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# Antimicrobial resistance and potential virulence of *Vibrio parahaemolyticus* isolated from water and bivalve mollusks from Bahia, Brazil

Irana Paim Silva[a], Camila de Souza Carneiro[a], Margarete Alice Fontes Saraiva[a], Thiago Alves Santos de Oliveira[a], Oscarina Viana de Sousa[b], Norma Suely Evangelista-Barreto[a,*]

[a] Center of Agricultural, Environmental and Biological Sciences, Federal University of Recôncavo da Bahia, Rua Rui Barbosa, 710, 44380-000, Cruz das Almas, BA, Brazil
[b] Marine Science Institute - Federal University of Ceará, Av. da Abolição, 3207, Meireles, 60165-081, Fortaleza, CE, Brazil

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ABSTRACT

The aim of the present study was to verify the antimicrobial susceptibility profile and virulence factors of *Vibrio parahaemolyticus* isolated from water and bivalve mollusks. A high percentage of *V. parahaemolyticus* was isolated *in natura*, processed bivalves tissues, and surrounding water (75%, 20%, and 59%, respectively). The most potential virulence phenotype in *V. parahaemolyticus* isolates was amylase production (97%) followed by DNase (83%), phospholipase (70%), β-hemolytic activity (57%). The *tdh* and *trh* genes were not detected. Besides, a high antimicrobial resistance was observed for ampicillin (97%), minimum inhibitory concentration [MIC] = 400 μg and cephalothin (93%, MIC ≤ 100 μg). The absence of expression of *tdh* and *trh* virulence genes excluded the toxigenic potential of *V. parahaemolyticus* isolates; however, the high prevalence of antimicrobial resistance among the environmental strains is a risk to human health.

## 1. Introduction

The species belonging to the genus *Vibrio* are Gram-negative bacteria native to marine and estuarine environments. These are generally mobile, mesophilic, and facultative anaerobes (Austin, 2010). Vibrios have epidemiological importance; at least 13 species are capable of causing infection in humans. Of these, the most important are *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* (Austin, 2010; Sousa et al., 2004).

*V. parahaemolyticus* is considered to be an emerging species because of its involvement in outbreaks after the consumption of contaminated food, especially, partially cooked fish and shellfish (Pereira et al., 2007). This microorganism causes three clinical conditions: gastroenteritis, septicemia, and infection. Gastroenteritis is usually accompanied by symptoms that include vomiting, diarrhea, headache, motion sickness, fever, and abdominal cramps (Alipour et al., 2014).

The infections caused by *Vibrio* are related to a variety of virulence factors such as cytotoxins, enterotoxins, lytic enzymes, and the production of a capsular polysaccharide (Cabrera-Rodríguez et al., 2008; Masini et al., 2007). Pathogenic *V. parahaemolyticus* strains can be identified based on the production of a thermostable direct hemolysin (TDH) and a thermostable related hemolysin (TRH), which are coded by the genes *tdh* and *trh*, respectively (Costa-Sobrinho et al., 2011).

A review, from 1988 to 1997 in the United States, on infections, reported that 88% of gastroenteritis and 91% of septicemia cases were related to the consumption of oysters (Daniels and Shafaie, 2000). In Chile, an epidemic outbreak during the summers of 2004 and 2005 caused over 10,000 cases of infections, most of which were associated with the consumption of mollusks (Food and Agriculture Organization/World Health Organization [FAO/WHO], 2011; Fuenzalida et al., 2006).

In 2006, the outbreaks of *V. parahemolyticus* infections associated with the consumption of raw shellfish resulted in a total of 177 cases in three states of the United States (Yoon et al., 2008). In Japan, North America, and Southeast Asia, *V. parahaemolyticus* has been responsible for the most cases of food poisoning due to seafood consumption (Hongping et al., 2011). This bacterium was responsible for gastroenteritis outbreak involving 26 subjects after consumption of raw crab in Brazil (Santos and Vieira, 2013).

Another problem associated with *V. parahaemolyticus* is the increasing prevalence of antimicrobial resistance in aquatic environments. Antimicrobial resistance is considered a consequence of evolution via natural selection of resistant bacterial populations, and often involves conjugation for the exchange of resistance genes (Carneiro et al., 2007). The occurrence and prevalence of antimicrobial resistant *V. parahaemolyticus* in seafood require urgent efforts and the need for a targeted policy on the use of antibiotics in aquaculture farming (Odeyemi and Stratev, 2016) since it is a major concern for human

* Corresponding author.
*E-mail address:* nsevangelista@yahoo.com.br (N.S. Evangelista-Barreto).

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health and veterinary practices throughout the world (Xie et al., 2017).

Considering the epidemiological importance of *V. parahaemolyticus* and the dangers of eating raw or undercooked shellfish, the present study aimed to verify the susceptibility profile and the virulence factors of *V. parahaemolyticus* strains isolated from water and bivalve mollusks collected from estuaries in the São Francisco do Conde region (Bahia, Brazil), which is an important natural oyster bed with an intense activity of harvesting.

## 2. Materials and methods

### 2.1. Sample collection and isolation of microorganisms

Fifteen samples of water were collected from three points (totalizing 45 water samples) along the estuary of the Subaé River (São Francisco do Conde region). The points were P1 (Vila Caeira) - S 12° 33′ 52.4″/W 038° 41′ 40.5″- river point consisting of several riverside communities; P2 (São Bento) - S 12° 35′ 35.8″/W 038° 41′ 47.7″- harvesting area of bivalve mollusks; and P3 (Cajaíba island) - S 12° 37′ 52.9″/W 038° 40′ 55.0″ - Baía de Todos os Santos (BTS) and harvesting area of bivalve mollusks. The water samples were collected at a depth of 50 cm in amber flasks (1000 mL) using aseptic technique.

The *in nature* mollusks totaled 30 samples, being the oyster (*Crassostrea rhizophorae*) consisted of 83 individuals, whereas mussel sample (*Mytella guyanensis*) were composed of 70 individuals. Mollusk processed was purchased from several merchants in the municipal market, in portions of 0.5 kg (totalizing 7.5 kg). Oysters and mussels (25 g) were processed by pre-enrichment in 225 mL of alkaline peptone water (APW) and incubated at 37 °C for 18 ± 2 h. Typical *V. parahaemolyticus* isolates (green or blue-green colonies) were selected from thiosulfate-citrate-bile-salts-sucrose (TCBS; Difco, Detroit, MI, USA) (Sousa et al., 2004). The phenotypic identification was made with the help of an identification key (Noguerola and Blanch, 2008).

### 2.2. Genotypic characterization

The DNA was extracted according to standard protocols (Sambrook et al., 1989). Multiplex PCR was carried out for the detection of virulence genes, *tdh* and *trh*. The forward (F) and reverse (R) primers used were F: 5′-gta aag gtc tct gac ttt tgg ac-3′ and R: 5′-tgg aat aga acc ttc atc ttc acc-3′ with 269 bp (*tdh*) and F: 5′-ttg gct tcg ata ttt tca gta tct-3′ and R: 5′-cat aac aaa cat atg ccc att tcc g-3′ with 500 bp (*trh*). The *V. parahaemolyticus* standard strain (IOC 17802) was used as a control. The amplification conditions used for the detection of virulence genes were according to the protocol of Bej et al. (1999).

### 2.3. Antimicrobial susceptibility

The antimicrobial susceptibility was determined by disk diffusion method (Clinical and Laboratory Standards Institute [CLSI], 2012) using commercially available antibiotic-containing disks (LABORCLIN, Brazil), nalidixic acid (NAL, 30 μg), ampicillin (AMP, 10 μg), gentamicin (GEN, 10 μg), cephalothin, ceftazidime (30 μg), ciprofloxacin (CLO, 5 μg), chloramphenicol (CLO, 30 μg), imipenem (10 μg), nitrofurantoin (NIT, 300 μg), sulfamethoxazole + trimethoprim (SUT, 25 μg), and tetracycline (TET, 30 μg). The antimicrobials tested are for human and veterinary medicine use, and used in the treatment of vibrios infections (Shaw et al., 2014).

Minimum inhibitory concentration [MIC] was determined in resistant strains according antimicrobial test disk diffusion (Kirby-Bauer) to a broth dilution (macro dilution) technique using Mueller-Hinton broth (Difco, Detroit, MI, USA). The multiple antimicrobial resistance index (MARI) was calculated as described by Clinical Laboratory Standards Institute (CLSI, 2012).

### 2.4. Plasmid-mediated antimicrobial resistance

The strains resistant to more than one antibiotics were submitted to plasmid curing technique (Molina-Aja et al., 2002) using Luria-Bertani broth (Difco, Detroit, MI, USA) supplemented with 0.85% NaCl and 100 μg.mL$^{-1}$ of acridine orange dye. Susceptibility was tested against antibiotics after the process. The strains with plasmid-mediated resistance profile were submitted to plasmid DNA extraction using "Plasmid Mini Kit I" (Omega/Bio-Tek, Norcross, GA, EUA). The plasmid profile analysis was visualized using an AlphaImager Mini System (Alpha Innotech, USA) after electrophoretic separation at 6 V·cm$^{-1}$ in a 0,8% agarose gel stained with Ethidium Bromide (10 mg.mL$^{-1}$). To determine the molecular weight was used molecular marker DNA Ladder kb.

### 2.5. Phenotypic virulence tests

For exoenzyme detection, the strains were grown on tryptic soy agar (TSA; Difco, Detroit, MI, USA) containing 1% NaCl and supplemented with 0.5% de gelatin (gelatinase), 5% skim powdered milk (casein), 1% egg yolk emulsion (phospholipase), 1% Tween 80 (lipase), 20% solution of sheep erythrocytes (hemolysin), 0.1% of starch (amylase), and agar DNase supplemented with 0.01% tolonium chloride (DNase), respectively. The formation of a transparent or opalescent halo indicated the positivity of the tests (Cabrera-Rodríguez et al., 2008; Hongping et al., 2011; Pereira et al., 2004; Wagatsuma, 1968).

## 3. Results and discussion

Forty-five isolates were phenotypically identified, including species that are clinically relevant namely *V. cholerae* (6 isolates) and *V. parahaemolyticus* (30 isolates) (Table 1). The abundance and distribution of these pathogens have been linked to environmental factors, most notably temperature and salinity (Johnson et al., 2012).

*V. cholerae* was isolated only from point P1 (Vila Caieira) due to the low salinity at this point in addition to a load of solid residues from the communities living on the banks of the River Subaé or its affluent. This bacterium is a cosmopolitan aquatic species that is capable of causing illness in humans. Its ability to survive in different environmental

**Table 1**
*Vibrio* spp. isolated in water and bivalve mollusks in the estuary of the river Subaé, São Francisco do Conde, Bahia, Brazil.

| Samples | Sources | Species | Prevalence % (N°/total of isolates)[a] |
|---|---|---|---|
| Water | Point 1[b] | *V. parahaemolyticus* | 24 (6/25) |
| | | *V. pelagicus* | 04 (1/25) |
| | | *V. alginolyticus* | 04 (1/25) |
| | | *V. cholerae* | 12 (3/25) |
| | Point 2[c] | *V. parahaemolyticus* | 28 (7/25) |
| | Point 3[d] | *V. coralliilyticus* | 04 (1/25) |
| | | *V. parahaemolyticus* | 16 (4/25) |
| | | *V. litoralis* | 04 (1/25) |
| | | *V. alginolyticus* | 04 (1/25) |
| Mollusk *in nature* | *M. guyanensis* | *V. parahaemolyticus* | 60 (3/05) |
| | | *V. cholerae* | 40 (2/05) |
| | Oysters | *V. parahaemolyticus* | 83 (5/06) |
| | | *V. cholerae* | 17 (1/06) |
| Mollusk processed | *M. guyanensis* | *V. parahaemolyticus* | 56 (5/09) |
| | | *V. ponticus* | 11 (1/09) |
| | | *V. litoralis* | 11 (1/09) |
| | | *V. metschnikovii* | 11 (1/09) |
| | | *V. crassostreae* | 11 (1/09) |

[a] Number of strains identified as *Vibrio* spp. from total of isolates.
[b] Vila Caeira.
[c] São Bento.
[d] Cajaíba Island.

niches is attributed to its adaptive responses that allow it to withstand nutrient deprivation, fluctuations in salinity and temperature, besides resisting predation by heterotrophic protists and bacteriophages (Lutz et al., 2013). In Brazil, from 2002 to 2003, *V. cholerae* O1 was isolated from the environmental samples in the regions of Alagoas and Pernambuco. In 2004, a cholera outbreak in Brazil led to 21 confirmed native cases. In 2005, five more native cases were registered, all from Pernambuco state (Secretária de Vigilância Sanitária [SVS], 2008).

From point P2 (São Bento), which is an area of bivalve mollusk extraction, only *V. parahaemolyticus* was isolated (Table 1). This organism is a major causative agent of seafood-borne gastroenteritis worldwide and is acquired by the consumption of raw or undercooked seafood, especially shellfish (Johnson et al., 2012; Mala et al., 2016). From P3 (Cajaíba island), a region with the greatest salinity (30 ppm), in addition to *V. parahaemolyticus*, other species like *V. crassostrea*, *V. alginolyticus*, *V. metschnikovii*, and *V. coralliilyticus* associated with zoonosis in aquatic animals were also identified (Austin, 2010).

*V. parahaemolyticus* was largely present in raw oysters (83% of samples analyzed), in raw and processed samples of *M. guayanensis* (approximately 65.0% of samples), and 44.0% of water samples analyzed.

The high percentage of these microorganisms in raw oysters analyzed is associated with estuarine regions and feeding form (filtration). As the collections were carried out at low tide, the oysters were found with closed valves, thereby decreasing the depuration process and raising the concentration of bacteria in the host tissue. The percentage of *V. parahaemolyticus* found in water can be explained as it is a halophilic bacterium found in marine and estuarine environments. Although *M. guayanensis* is also a filtrate, other than the oyster, its habitat is the substrate. *Vibrio* is generally found in higher numbers in the water column due to the ambient temperature.

In Brazil, *V. parahaemolyticus* is present in such an abundance that the bivalve mollusks fall in the risk group. The raw oysters are consumed on a large scale in the north-eastern region of Brazil. The lack of epidemiological data in the country has resulted in the absence of knowledge of the real number of food poisoning outbreaks involving these foodstuffs. In the United States, from May to August 2013, *V. parahaemolyticus* accounted for food poisoning outbreaks in 13 states and the outbreaks were predominated by the O4:K12 serotype (Centers for Disease Control and Prevention [CDC], 2013). In Ceará state of Brazil, this bacterium accounted for an outbreak of gastroenteritis involving 26 individuals due to the ingestion of raw crabmeat in a restaurant in the capital city (Santos and Vieira, 2013).

A high number of bacterial species were observed in the processed mollusks in comparison with the same organism in the raw form. Four of these species are associated with zoonosis, whereas one is of clinical importance (Table 1). Their presence may be attributed to hygienic and sanitary failures during the bivalve mollusk processing and cross-contamination at the commercialization locations. In addition, storage time and temperature are other factors that contribute to increasing the microbial load in the processed foods.

The reduction in the prevalence of *V. parahaemolyticus* may be associated with cooking, freezing, and storage conditions, which are considered efficient in eliminating these microorganisms from mussel samples (Cordeiro et al., 2007).

### 3.1. Antimicrobial susceptibility profile

Tetracycline, gentamicin, nalidixic acid, ciprofloxacin, and nitrofurantoin were found to be the most efficient in the elimination of *V. parahaemolyticus* strains isolated in the samples (Table 2). Susceptibility to tetracycline was satisfactory as it is one of the preferred drugs in the treatment of infections caused by *Vibrio* spp. (Han et al., 2007). The efficiency of tetracycline is thought to be related to its ability to inhibit protein synthesis of the pathogenic extracellular enzymes, e.g. proteases and lipases (Elmahdi et al., 2016).

Similar results were reported by Costa et al. (2015) in their study with fresh and frozen oysters. The authors suggested a consideration of chloramphenicol, nalidixic acid, ciprofloxacin, tetracycline, and gentamicin and reported that they should be selected to treat diseases caused by *V. parahaemolyticus*. Mala et al. (2016) found that all strains isolated from cockles (*Anadara granosa*) were susceptible to ampicillin, cefotaxime, gentamicin, norfloxacin, ofloxacin, tetracycline, and trimethoprim-sulfamethoxazole.

All isolates identified as *V. parahaemolyticus* showed resistance to ampicillin, cephalothin, chloramphenicol and imipenem with a MIC varying from 20 to > 400 μg (Fig. 1). All bacterial isolates were resistant to ampicillin, presenting a maximum MIC of > 400 μg, whereas the test disc concentration was 10 μg (Table 3). Ampicillin has widely accepted as a drug of the first choice in the treatment of foodborne illnesses; however, it exhibits a low efficiency against *Vibrio* spp. (Dewaal and Grooters, 2013; Han et al., 2007; Table 3). The cephalothin-resistant isolates presented an MIC (≤100 μg) higher than the test disc used (30 μg). This suggests that the first generation cephalosporins may have been misused widely, thereby reducing their susceptibility and efficiency in the treatment of *V. parahaemolyticus* infection (Yu et al., 2016). For imipenem, the isolates presented an MIC (20 μg); however, imipenem is a compound of restricted use in hospital units. The presence of resistant strains serves as a warning to the responsible authorities regarding the use and disposal of this compound into the environment. Zanetti et al. (2001) reported that 88.9% of the isolates from marine environments showed resistance to antibiotic (MIC > 64 μg) due to β-lactamase production.

Xie et al. (2017) while evaluating the isolates of *V. parahaemolyticus* in aquatic products detected resistance to streptomycin with rate of 90.53% (86/126), ampicillin (33.68%), cephalothin (30.53%), and cefazolin (28.42%), while the resistance rate of clinical isolates was of 93.55% to streptomycin, followed by ampicillin (87.10%), cefazolin (64.52%), cephalothin (45.16%), and kanamycin (45.16%). Some studies that were conducted to characterize the antibiotic susceptibility profile of *Vibrio* spp. indicated that *V. parahaemolyticus* and *V. vulnificus* have developed multiple antibiotic resistances, which may lead to the failure of the available treatment options for common infections (Elmahdi et al., 2016).

Multiple resistance was observed in 75.0% of the isolates from raw bivalve mollusks (multiple antibiotic resistance index [MARI], 0.18 to 0.36), 59% in the water isolates (MARI, 0.18) and 20% from the processed *Mytella guyanensis* isolates (MARI, 0.27) (Table 3). The resistance in microorganisms to antimicrobials can be attributed to the inherent cell characteristics and is generally determined by chromosomal genes (Costa et al., 2008). The significance of the studying microbial resistance in indigenous aquatic microorganisms lies in the fact that it indicates the extent of anthropic action, especially when the antimicrobials are released into the sewers through human waste and even corpses (Baquero et al., 2008). High multiple resistance index raises doubts about the effectiveness of antimicrobial agents commonly used in the treatment of gastroenteritis caused by *Vibrio* spp. (Costa et al., 2015).

In Malaysia, MAR index of 0.93 was found in *V. parahaemolyticus* strains in fish samples (Noorlis et al., 2011). Daramola et al. (2009) also reported high MAR index values (0.63) in *V. parahaemolyticus* strains from environmental samples in England.

Plasmid-mediated resistance was observed in 25%, 20%, and 12% of the *V. parahaemolyticus* strains isolated from raw bivalve mollusks, processed *Mytella guyanensis*, and water, respectively, especially for cephalothin, chloramphenicol, and ceftazidime (Table 2). The molecular weight of the plasmids present in the strains ranged from 3.0 to 5.0 kb, and up to two plasmids were present in some strains (1.5 and 3.0 kb). The lack of basic sanitation and continuous discharge of domestic effluents into the river Subaé affect the native microbiota of the environment; some strains may undergo mutations, receive plasmids or transposons, and may pass the antibiotic resistance after acquisition to

**Table 2**
Percentage of *Vibrio parahaemolyticus* isolated ($n$ = 30) resistant to different a antimicrobials from water, *in nature* and processed bivalve mollusks in the estuary of the river Subaé, São Francisco do Conde, Bahia, Brazil.

| Antimicrobials | *Vibrio parahaemolyticus* (30)[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Water (17) | | | Bm[b] (8) | | | Pb[c] (5) | | |
| | S[d] | I[e] | R[f] | S | I | R | S | I | R |
| Aminoglycosides | | | | | | | | | |
| - Gentamicin | 100 | 0 | 0 | 100 | 0 | 0 | 100 | 0 | 0 |
| β-lactam | | | | | | | | | |
| - Ampicillin | 0 | 24 | 76 | 0 | 12 | 88 | 0 | 20 | 80 |
| - Cephalothin | 12 | 23 | 65 | 0 | 37 | 63 | 0 | 80 | 20 |
| - Ceftazidime | 53 | 35 | 12 | 75 | 0 | 25 | 60 | 20 | 20 |
| - Imipenem | 94 | 6 | 0 | 100 | 0 | 0 | 100 | 0 | 0 |
| Phenicols | | | | | | | | | |
| - Chloramphenicol | 100 | 0 | 0 | 75 | 0 | 25 | 80 | 0 | 20 |
| Nitrofuran | | | | | | | | | |
| - Nitrofurantoin | 100 | 0 | 0 | 100 | 0 | 0 | 100 | 0 | 0 |
| Quinolones | | | | | | | | | |
| - Nalidixic acid | 100 | 0 | 0 | 100 | 0 | 0 | 100 | 0 | 0 |
| - Ciprofloxacin | 100 | 0 | 0 | 100 | 0 | 0 | 100 | 0 | 0 |
| Sulfonamides | | | | | | | | | |
| - Sulfamethoxazol + trimethoprim | 100 | 0 | 0 | 87 | 0 | 12 | 100 | 0 | 0 |
| Tetracyclines | | | | | | | | | |
| - Tetracycline | 100 | 0 | 0 | 100 | 0 | 0 | 100 | 0 | 0 |

[a] Isolated.
[b] Bm (Bivalve mollusk in nature, oyster and mussel).
[c] Pb (Processed bivalve mollusk).
[d] S (Susceptible).
[e] I (Intermediate resistance).
[f] R (Resistant).

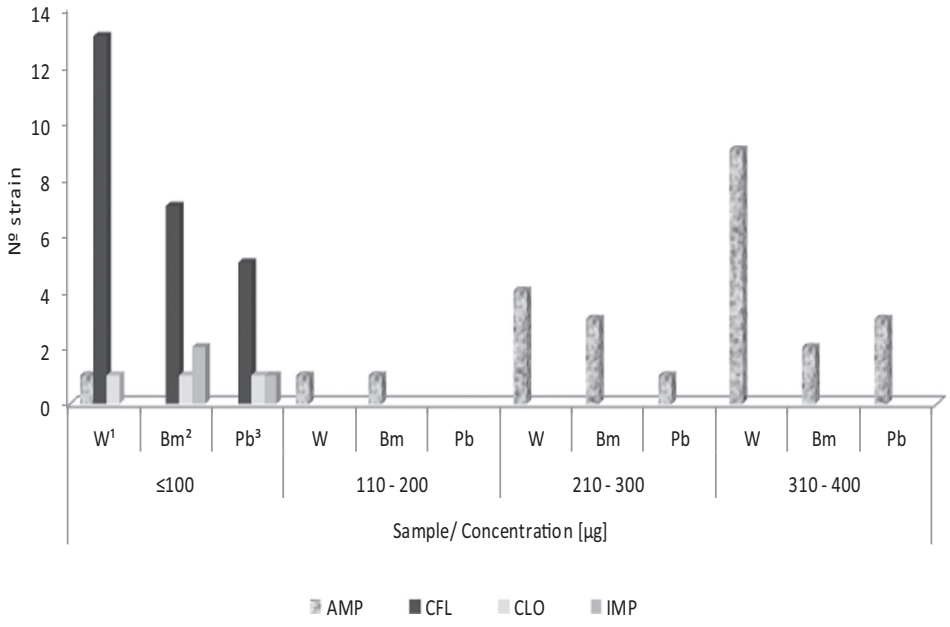


**Fig. 1.** The Minimum Inhibitory Concentration [MIC] of *Vibrio parahaemolyticus* isolated from water, *in nature* and processed bivalve mollusks in the estuary of the river Subaé, São Francisco do Conde, Bahia, Brazil. [1]Water; [2]Bivalve mollusk *in nature*; [3]Processed bivalve mollusk; Ampicillin (AMP); Cephalothin (CFL); Chloraphenicol (CLO); Imipenem (IMP).

the future strains (Costa et al., 2008). A study with shellfish in Kerala, India showed that plasmid frequency in mollusks and crustaceans was 40% and 20%, respectively, and the molecular weight ranged from 5.98 to 19.36 kb, respectively, although a high chromosome resistance was observed (Manjusha and Sarita, 2013).

### 3.2. Phenotypic virulence factors

The recurrent patterns of exoenzymes and hemolytic activity in the *V. parahaemolyticus* strains were as follows: amylase > DNase > phospholipase > hemolysin > gelatinase > lipase > caseinase > urease. All the strains were positive for at least one virulence factor tested. Some isolates displayed positivity up to five tests (Table 4). Costa et al. (2013) reported enzymatic profiles for *Vibrio* strains as: DNase > amylase > gelatinase > lipase > phospholipase > caseinase. Exoenzymes may be involved in different functions in the microorganisms, several studies have shown their involvement in increasing the pathogenicity in vibrios (Costa et al., 2013; Cabrera-Rodríguez et al., 2008; Masini et al., 2007). The related to the bacteria causing infections in humans are categorized depending on their different abilities: collagen hydrolysis (gelatinases), bacterial

**Table 3**
Multiple microbial and plasmid resistance of *Vibrio parahaemolyticus* isolates from water and bivalve mollusks in the estuary of the river Subaé, Francisco do Conde, Bahia, Brazil.

| Sources | No.[a] | Antimicrobials | MARI[b] | Plasmid resistence |
|---|---|---|---|---|
| Water | 1 | CAZ[c] | – | CAZ |
| | 1 | CAZ | – | – |
| | 9 | AMP[d], CFL[e] | 0.18 | – |
| | 4 | AMP | – | – |
| | 1 | CFL | – | – |
| | 1 | CFL, CLO | 0.18 | CFL, CLO |
| Mollusk *in nature* | 4 | AMP, CFL | 0.18 | – |
| | 2 | AMP | – | – |
| | 1 | AMP, CFL | 0.18 | CFL |
| | 1 | AMP, CFL, CLO[f], SUT[g] | 0.36 | CLO, SUT |
| Mollusk processed | 1 | AMP, CFL, CAZ | 0.27 | CAZ |
| | 3 | AMP | – | – |
| | 1 | CLO | – | – |

[a] Number of strains.
[b] Antimicrobial Multiple Resistance Index.
[c] Ceftazidime.
[d] Ampicillin.
[e] Cephalothin.
[f] Chloramphenicol.
[g] Sulfamethoxazol + trimethoprim.

toxicity (caseinases), membrane lipid degradation (lipases), capacity to act as hemolysin (phospholipases), and hydrolysis of phosphodiester bonds in the DNA structure (Costa et al., 2013). Proteases, lipases, and cytotoxin production are common to *Vibrio* genus and are responsible for the conditions of hemorrhagic edema and may cause damage to the host's defense system, there by favoring the development of infectious processes (Cabrera-Rodríguez et al., 2008). Exoenzymes present in microorganisms isolated from water and foods pose a risk to the communities that use the estuary for leisure and/or food extraction because they increase the chances of infection by the microorganism.

Enzymatic characterization of *Vibrio* spp. in a coastal town in Italy from marine environment with low anthropogenic impact revealed a high percentage of gelatinases (86%), lipases (54%), proteases (14%), ureases (7%), and those with hemolytic activity (3%) (Masini et al., 2007). Costa et al. (2013) determined the virulence profile of *V. parahaemolyticus* isolates in fresh and frozen oysters and detected 100% activity for DNase and 97.5% for amylase.

The Kanagawa phenomenon (β-hemolysis) was observed in 57% in *V. parahaemolyticus* strains (Table 4), showing that the isolates are capable of causing hemolysis of human erythrocytes, thereby posing a risk for people who consume mollusks *in nature* or use the water of the estuary for recreation.

**Table 4**
Phenotypic virulence profile in *Vibrio parahaemolyticus* from water and bivalve mollusks in the estuary of the river Subaé, São Francisco do Conde, Bahia, Brazil.

| Exoenzymes | *Vibrio parahaemolyticus* (% of positive isolates) | | | |
|---|---|---|---|---|
| | W[a] (17) | Bm[b] (8) | Pb[c] (5) | Total (%) |
| Lipase | 12 | 25 | 0 | 13 |
| Phospholipase | 82 | 37 | 80 | 70 |
| Gelatinase | 41 | 62 | 20 | 43 |
| Caseinase | 0 | 0 | 20 | 07 |
| DNase | 13 | 100 | 80 | 83 |
| Amylase | 100 | 100 | 80 | 97 |
| Urease | 0 | 0 | 0 | 0 |
| β-hemolysin | 53 | 50 | 80 | 57 |

[a] Water.
[b] Bivalve mollusk *in nature*.
[c] Processed bivalve mollusk.

In Asia and Japan, the bacterium is recognized as the main cause of foodborne illnesses because of high fish consumption (Yang et al., 2008). In China, the β-hemolysin test is used routinely to detect pathogenic *V. parahaemolyticus* strains (Hongping et al., 2011). In environmental samples, the presence of Kanagawa-positive strains has been observed in only 1–3% of the isolates, whereas in the strains of clinical origin, the detection percentage is 90% (Vongxay et al., 2008).

The capacity to hydrolyze urea was not observed in the *V. parahaemolyticus* isolates (Table 4). Urea hydrolysis is directly associated with the presence of *trh* gene (Nair et al., 2007). However, the *tdh* and *trh* genes associated with the virulence in *V. parahaemolyticus* were not detected; strains isolated from environmental samples were generally non-toxigenic (Elmahdi et al., 2016). *V. parahaemolyticus* pathogenicity in clinical and environmental samples was reported, and it was characterized by 191 isolates from shellfish, out of which, only 2% presented the *tdh* gene and 4% the *trh* gene (Vongxay et al., 2008).

No relationship was observed between the presence of *trh* gene and the positivity for the Kanagawa phenomenon in the *V. parahaemolyticus* isolates, which may be associated with the existence of other virulence factors, for example, type 3 secretion systems (T3SSs) that release bacterial effectors into the host's cytoplasm and lead to cell lysis. The T3SS1 is found in all strains of *V. parahaemolyticus*, whereas T3SS2 is present only in the strains positive to the Kanagawa phenomenon. These systems are involved in cytotoxicity/enterotoxicity of eukaryotic receptors (Caburlotto et al., 2016; Mala et al., 2016). Besides, studies have shown that regardless of the TDH production, the bacterium alters the epithelial barrier of the host by introducing rearrangements in the cytoskeleton, a pro-inflammatory response, and/or cell death due to the involvement of other virulence factors (Nair et al., 2007).

A study conducted in Italy showed that about 10% of the clinical strains contained neither the gene *tdh* nor *trh* in cases of infection (Ottaviani et al., 2013). Besides this, Pereira et al. (2004) in their study reported Kanagawa-negative strains to be responsible for gastrointestinal infection in humans.

In a study on the prevalence of pathogenic *Vibrios* in crustaceous samples in Italy, the presence of *tdh* gene was identified in three isolates with a prevalence of 1.4% (Caburlotto et al., 2016). The prevalence of virulence genes in clinical samples was found to be higher than in the environmental samples (Mala et al., 2016).

*V. parahaemolyticus* associated with food poisoning outbreaks involving oysters in Spain also reported an absence of urease and *trh* genes (Lozano-León et al., 2003). Vongxay et al. (2008) characterized *V. parahaemolyticus* pathogenicity in clinical and environmental samples and reported that out of 191 isolates from shellfish, only 2% presented the *tdh* gene and 4% the *trh* gene.

## 4. Conclusion

The present study demonstrated that the natural bed of mollusks serve as sources of *V. parahaemolyticus* and other *Vibrio* spp. The bivalves in these areas can represent a potential reservoir of resistant genes that can be transmitted to humans through consumption of sea food and when these genes are mobile, being carried on plasmid their spread can be rapid by conjugation. In spite of the absence of *tdh* and *trh* genes, the *V. parahaemolyticus* strains carry markers of virulence and revealed high rates of resistance to antimicrobial drugs that could be ineffective for treating their infections. These bacterial lineages can be concentrated by mollusks, thereby posing a health risk to the people those consume them.

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