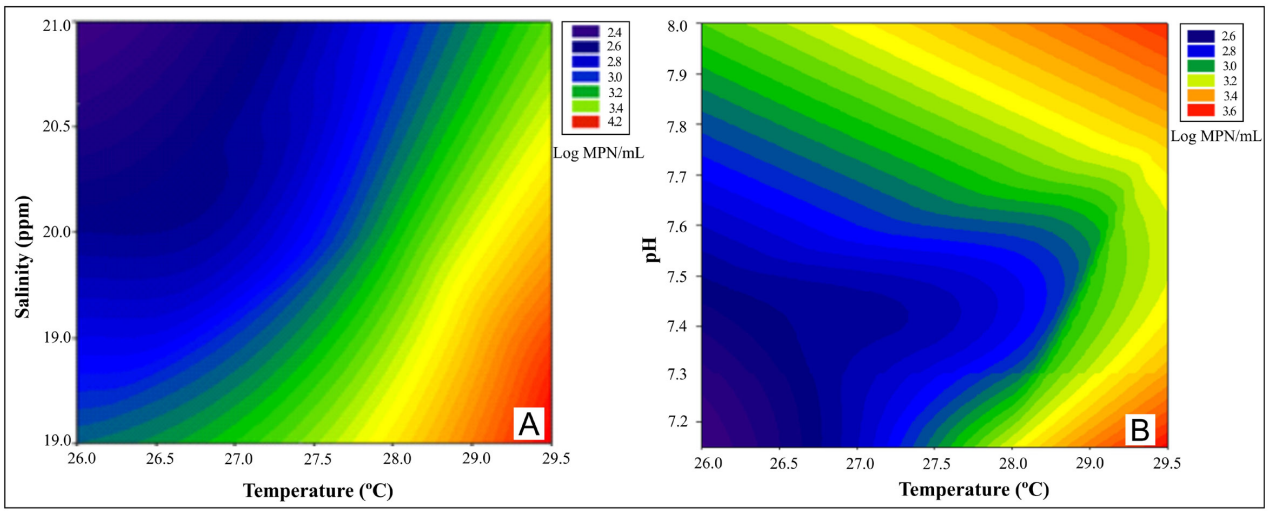
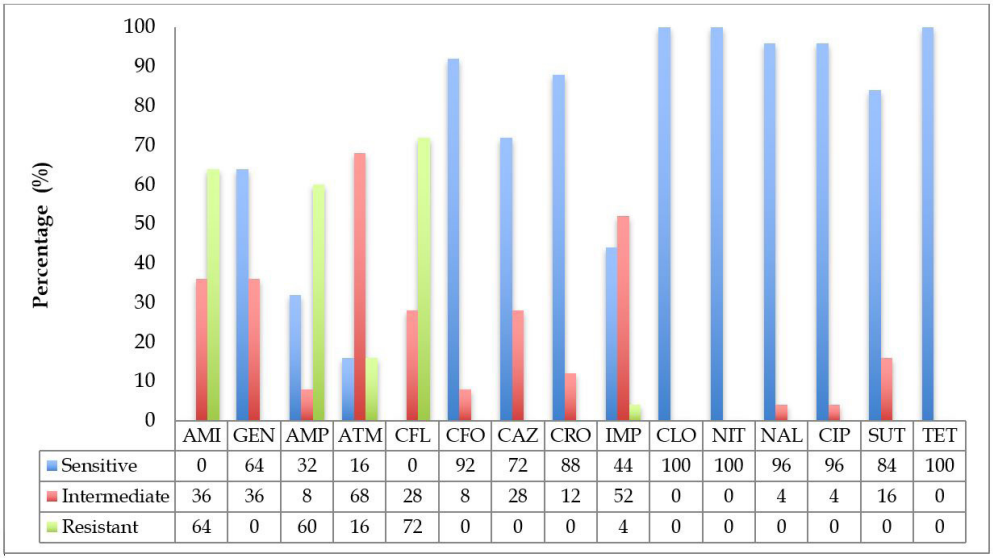


Figure 4. The plot between the environmental parameters of water and the density of *Vibrio* spp. in the area of cultivation (E2), Baixo Sul da Bahia. (A) Salinity x temperature and (B) pH x temperature.



	AMI	GEN	AMP	ATM	CFL	CFO	CAZ	CRO	IMP	CLO	NIT	NAL	CIP	SUT	TET
Sensitive	0	64	32	16	0	92	72	88	44	100	100	96	96	84	100
Intermediate	36	36	8	68	28	8	28	12	52	0	0	4	4	16	0
Resistant	64	0	60	16	72	0	0	0	4	0	0	0	0	0	0

Figure 5. Percentage of antimicrobial resistance in strains of *Vibrio parahaemolyticus* isolated from water samples and cultivation oysters and extractivism oysters, in two estuaries of Baixo Sul, Bahia. Amikacin (AMI); Gentamicin (GEN); Ampicillin (AMP); Aztreonam (ATM); Cephalothin (CFL); Cefoxitin (CFO); Ceftazidime (CAZ); Ceftriaxone (CRO); Imipenem (IMP); Chloramphenicol (CLO); Nitrofurantoin (NIT); Nalidixic acid (NAL); Ciprofloxacin (CIP); Sulfazotrin (SUT); Tetracycline (TET).

Table 1. Multi-resistance profile, MAR index, and plasmid resistance of *V. parahaemolyticus* in samples of water and cultivation oysters and extractivism oysters, in two estuaries of Baixo Sul, Bahia.

Source	N°	Microbial resistance	MAR	Plasmid resistance
Water (n=8)	3	CFL, AMI	0.13	-
	2	CFL, ATM, AMI	0.20	CFL
	1	CFL, SUT, GEN, ATM, AMI, IMP, AMP	0.46	SUT, GEN, IMP
	1	CRO, CFL, CAZ, CFO, GEN, ATM, AMI, IMP, AMP	0.60	CAZ, CFO, GEN
	1	CFL, CFO, GEN, ATM, AMI, IMP, AMP	0.46	ATM
Growing oysters (n=7)	1	CRO, CFL, CAZ, ATM AMI, IMP, AMP	0.46	-
	1	CFL, ATM, AMI	0.20	-
	1	CFL, AMI, AMP	0.20	-
	1	CFL, SUT, ATM, AMI	0.26	CFL, SUT, ATM
	1	CIP, CRO, CFL, CAZ, ATM, AMI, IMP, NAL, AMP	0.60	CIP, NAL
	1	CFL, GEN, ATM, AMI, IMP, AMP	0.40	GEN, ATM
Natural bank oysters (n=10)	1	CFL, CAZ, GEN, ATM, AMI, IMP, AMP	0.46	GEN
	2	CFL, GEN, ATM, AMI, IMP, AMP	0.40	GEN, ATM, IMP
	2	CFL, CAZ, GEN, ATM, AMI, IMP, AMP	0.46	CAZ, GEN, ATM, AMI
	1	CFL, ATM, IMP, AMP	0.26	-
	1	CFL, CAZ, ATM, AMI, IMP, AMP	0.40	ATM
	1	CFL, ATM, AMI, AMP	0.26	-
	1	CFL, SUT, ATM, AMI	0.26	ATM, AMI
	1	CFL, SUT, ATM, AMI, AMP	0.33	SUT, ATM
	1	CFL, ATM, AMI, IMP, AMP	0.33	ATM, IMP

N°: number of strains; MAR: Multiple Antimicrobial Resistance Index; CIP: Ciprofloxacin; CRO: Ceftriaxone; CFL: Cephalothin; SUT: Sulfazotrin; CAZ: Ceftazidime; CFO: Cefoxitin; GEN: Gentamicin; ATM: Aztreonam; AMI: Amikacin; IMP: Imipenem; NAL: Nalidixic acid; AMP: Ampicillin.

A multi-resistance profile was observed in 100% of the *V. parahaemolyticus* strains (n = 25), mainly amikacin, ampicillin, cephalothin, aztreonam, and imipenem, with multi-resistance varying for 7 to 9 antimicrobials (MAR = 0.46-0.60) in 32% of the isolates (Table 1). In 68% (17/25) of the strains, resistance was mediated by R-plasmids, and, of those, for 41% of these strains, resistance was observed for 3-4 antimicrobials (Table 1). High prevalence plasmids-mediated resistance (80%) was observed in strains from extractive oysters when compared to oyster culture strains (57%) and water (62.5%). Of the antimicrobials tested, gentamicin and aztreonam showed a higher frequency of plasmid-mediated resistance, especially in extractive oyster strains (Table 1).

Despite the high resistance to the wide-spectrum β-lactam aztreonam, no production of ESBL enzymes was observed in *V. parahaemolyticus* strains. Of the three types of genes (*bla*TEM, *bla*SHV, and *bla*CTX-M) studied, only the blaTEM gene was detected.

DISCUSSION

In the oysters from cultivation samples, a greater variation of bacteria between the samplings ($p \leq 0.05$) (Figure 3A) is attributable to its filtration feed process, which contributes to the accumulation of microorganisms in the tissues, where the gills, glands, and digestive tissues are the best site for detection of *V. parahaemolyticus* (YU *et al*., 2016). The microbiological analysis in oysters has been shown to be safer than that in water in relation to the sanitary quality of the food, since water represents the environmental condition at the time of collection and, in oyster samples, these retain the microorganisms longer (SANDE *et al*., 2010).

The occurrence of *Vibrio* genus is not always associated with the presence of indicator microorganisms, rather with the physicochemical alterations of the environment. The temperature (29 °C) was the parameter that most influenced the density of *Vibrio* spp. when the salinity was approximately 19 ppm (Figure 4A) and the pH was around 8.0 (Figure 4B). The Northeastern coast favors the proliferation of *Vibrio* throughout the year due to the optimal environmental conditions of bacterial growth, with salinity varying from 15 to 25 ppm, pH of 5-11, and temperature > 21 °C (ANACLETO *et al*., 2013). In Bahia, in the summer months when the temperatures reach 35 °C, the supply of raw oysters increases on beaches and the lack of notification of gastroenteritis data does not allow consumers to be aware of the actual number of cases involving these foods. According to the data from the Ministry of Health in 2017, between 2000 and 2017, of the 12,503 cases of outbreaks of food-borne diseases, 46.82% were not identified (BRASIL, 2018).

Brazil has not established microbiological parameters for the presence of *Vibrio* spp. or *V. parahaemolyticus* in oysters, although, in countries such as New Zealand and Japan, the maximum level allowed for *V. parahaemolyticus* in raw consumed shellfish is 2 log CFU g^{-1} or 100 CFU g^{-1} (LEE *et al*., 2008), and in the United States and Canada, oysters may only be marketed if they contain <4 CFU g^{-1} or 10,000 CFU g^{-1} of the bacteria (CDC, 1998). The Portaria SVS/MS nº 451 of 1997 (BRASIL, 1997) established limits of 3.70 log CFU g^{-1} or 5,000 CFU g^{-1} for *V. parahaemolyticus* in raw fresh fish, which was revoked by the resolution RDC nº 12 of 2001 (BRASIL, 2001). The absence of *V. parahaemolyticus* as an indicator of quality in bivalve mollusks was also observed in the Program of Hygienic-Sanitary Control of Bivalve Mollusks (PNCMB) which, when establishing the monitoring of contaminating microorganisms, contemplates only *E. coli* and marine biotoxins as a preventive measure of harmful effects on the consumer health (BRASIL, 2012). It is believed that the low incidence of cases involving *V. parahaemolyticus* justifies the absence of bacterium in Brazilian food regulations. However, in a country where under-reporting of food outbreaks cases is high and where the involvement of an etiologic agent in 79.3% of food outbreaks cases remains unknown (BRASIL, 2018), it is necessary that public health institutions reconsider the inclusion of *V. parahaemolyticus* in food from fisheries, mainly in raw bivalve mollusks.

KLEIN *et al*. (2014) found that the wide distribution of *Vibrio* spp. in aquatic environments tends to increase the risk to humans as agents in food outbreaks, especially when harboring pathogenic species such as *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus* and *V. cholerae.*

The filtering characteristic of bivalve mollusks makes the choice of cultivation area essential for the health of cultivated organisms. In the present study, on when comparing the risk of oyster intake by *Vibrio* spp. obtained in natural banks with oysters obtained in the culture system, a higher density of bacteria in oysters were obtained from extractivism (E1) (Figure 3). This is due to the proximity of natural banks in the town of Taperoá, a municipality with a population 40-times greater than that of the town of Torrinhas (E2) - a quilombola community composed of 450 inhabitants (IBGE, 2015). The lack of a sewage treatment system in most Brazilian municipalities directly affects the environmental quality of the estuaries due to the release of domestic wastes, as observed in the estuary of the river Una (SANTOS *et al*., 2015; BONDIOLI *et al*., 2017), which contributes to the proliferation and maintenance of *Vibrio* in the water due to the increase of debris and nutrients (COSTA *et al*., 2009).

The gene *tl*, in addition to being a specific marker for the identification of *V. parahaemolyticus*, codes for thermolabile hemolysin (TLH). Although the contribution of hemolysin to the pathogenicity of this species remains unknown, its expression is regulated positively under conditions that mimic the human intestine (KLEIN *et al*., 2014). Correlation with the gene *tl* and the *V. parahaemolyticus* species was also reported by LEAL *et al*. (2008) in clinical isolates from patients in Pernambuco, Ceará, and Alagoas when observing the amplification of gene in all strains of *V. parahaemolyticus*.

Other species of *Vibrio* may also be able to amplify the gene *tl*, although these species present a smaller amplicon and different phenotypic characteristics of *V. parahaemolyticus*, such as positive sucrose colonies on TCBS agar and positivity for the Voges-Proskauer test (YÁÑEZ *et al*., 2015), which was not observed in the strains in the present study during the additional tests.

The absence of the virulence genes *tdh* and *trh* and the absence of the Kanagawa phenomenon (β-hemolysis) in the strains of *V. parahaemolyticus* allowed the establishment of a phenogenotypic relation of the isolates. In environmental samples, the detection of the *tdh* and *trh* genes was low (only 1-3% of the isolates), whereas, in clinical strains, the detection percent was 90% (VONGXAY *et al*., 2008). Similar results were also reported by COSTA SOBRINHO *et al*. (2011), who observed high density of *V. parahaemolyticus* in oysters commercialized in the retail trade in São Paulo and did not detect strains positive for the *tdh* and *trh* genes. However, GUTIERREZ-WEST *et al*. (2013) reported a high frequency of virulence genes *tdh* (48%) and *trh* (8.3%) in estuarine *V. parahaemolyticus* strains. For the authors, although this is a poorly impacted estuary, this fact demonstrates the existence of other ecologically relevant functions for these genes, including perhaps infection by other hosts, where the products of these genes are important.

The absence of the gene *ure* in the *V. parahaemolyticus* strains demonstrates a correlation between the presence of the gene *trh* and gene *ure* encoding the urease enzyme, which has been proposed as an additional virulence marker for some strains of *V. parahaemolyticus* (YEUNG and BOOR, 2004). Even the pathogenicity of *V. parahaemolyticus* strains showing no virulence with the genes studied, should not be ruled out, since other virulence factors may be present in this and other *Vibrio* species not studied in the present study, such as the secretion of type 3 (T3SSs), which contains pathogenicity factors that causes lysis of the infected host cell (CECCARELLI *et al*., 2013). Thus, continuous monitoring of *V. parahaemolyticus* in fishing grounds is necessary for the better understanding of the ecological behavior of the species, since these strains act as reservoirs of known or undiscovered virulence genes, but involved in human pathogenicity, that can be disseminated in the marine environment (CABURLOTTO *et al*., 2009).

The risk of seafood outbreaks involving *V. parahaemolyticus* is aggravated when the selective pressure of antimicrobial residues and the release of resistant Enterobacteriaceae family bacteria into the rivers and estuaries directly affect natural microbial populations such as vibrios. The high antimicrobial resistance observed in aquatic isolates of *V. parahaemolyticus* is due to their ability to exchange genetic determinants (XIE *et al*., 2017). This fact is aggravated by the high intermediate resistance index for β-lactams, such as imipenem (52%), aztreonam (68%) (monobactam), and ceftriaxone of the third-generation, ceftriaxone (12%) (Figure 5). Most of the antimicrobials tested in the present study are recommended for the treatment of infections caused by *Vibrio* spp. Third-generation cephalosporins are considered to provide some of the best defenses against serious infections by these microorganisms, and the presence of resistant isolates, even

in small percents, puts future treatments for vibrios infections at risk (SHAW *et al*., 2014).

One of the factors that contributed to the reduction in the efficacy of treatment with first-generation antimicrobials such as ampicillin and cephalothin, has been its wide use in aquaculture (ROCHA *et al*., 2016). High antimicrobial resistance to ampicillin and cephalothin has also been reported by LOU *et al*. (2016) and ROCHA *et al*. (2016), respectively. Already WANG *et al*. (2015) warned against the use of antimicrobials in cultivated areas, reporting that more >40% of *V. parahaemolyticus* strains isolated from abalones showed antimicrobial resistance, especially in areas of shrimp farming.

For TANIL *et al*. (2005), MAR index of >0.2 is due to contamination from high-risk sources, which puts human health at risk. The high index of multiple antimicrobial resistance with MAR >0.20 in 88% of *V. parahaemolyticus* strains demonstrates the anthropic influence in the study areas (Table 1), which has contributed to the fact that non-pathogenic environmental bacteria are becoming the largest reservoir of resistance to broad-spectrum antimicrobials (MANJUSHA and SARITA, 2013) along with becoming a risk to the new-generation antimicrobial agents for controlling the pathogenicity of *V. parahaemolyticus* (YU *et al*., 2016), since, after the death of the bacterium and the consequent rupture of the cell wall and membranes, genetic elements like plasmids and integrons are disseminated in the aquatic environment for incorporation in other bacteria (ROCHA *et al*., 2016).

Since *V. parahaemolyticus* strains show high intermediate resistance or resistance to the two aminoglycosides amikacin and gentamicin and to cephalosporins (antimicrobials usually used in the treatment of vibrios infections), it is recommended that, in cases of infections caused by these microorganisms, treatment with tetracycline, quinolones (nalidixic acid), and fenicol (chloramphenicol) is the best chemotherapeutic option in this region.

Although the production of β-lactamase enzymes (ESBL) in the strains of *V. parahaemolyticus* is still not frequent, the high resistance to second- and third-generation β-lactams observed in the present study demonstrates that antimicrobial resistance in the environmental strains needs to be monitored. In the present study, it was observed that there were no significant differences between the two strains of *V. parahaemolyticus tdh* and *trh*-negative and ESBL-producing strains in Hong Kong (WONG *et al*., 2012). The production of β-lactamase enzymes has been a feature observed mainly in enterobacteria (MEYER and PICOLI, 2011); however, self-transmissible plasmids from Gram-negative enterobacteria are responsible for the production of ESBL in strains of *V. parahaemolyticus* isolated from fish in Hong Kong (WONG *et al*., 2012).

In the microorganisms with >300 ESBL subtypes, the genes that encode TEM, CTX, or SHV are most commonly studied (BUSH and JACOBY, 2010). The presence of the *bla*TEM gene in the strains of *V. parahaemolyticus* was satisfactory because it is a narrow-spectrum β-lactamase, which hydrolyzes first-generation penicillin and cephalosporin (BUSH and JACOBY, 2010). The strains of *V. parahaemolyticus* also bearing *bla*TEM gene were reported by ROJAS *et al*. (2011) in the samples of bivalve mollusks at different points of sale in the State of São Paulo. According to the authors, it is necessary that continuous monitoring of halophilic vibrios in hospital areas near the coastal regions and in places of consumption of seafood be conducted to notify the incidence of vibrioses in the population.

CONCLUSION

Anthropogenic action in the Baixo Sul da Bahia estuaries has affected the resident microbiota, contributing thereby to the increase in multidrug resistance mediated by plasmids in *V. parahaemolyticus* strains. This fact represents a risk for raw oyster ingestion in the region with the transfer of resistant plasmids to the intestinal microbiota of the consumers. Thus, it is necessary to create a regulation for food and environment with specified limits to the presence of *V. parahaemolyticus* in seafood as well as guidelines aimed at the commercialization of these organisms from extractivism, since what is currently more focused on the production areas. It is believed that the adoption of Good Manipulation Practices together with educational actions promoted by the Sanitary and Epidemiological Surveillance can help regulate the quality of the products marketed by the fishing production chain in order to minimize the risks of food outbreaks.

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