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Multidrug-resistant *Vibrio* associated with an estuary affected by shrimp farming in Northeastern Brazil

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ABSTRACT

Bacteria of genus *Vibrio* with multidrug resistance in shrimp farm environment were recurrent. Thus, the aim of this study was to evaluate the antimicrobial resistance profile of 70 strains of *Vibrio* isolated from water and sediment of Acaraú estuary, Ceará, Brazil. In order to achieve this goal, disk diffusion technique was used with the following antimicrobial agents: ampicillin (Amp), aztreonam (Atm), cephalothin (Cef), cefotaxime (Ctx), ceftriaxone (Cro), ciprofloxacin (Cip), chloramphenicol (Clo), florfenicol (Flo), nitrofurantoin (Nit), gentamicin (Gen), oxytetracycline (Otc), tetracycline (Tet), streptomycin (Str), nalidixic acid (Nal), and sulfazotrim (Sut). All *Vibrio* strains were resistant to at least one antimicrobial agent, being verified as 17 multidrug-resistant profiles. All strains resistant to Otc and Tet were characterized to exhibit plasmidial resistance. Therefore, *Vibrio* strains from Acaraú estuary pose a risk to public health and aquatic culture.

1. Introduction

Detection of bacteria with multiple resistance to antimicrobials (MRA) in aquatic environment has been a growing worldwide concern (Lawrence and Jeyakumar, 2013). In this context, the interaction between clinically relevant bacteria and MRA bacteria, associated with overuse of antimicrobials for human and veterinary purposes, is considered a serious environmental problem (Nascimento and Araujo, 2014; Berendonk et al., 2015).

Bacterial resistance may have chromosomal origin or be mediated by plasmids (Coutinho et al., 2014). Chromosomal resistance is an inherent characteristic of the bacteria and, when detected, assumes remarkable significance in the prophylactic treatment (Dzidic et al., 2008; Davies and Davies, 2010). By contrast, plasmid-mediated resistance poses a risk to public health because of the transfer of mobile genetic elements in bacteria to the same species or different species, which increases its spread (Svara and Rankin, 2011; Schultsz and Geerlings, 2012).

The intensive use of antimicrobials is a major cause for the introduction of bacterial resistance (Martinez, 2009). Farming activities of marine organisms, such as shrimp farming, require a large amount of antimicrobial substances that are wrongly administered as growth promoters (Baquero et al., 2008; Kuemmerer, 2009). In addition, wastewater disposal without prior treatment in estuarine waters may change the microbial diversity of that environment and promote the spread of antimicrobial resistance (Barraza-Guardado et al., 2013).

Bacteria of genus *Vibrio* are endemic in marine and estuarine environments, and its detection in shrimp farming environment is recurrent (Vieira et al., 2010; Shaw et al., 2014). Strains with MRA have been reported for species such as *Vibrio harveyi* and *Vibrio alginolyticus*, which are recognized as opportunistic pathogens in cultured marine shrimp (Kitaoka et al., 2011; Parvathi et al., 2011). Furthermore, MRA *Vibrio* may be transmitted from farmed organisms to humans when the bacteria-containing fish is consumed without cooking or in kind (Costa et al., 2015a; Raissy et al., 2012).

Considering the importance of shrimp farming activity in Northeastern Brazil, it was aimed to verify the antimicrobial susceptibility profile of *Vibrio* strains of estuarine environment with recognized activity of creation of the marine shrimp *Litopenaeus vannamei*.

2. Materials and methods

2.1. Source of Vibrio strains

A total of 70 strains of *Vibrio* were selected from bacterial collection of the Fishing and Environmental Microbiology Laboratory, Federal University of Ceará, Brazil (Table 1). The strains were isolated from water and sediment of Acaraú estuary (local shrimp farming) in Ceará (Fig. 1). Bacterial isolates were grown previously on thiosulfate citrate bile and sucrose (TCBS) agar and identified according to the method proposed by Noguerola and Blanch (2008). The phenotypic identification was confirmed by analysis of 16S rDNA sequencing.

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Table 1
Source and nomenclature of strains of *Vibrio* species isolated from the estuary of Acaraú, Ceará, Brazil.

Species	Source of strains		Total
	Water	Sediment	
Vibrio calviensis	V01		1
Vibrio cholerae	V02	V03, V04	3
Vibrio corallilyticus	V05, V06, V07, V08, V09, V10, V11, V12, V13, V14	V15, V16, V17, V18, V19, V20, V21, V22	18
Vibrio diabolicus	V23	V24, V25	3
Vibrio fortis	–	V26	1
Vibrio gigantis	V27	–	1
Vibrio harveyi	–	V28	1
Vibrio litoralis	V29	–	1
Vibrio logei	V30	–	1
Vibrio mimicus	V31, V32, V33, V34, V35, V36, V37, V38, V39, V40, V41, V42, V43	V44, V45, V46, V47, V48, V49, V50, V51, V52, V53, V54, V55, V56, V57, V58, V59, V60, V61, V62	32
Vibrio parahaemolyticus	V63, V64	–	2
Vibrio proteolyticus	V65, V66	V67, V68	4
Vibrio ruber	–	V69	1
Vibrio rumoiensis	–	V70	1
Total	33 (47.1%)	37 (52.9%)	70

2.2. Antimicrobial susceptibility test

The antimicrobial susceptibility test was performed according to the recommendations of Clinical Laboratory and Standards Institute (CLSI, 2015), using the disk diffusion method (Bauer et al., 1966). *Vibrio* strains were grown on trypticase soy agar (TSA) supplemented with 1% (*w*/*v*) of NaCl and incubated at 35 °C for overnight. After incubation, the strains were adjusted to a concentration of 1×10^8 CFU mL^{-1}, corresponding to turbidity 0.5 of McFarland scale in a saline solution of 1% (*w*/*v*) of NaCl. The strains were inoculated onto plates containing Mueller–Hinton (MH) agar supplemented with 1% (*w*/*v*) NaCl, in which the antimicrobial agent disks were applied. Antimicrobial agents were selected based on recognized use in shrimp industry and divided into three mechanisms of action groups: Group I — inhibitors of cell wall synthesis: ampicillin (Amp 10 μg), aztreonam (Atm 30 μg), cephalothin (Cef 30 μg), ciprofloxacin (Cip 5 μg), ceftriaxone (Cro 30 μg), and cefotaxime (Ctx 30 μg); Group II — inhibitors of protein synthesis: chloramphenicol (Clo 30 μg), florfenicol (Flo 25 μg), nitrofurantoin (Nit 300 μg), gentamicin (Gen 10 μg), oxytetracycline (Otc 30 μg), tetracycline (Tet 30 μg), and streptomycin (Str 10 μg); Group III — inhibitors of nucleic acid synthesis: nalidixic acid (Nal 30 μg) and sulfazotrim (Sut 25 μg).

2.3. Plasmid curing

Vibrio-resistant strains were subjected to plasmid curing technique by the action of acridine orange dye at a concentration of 100 μg mL^{-1} (Sigma), according to Molina-Aja et al. (2002). After exposure to the mutagen, the strains were submitted to the antimicrobial susceptibility test to detect initially resistant antimicrobials. Resistance was considered potentially chromosomal when permanence of the resistance phenotype to certain antimicrobial was observed after curing, otherwise it was characterized as plasmid.

3. Results and discussion

Among the *Vibrio* strains isolated from water, 16 (48.5%) were resistant to Cef, 12 (36.4%) to Flo, 11 (33.3%) to Amp, 10 (30.3%) to Nit, 4 (12.1%) to Otc, and 4 (12.1%) to Tet (Table 2). However, for sediment strains, 15 (40.5%) was resistant to Amp, 11 (29.7%) to Flo, 5 (13.5%) to Nit, 4 (10.8%) to Otc, 3 (8.1%) to Atm, 2 (5.4%) to Tet, 1 (2.7%) to

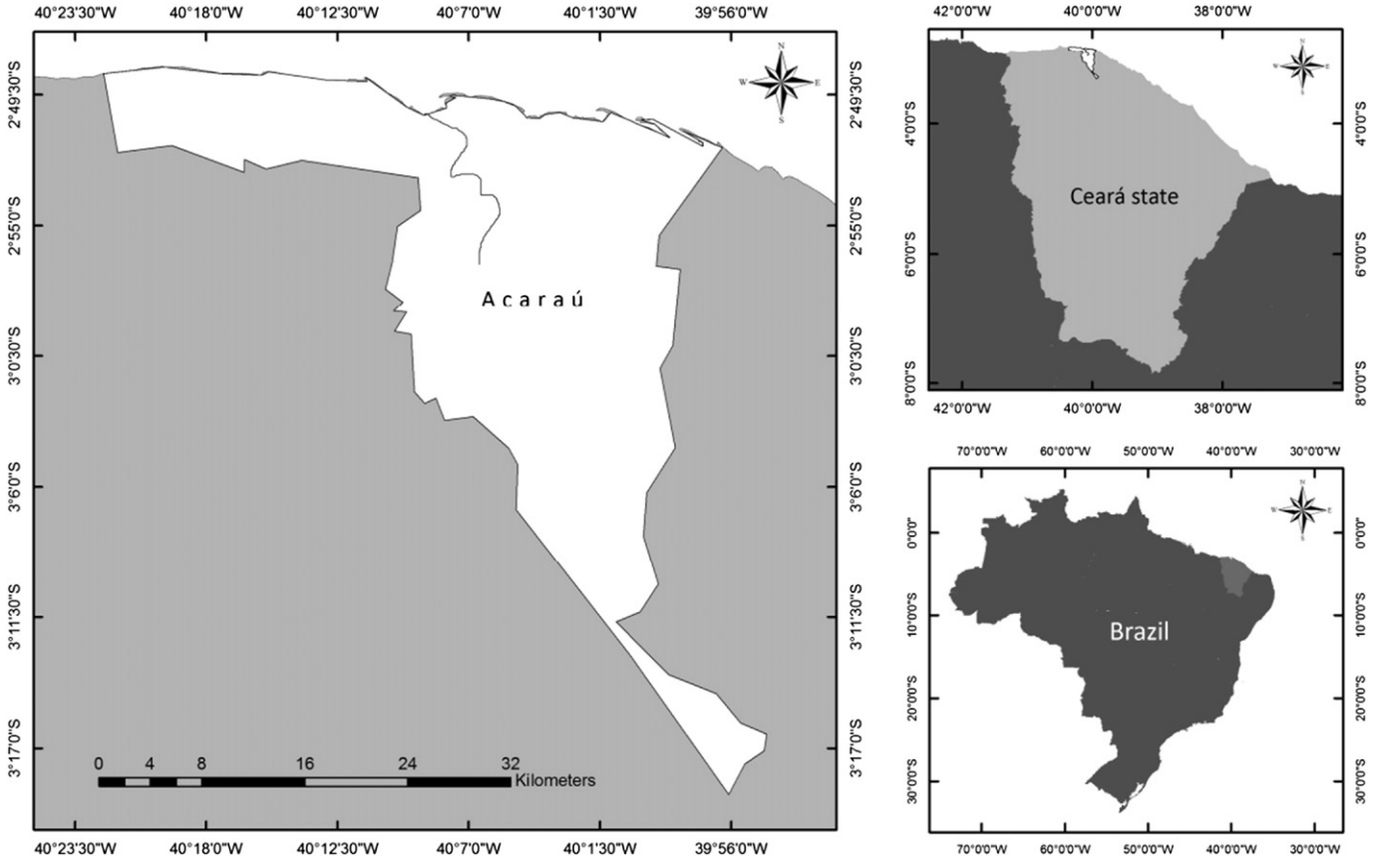

Fig. 1. Sample location of water and sediment in Acaraú estuary, Northeastern Brazil.

Cef, and 1 (2.7%) to Clo (Table 2). All *Vibrio* strains from water and sediment were resistant to at least one of the tested antimicrobials.

Resistance to antimicrobial inhibitors of nucleic acid synthesis Nal and Sut was observed (Group III) (Table 2). However, a significant number of strains showed resistance to cell wall synthesis (Group I, $n = 45$) and protein synthesis antimicrobial inhibitors (Group II, $n = 47$), of which the major recurring resistance in each group was to Flo and Cef, respectively (Table 2).

In a study by Costa et al. (2015b), the authors found *Vibrio*, isolated from the marine shrimp hemolymph, with resistance to ampicillin and cephalothin, suggesting initial resistance in shrimp aquaculture environment. Holmstrom et al. (2003) considered that tetracycline and oxytetracycline resistance in bacteria isolated from shrimp farming is disordered by the use of these antimicrobials during cultivation.

A total of 23 resistance profiles were determined, of which 6 and 17 were unique and multidrug resistances, respectively. In sum, 11 multidrug-resistant profiles were observed in *Vibrio* strains from water and nine in strains from sediment (Table 3). The profile Otc + Tet was not considered multidrug resistant, because antimicrobials tetracycline and oxytetracycline belong to the same chemical class, and thus have the same mechanism of action (Magiorakos et al., 2012).

The profile Cef + Flo was the most frequent among isolates, of which five strains were from water: *Vibrio coralliilyticus* (V07, V08, V09) and *V. mimicus* (V40, V41); and three from sediment: *V. coralliilyticus* (V17), *V. harveyi* (V28), and *V. mimicus* (V51). *V. coralliilyticus* has been found to be a unique pathogen of coral *Pocillopora damicornis* and its pathogenic potential in shrimp has not been evidenced. However, multidrug-resistant strains have been reported in farmed shrimp (Albuquerque et al., 2013), which, according to Kimes et al. (2012), is probably a temperature-dependent pathogen. Other pathogenic *Vibrio* strains, such as *V. harveyi* and *V. mimicus*, have also been reported with multidrug-resistant profile in this study. According to Chaterjee and Haldar (2012), *V. harveyi* is an opportunistic shrimp pathogen related to *Vibrio* infections with high mortality.

All unique resistance profiles were common to strains from water and sediment; however, only three multidrug-resistant profiles were observed in both water and sediment isolates: Amp + Cef, Cef + Flo, and Amp + Clf + Flo (Table 3). The profile Amp + Cef + Flo + Nit was observed for three strains of different species isolated from water: *V. cholerae* (V02), *V. coralliilyticus* (V05), and *V. parahaemolyticus* (V64). By contrast, the profile Amp + Flo + Nit + Otc + Tet was observed for only one strain isolated from water: *V. parahaemolyticus* (V63). *V. parahaemolyticus* has been found to be a causative pathogen of acute hepatopancreatic necrosis disease (AHPND), which in most cases leads to death of all affected shrimp in <1 week (Soto-Rodriguez et al., 2012). In this study, two strains of this pathogen showed multidrug-resistant profiles (Amp + Flo + Nit + Otc + Tet and Amp + Cef + Flo + Nit), which pose a potential risk to the aquatic health of the farmed shrimp.

It was possible to verify, in both strains from water and sediment, that the resistance genotype was considered to exhibit plasmid nature for all resistant strains to Otc and Tet (Table 4). According to Pote et al. (2003), bacterial plasmids and other genetic structures remaining in the soil can be carried by groundwater for a longer period of time and still appeared biologically active when incorporated into new bacterial strains. Guglielmetti et al. (2009) demonstrated that the tetracycline-resistant plasmid can be easily spread among bacteria isolated from cultured organisms and human pathogenic bacteria.

The presence of bacterial strains with resistance mediated by plasmids reinforces the importance of ecological monitoring of environmental bacteria in areas with proven human impact. According to Thavasi et al. (2007), resistance plasmids can be shared among bacteria of the same species or different species, thus favoring dissemination of resistance genotype in the environment. In addition, after bacterium death and consequent disruption of cell wall and membranes, genetic structures such as plasmids and integrons are provided in the aquatic environment and may be incorporated in other bacteria (Mao et al., 2014).

According to Czekalski et al. (2014), the presence of bacterial strains with resistance mediated by plasmids suggests an ongoing source of bacterial contamination in the environment, for example domestic sewage without treatment. In the area of our study, it is assumed what shrimp farming activity exerts a determining factor to perpetuation of multidrug-resistant bacteria in the estuarine environment. Tetracycline-resistant plasmid was observed for one *V. parahaemolyticus* strain (V63). Han et al. (2015) considered that the presence of tetracycline-resistant plasmids has a strong association with AHPND in farmed shrimp. Other nonpathogenic *Vibrio* also showed plasmid-resistant profiles, representing a risk due to plasmids that confers that antimicrobial

Table 2
Number of susceptible, intermediate, and resistant strains isolated from water and sediment of Acaraú estuary, Northeastern Brazil.

Antibiotic per mechanism of action group	Water ($n = 33$)			Sediment ($n = 37$)		
	R	I	S	R	I	S
Group I — inhibitors of cell wall synthesis						
Ampicillin	11	5	17	15	5	17
Aztreonam	–	1	32	3	1	33
Cephalothin	16	1	16	1	4	23
Ciprofloxacin	–	–	33	–	–	37
Ceftriaxone	–	–	33	–	1	36
Cephotaxin	–	1	32	–	2	35
Group II — inhibitors of protein synthesis						
Chloramphenicol	–	–	33	1	1	35
Florfenicol	12	–	21	11	–	26
Gentamicin	–	–	33	–	–	37
Nitrofurantoin	10	1	22	5	–	32
Oxytetracycline	4	2	27	4	1	32
Tetracycline	4	4	25	2	2	33
Streptomycin	–	–	33	–	–	37
Group III — inhibitors of nucleic acid synthesis						
Nalidixic acid	–	–	33	–	–	37
Sulfazotrim	–	–	33	–	–	37

R: resistant; I: intermediate resistance; S: susceptible.

Table 3
Resistance profiles of *Vibrio* strains isolated from water and sediment of an estuary of Northeastern Brazil.

Resistance type (23 profiles)	Water ($n = 33$)	Sediment ($n = 37$)
Amp	2	7
Cef	3	4
Flo	1	3
Nit	2	1
Otc	1	1
Amp + Cef*	2	2
Amp + Flo*	1	–
Amp + Nit*	–	1
Amp + Nit*	1	–
Cef + Flo*	5	3
Cef + Nit*	1	–
Flo + Nit*	1	–
Otc + Tet	1	1
Amp + Atm + Flo*	–	1
Amp + Atm + Nit*	–	1
Amp + Cef + Flo*	1	1
Clo + Otc + Tet*	–	1
Nit + Otc + Tet*	1	–
Cef + Flo + Nit*	1	–
Amp + Atm + Flo + Nit*	–	1
Amp + Flo + Nit + Otc*	–	1
Amp + Cef + Flo + Nit*	3	–
Amp + Flo + Nit + Otc + Tet*	1	–
Total	28	29

* Multidrug-resistant profile.

Table 4
Number of *Vibrio* strains after plasmid curing by resistance source.

Antimicrobial	Water			Sediment		
	N	Chromosomal	Plasmid	N	Chromosomal	Plasmid
Ampicillin	11	1	10	15	3	12
Aztreonam	–	–	–	3	1	2
Cephalothin	16	2	14	1	0	1
Chloramphenicol	–	–	–	1	0	1
Florfenicol	12	2	10	11	1	10
Nitrofurantoin	10	2	8	5	1	4
Oxytetracycline	4	0	4	4	0	4
Tetracycline	4	0	4	2	0	2

N: number of resistant strains per antimicrobial before plasmid curing.

resistance may be shared for species with recognized pathogen to farmed shrimp.

Chromosomal resistance is an innate characteristic of bacterial strains that is important for choice of the most effective antimicrobials for treating bacterial diseases (Giedraitiene et al., 2011). However, the interaction between environmental bacteria, which have multidrug-resistant genes, and known bacterial pathogens to human and farmed aquatic organisms poses a risk to public health and environment.

4. Conclusion

Vibrio strains were detected with multiple antimicrobial resistance in water and sediment of Acaraú estuary, Northeastern Brazil. Characterization of strains with multiple antibiotic-resistant plasmids poses a risk to public health, mainly in cultivation regions of marine shrimp *L. vannamei*.

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