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Full Length Research Paper

Vibrio cholerae non-O1 in bivalve mollusks harvesting area in Bahia, Brazil

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The aim of this study was to characterize the antimicrobial resistance and pathogenicity potential of *Vibrio cholerae* isolates originated from water samples and bivalve mollusks. The strains were subjected to phenotypic identification and molecular confirmation using the species-specific initiator *(OmpW)*; minimum inhibitory concentration (MIC) was determined; and the production of metallo-β-lactamases (MβLs) and virulence potential of the strains by using the initiator *ctxAB* (cholera toxin), *tcp* (toxin co-regulator pilus), *rfb*O1 (serogroup O1) and *zot* (zonula occludens toxin) were investigated. Six isolates of the bacterium (three from water and three from bivalve mollusks) were confirmed through the biochemical and specific gene detection tests. The isolates presented a high susceptibility toward the tested antimicrobials (91%) (10/11). One of the strains from water showing resistance to imipenem (MIC 20 µg), and producing MβLs did not show any involvement of plasmids. The genes related to the virulence were not detected; and all of the *V. cholerae* isolates belonged to the non-O1 serotype. However, the presence of an imipenem-resistant and MβLs-producing *V. cholerae* in a river mouth aquatic environment, which is a natural aviary of bivalve mollusks, represents a risk to the health of the population and alarms the public health agencies.

Key words: Mollusc, public health, antibiotic resistance.

INTRODUCTION

Vibrio cholerae is the causative agent of cholera, which inhabits aquatic environments. Water has a significant role in its transmission and epidemiology of this disease leading to outbreaks at endemic, epidemic, and pandemic levels (Goel et al., 2010).

Based on somatic antigen (O antigen), *V. cholerae* is classified into serogroups or serovars, and a total of 206 serogroups of *V. cholerae* have been identified so far. The toxigenic serogroups O1 and O139 have been found to be directly associated with epidemic (O1 and O139) and pandemic (O1) cholera (Raychoudhuri et al., 2009). The O1 serogroup can be further classified into two biotypes, classical and El Tor. El Tor was disseminated around the world the etiological agent behind current

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pandemic (Espiñeira et al., 2010).

V. cholerae has frequently been isolated from environmental samples, which mainly consists of serogroups, non-O1/ non-O139. These serogroups may cause diarrheal diseases less severe than cholera and do not present epidemic potential (Wong et al., 2012). Although they normally do not produce a cholera toxin, they carry other virulent factors involved in the pathogenicity, including the production of hemolysins, proteases, hemagglutinins, and may have multiple drug resistance (Oufdou and Mezrioui, 2012). It is reported that in aquatic environment, one serogroup can be converted to another by homologous recombinations, or by mutation and/or rearrangement, as noticed in O139 lineage (Blokesch and Schoolnik, 2007).

Gram-negative bacteria may acquire, maintain and express new genetic information and become resistant to one or several antimicrobials (Walsh et al., 2002). The extensive and/or inappropriate use of antimicrobials in humans and animals, agriculture and aquaculture as well as improper disposal of antimicrobials contributes in the development of resistant bacteria in the environment (Manjusha and Sarita, 2013).

The study of resistance to antimicrobial agents in indigenous aquatic microorganisms is important since it indicates degree of modification of ecosystems by man. The release of antimicrobials in municipal sewage system, surface water, groundwater, soil sediments, and mud samples exerts a selective pressure on the environmental microorganisms, and thus contributes in the proliferation of resistant microbes (Baquero et al., 2008).

Metallo-β-lactamase, an enzyme produced by bacteria, hydrolyzes carbapenems, penicillin, and cephalosporin. The main members of MβL family include imipenemase (IMP) and Verona imipenemase (VIM). VIM is well known and commonly found in several bacterial species. It has eleven variants reported all over the world, especially in Europe and Asia (Villegas et al., 2006). In this study, *V. cholerae* strains were characterized according to the presence of genes causing virulence and resistance to antimicrobials. These strains were isolated from the river waters in Recôncavo da Bahia (Brazil), an area where bivalve mollusks are harvested.

MATERIALS AND METHODS

Isolation and identification

We analyzed six bacterial isolates suspects of *V. cholerae* from water samples and bivalve mollusks (*Crassostrea rhizophorae* and *Mytella guyanensis*) harvested from the river mouth of São Francisco do Conde, Bahia (S 12° 33' 52.4"/ W 038º 41' 40.5"). These samples belong to the microbial specimen collection of the Food and Environmental Microbiology Laboratory, at the Nucleus for Studies of Fishing and Aquaculture (NEPA), of the Federal University of Recôncavo da Bahia. The strains were stored in agar stock at 15°C.

Initially, the isolates were re-isolated and biochemically verified. The isolates were grown in BHI broth containing 1% NaCl (pH 8.5) at 37ºC for 24 h and then inoculated in Petri plates containing thiosulfate citrate bile salts sucrose (TCBS) agar. Biochemical identification was carried out with the help of the biochemical keys proposed by Noguerola and Blanch (2008). Molecular identification was performed with the help of PCR by using primers directed to *ompW* gene (external membrane protein) (304 bp); forward: 5' - CAC CAA GAA GGT GAC TTT ATT GTG- 3' and reverse: 5' - GGT TTG TCG AAT TAG CTT CAC C - 3' (Goel et al., 2007).

Total DNA extraction

The genomic DNA was extracted by using modified protocol proposed by Sambrook et al. (1989). Initially, 2 mL of culture cultivated in tryptone soy broth (TSB) containing 1% NaCl at pH 8.5 for 24 h was transferred to microtubes and frozen. After being frozen for 30 min, the material was defrosted and centrifuged at 5.0 rpm for 10 min. The supernatant was discarded; 1 mL of sterile water was added to the pellet and centrifuged again.

The pellet was re-suspended in 500 µL of extraction buffer (0.15 M NaCl, 50 mM Tris-HCL, 10 mM EDTA, 2% SDS, pH 8.0) supplemented with lysozyme and incubated in water bath at 65ºC for 1 h. Then 0.5 mL of chloroform: isoamyl alcohol (24:1) and 0.5 mL of potassium acetate (0.5 M) was added and centrifuged at 10.0 rpm for 15 min. The supernatant was collected and transferred to a new microtube and 1 volume of ice-cold isopropyl alcohol was added and centrifuged at 10,000 rpm for 15 min. The pellet was washed twice with cold 80% v/v ethanol, centrifuged and dried overnight. The DNA was re-suspended in 0.1 mL of TE buffer (10 mM Tris-HCl; 1 mM EDTA) and kept at -20°C.

Polymerase chain reaction (PCR) amplification

To confirm *V. cholerae* species and to detect virulence factors, the DNA was amplified using multiplex PCR technique with the help of specific primers. The specificity of the multiplex PCR was determined by using a standard *V. cholerae* strain (ATCC 19782). The PCR was performed in 25 µL reaction mixture containing target DNA. The amplification conditions used for detection of virulence factors were as follows: Initial denaturation at 94°C for 1 min, followed by 30 cycles of amplification, annealing at 59°C for 1 min, and extension at 72°C for 2 min. Before initiation of the first cycle, the reaction mixture was heated (10 min, 94°C) to complete denaturation of the template, after the last cycle, the reaction mixture was subjected at 72°C for 10 min for final extension (Goel et al., 2007).

The PCR amplified products were separated by using 1.5% agarose gel (at 150 V, 400 mA for 60 min) and visualized using transilluminator. The gels were photographed using digital documentation system Kodak EDAS290.

Detection of the virulence genes

For the detection of the virulence genes, the DNA was subjected to multiplex PCR using primers specifics for *ctxAB* (cholera toxin) (forward: 5' - GCC GGG TTG TGG GAA TGC TCC AAG - 3' and reverse: 5' - GCC ATA CTA ATT GCG GCA ATC GCA TG - 3'); *tcp* (toxin co-regulator pilus) (forward: 5' - CGT TGG CGG TCA GTC TTG - 3' and reverse: 5' - CGG GCT TTC TTC TTG TTC G - 3'); *rfbO1* (somatic antigen) (forward: 5' - TCT ATG TGC TGC GAT TGG TG - 3' and reverse: 5' - CCC CGA AAA CCT AAT GTG AG - 3'); *zot* (zonula occludens toxin) (forward: 5'- TCG CTT AAC GAT GGC GCG TTT T - 3' and reverse: 5' - AAC CCC GTT TCA CTT CTA CCC A - 3') (Goel et al., 2007).

Table 1. Occurence of *ompW* specific gene and *ctxAB, tcp, zot* and *rfbO1* virulence genes of *Vibrio cholerae* isolates tested by multiplex PCR amplification.

Isolates	Specific gene		Virulence profile		
	***ompW* (304 bp)**	***ctxAB* (536 bp)**	***Tcp* (805 bp)**	***Zot* (947 bp)**	***rfbO1* (638 bp)**
Vc5	+	-	-	-	-
Vc6	+	-	-	-	-
Vc7	+	-	-	-	-
Vc8	+	-	-	-	-
Vc9	+	-	-	-	-
Vc10	+	-	-	-	-
V. cholerae ATCC 19782	+	+	+	+	+

Antimicrobial susceptibility

The antimicrobial susceptibility was performed by disk diffusion method (CLSI, 2010) using commercially available antibiotic-containing disks (Laborclin, Brazil): nalidixic acid (30 µg), ampicillin (10 µg), gentamicin (10 µg), cefalotin (30 µg), ceftazidime (30 µg), ciprofloxacin (30 µg), chloramphenicol (30 µg), imipenem (10 µg), nitrofurantoin (300 µg), sulfamethoxazole + trimethoprim (25 µg), and tetracycline (30 µg). Bacterial strains *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were used as reference controls.

The minimum inhibitory concentration (MIC) of antimicrobials was determined for the strains showing antibiotic resistance. We applied the method of increasing antimicrobial dilution in Mueller-Hinton broth (HiMedia) with the concentrations of 15, 20 and 25 µg, starting from the concentration immediately higher in the commercial discs (CLSI, 2010).

Detection of the resistance mediated by R-plasmids

The presence of R-plasmids was tested for the strains showing an antimicrobial resistant profile. We used acridine orange (AO) (Merck) at a concentration of 100 µg mL^{-1} as a curing agent. After culturing at 37°C for 24 h, the samples were aliquoted (200 µL) to the tubes containing Luria Bertani (LB) broth (control) and LB plus AO and incubated them at 37°C for 24 h. These cultures were submitted again to the antibiogram (Molina-Aja et al., 2002). We extracted plasmid DNA from the strains with antimicrobial resistance by using the Plasmid Mini Kit I (Omega/Bio-Tek®, GA) and separated it on 0.8% agarose gel. The gels were digitally photographed by an L-pix system (Locus Biotechnology).

Determination of the production of metallo-β-lactamases (MβLs)

The cultures having imipenem resistance were evaluated for the production of MβL enzymes by disk approximation test using 2-mercaptopropionic acid (Sigma-Aldrich, Buchs, United States) close to a ceftazidime disk, according to the protocols proposed by the Yong et al. (2002), Lee et al. (2003) and CLSI (2010). After standardization (10^8 CFU mL^{-1}), the inoculum was suspended in 17 mL of Mueller-Hinton agar. Then the discs containing imipenem (10 µg), ceftazidime (30 µg), 3 µg of a 2-MPA (1.2 g mL^{-1}), or 10 µL of 0.5 M EDTA (pH 8) were positioned on the plate. The discs of EDTA and MPA were placed 15 and 20 mm apart (center to center), respectively, to that of the antimicrobial containing disc. The plates were incubated at 37°C for 18 h. The presence of an inhibition zone or increasing in its size around the discs containing imipenem and/or ceftazidime located close to the disc containing MPA and/or EDTA was considered positive for the MβL production.

RESULTS AND DISCUSSION

The *V. cholerae* isolates were identified based on their phenotype and further confirmed by the presence of *ompW* (Table 1). This indicates the efficiency of the dichotomous key based on the morphology of the cells and biochemical evidence. The *ompW* specific gene is highly conserved among *V. cholerae* strains belonging to different serotypes and biotypes (Goel et al., 2007).

The state of Bahia is in the third position of largest domestic producer of marine extractive fishing in the country, it is the first producer of the region northeast (Brazil, 2010). In the Baia de Todos os Santos, Recôncavo region da Bahia, area of this study, the activity collect shellfish is intense, especially bivalve mollusks. The risk of consumption of these organisms is due to ingestion of raw oysters not debugged or had mild heat treatment. This places the oysters on top of foods responsible for foodborne illnesses.

Another problem for the state is the serious problems of sanitation and industrial impacts are increasing on an increasing scale, risk environmental quality. The presence of pollutants not only endangers the health of the population, as commits to fishing activity, that in most cases it is the only source of income for many fishermen and seafood restaurants in the region.

The absence in region of virulent strains of *V. cholerae* is satisfactory, because it is a region that has suffered from the action antrópica, moreover supplies seafood in region. The lack of real epidemiological data makes extraction of areas of bivalve mollusks need to be monitored periodically, whereas cases of cholera occur in isolation in some regions of the country.

In 2012, according to the World Health Organization, 245.393 cases of cholera were reported in 48 countries

Table 2. Resistance microbial, determination of minimum inhibitory concentration (MIC), enzyme detection metallo-β-lactamases (MβLs) and R-plasmids presence in *Vibrio cholerae* isolates from water and bivalve mollusks in the estuary of the river Subaé, São Francisco do Conde, Bahia, Brazil.

Antimicrobial	[µg]	*Vibrio cholerae* (n=6)								
		Water (3) (%)			Bm (3) (%)			MIC (µg/mL)	MβLs	R-plasmids
		S	I	R	S	I	R			
Gentamicin	10	100	0	0	100	0	0	-	-	-
Ampicillin	10	100	0	0	100	0	0	-	-	-
Cephalothin	30	100	0	0	100	0	0	-	-	-
Ceftazidime	30	100	0	0	100	0	0	-	-	-
Imipenem	10	67	0	33	100	0	0	20 µg	+	-
Chloraphenicol		100	0	0	100	0	0	-	-	-
Nitrofurantoin	300	100	0	0	100	0	0	-	-	-
Nalidixic acid	30	100	0	0	100	0	0	-	-	-
Ciprofloxacin	30	100	0	0	100	0	0	-	-	-
Sulfamethoxazol+trimethoprim	25	100	0	0	100	0	0	-	-	-
Tetracycline	30	100	0	0	100	0	0	-	-	-

S, Susceptible; I, intermediate; R, resistant; Bm, Bivalve mollusks; MβLs, Metallo-β-lactamases.

across the globe (WHO, 2009). The largest number of cases has been observed in African countries (WHO, 2013). In America, only six cases have been reported. In Brazil, there has been no registered case of indigenous cholera since 2005; despite of increased number of *V. cholerae* O1 has been isolated from environmental samples.

The 26 confirmed cases have been registered in Brazil during the outbreak between 2004 and 2005 in the state of Pernambuco. The Northeastern region had large number of cases due to its climate and poor sanitation (SVS, 2008).

Virulence profile

We did not detect the genes *ctxAB, tcp, rfbO1* and *zot* in the isolates of *V. cholerae* (Table 1). The pathogenesis of the infections caused by vibrios is complex due to a variety of virulence factors, such as cytotoxins, enterotoxins and lithic enzymes (Masini et al., 2007). This does not overrule the possibility of the strains causing gastroenteritis, given their virulence is associated to several mechanisms. Similar results were reported by Wong et al. (2012) for environmental samples.

In drinking ground water samples analyzed in India, was detected the presence of the gene *ompW* in all the isolates, but could not find any evidence of the genes *rfbO1*, *tcp*, *ctxAB* and *zot*. The majority environmental isolates belonging to serogroups non-O1 and non-O139 are not toxigenic in general (Tamrakar et al., 2009). The gene of cholera toxin (*ctx*) is essential to cause the disease cholerae, and present only in *V. cholerae* strains O1 and O139 (Goel et al., 2007).

However serogroups non-O1 and non-O139 of environmental origin can be considered strains without risk, it is known that the aquatic environment can serve as a reservoir for the emergence of pathogenic strains from populations not pathogenic, due acquisition of genetic mobile materials, thus creating new pandemic strains (Islam et al., 2013).

Profile of antimicrobial susceptibility

The *V. cholerae* strains showed 91% (10/11) susceptibility to the drugs commonly used by the Brazilian population (Table 2). The bacterial resistance profiles are closely related to the environmental conditions and pressures to which the strain has been exposed

The susceptibility of the *V. cholerae* to tetracycline is satisfactory considering it is one of the first drugs chosen to treat infections caused by vibrios (Han et al., 2007). Fluoroquinolones (ciprofloxacin) and aminoglycoside (gentamicin) are used to treat infections caused by *V. cholerae* O1 and O139 (Okuda et al., 2007). However, the appearance of antibiotic resistant *V. cholerae* strains has restricted its use in to patients with severe dehydration (Kitaoka et al., 2011).

In Iran, Raissy et al. (2012) observed resistance to gentamicin in 83.3% strains and tetracycline in 18.1% strains of *V. cholerae* isolated from fish. Zanetti et al. (2001) reported 88.9% of the isolates in a marine environment to be resistant to ampicillin (MIC > 64 mg) due to the production of β-lactamases.

In this study, antimicrobial resistance to imipenem with an MIC of 20 µg (water isolate) was observed (Table 2). This fact suggests that along the course of Subaé River

until its mouth, wastewater from hospitals may be discharged in the river, causing an antibiotic contamination. Imipenem is a broad spectrum antibiotic used in the treatment of infections caused by β-lactamase producing enterobacteria. The occurrence of enzymes capable of inactivating carbapenems has increased the microbial resistance, thus limiting the options available for treatment (Ikeda et al., 2012).

The presence of the *V. cholerae* strains resistant to carbapenems poses a risk for the propagation of the microbial resistance in the environmental bacteria, when the aquatic environment is efficient in the selection of the resistant bacterial populations via exchange of resistance genes carried on mobile genetic elements (Kitaoka et al., 2011).

The study of microbial resistance is relevant from the point of view of public health. In purview of this, it is important to monitor the extent to which ecosystems is modified by man, especially when antimicrobials are released in municipal sewage system with urine and feces (Baquero et al., 2008).

The Brazilian authorities, aiming to minimize the problem of the antimicrobial resistance in the country, have limited the use of antibiotics by prescription only. However, flaws in the treatment of the sewage stations besides implication of animal prophylaxis have also contributed to the discharge of antibiotic residues into water bodies.

While finding the genetic origin of imipenem resistance, an active involvement of plasmids could not be detected in the extraction of plasmid DNA (Table 2); one possibility for this observation is that plasmids might have been lost during the storage of the isolates. According to Smitt and Bidochka (1998), the growth conditions, storage, and environmental conditions may cause changes in the plasmid, and may influence its stability.

In a study in Kerala, India, the antimicrobial resistance mediated by plasmids in vibrios was reposted from seafood. However, in several studies, no strains with plasmids were reported; this indicates occurrence of potential chromosomal resistance (Manjusha and Sarita, 2013).

Detection of metallo-β-lactamases (MβLs)

The *V. cholerae* isolates showing resistance to imipenem also showed ability to produce MβLs enzymes (Table 2). These enzymes make the bacteria resistant to a large number of antimicrobials, especially carbapenems (Padhi, 2011). Carbapenems belonging to a class of antibiotics reserved to treat severe infections caused by organisms are already resistant to the latest penicillins and cephalosporins (Fritsche et al., 2005).

V. cholerae has developed an ability to overcome the antimicrobial effect due to the presence of efflux pumps that act on several classes of antimicrobials and produce enzymes that may hydrolyze complex antimicrobials. In addition, *V. cholerae* has a strong capacity to share resistant genes for antimicrobials through integrons and plasmids (Mandal et al., 2012).

Due to the capacity to inactivate several microbial agents, an increase in the prevalence of this microorganism would drastically compromise its ability to efficiently treat hospital infections or the ones acquired in the community mostly caused by Gram-negative bacillus. These enzymes, due to posing a global threat via their resistance mechanism, are among the greatest concerns for the medical community.

The presence of the enzyme New Delhi metallo-β-lactamase (NDM-1) in *V. cholerae* and *Shigella boydii* in the environment has been reported. The blaNDM-1 gene has very high ability of genetic exchange between the environmental bacterial species. The increase in *V. cholerae* strains resistant to the prescription or excessive use of antimicrobials, as well as the absence of adequate monitoring (Padhi, 2011).

The Antimicrobial Surveillance Program (SENTRY) has documented a world-wide raise in the index of occurrence and number of types of MβLs; this increase is a matter of concern, especially in Asia, Europe and Latin America (Fritsche et al., 2005). In Korea, approximately 10 to 50% of the resistance to imipenem in *Pseudomonas aeruginosa* and *Acinetobacter* spp. is due to the production of MβLs, which can spread very quickly to several species of Gram-negative bacillus via transferring genes for MβLs enzymes of the type *Verona imipenemase-2* and *Imipenemase-1* (Yong et al., 2002). In Brazil we do not have data *Vibrio cholerae* strains metallo-β-lactamase positive only was reported clinical cases of *Pseudomonas aeruginosa* and *Acinetobacter* spp. The chelating agents (EDTA/MPA) are efficient in inhibiting MβLs enzymes. The inhibition of these enzymes by EDTA is an important characteristic used to distinguish MβLs from other β-lactamases (Lee et al., 2003). According to the same authors, imipenem-ethylene diamine tetra acetic acid (IPM-EDTA) and ceftazidime-mercapto propionic acid (CAZ-MPA) are the simple methods to detect MBL-producing strains using simple disc for the diffusion of MβLs.

Conclusion

The strains of *V. cholerae* originating from São Francisco do Conde, Bahia, did not have genes related to the toxicity of these bacteria and were susceptible to most antimicrobials evaluated. However, considering the genetic plasticity of these bacteria in the aquatic environment, the presence of metallo-β-lactamase enzymes is an indicator of the risky situation to which the population is exposed. The monitoring of these environments may be an efficient strategy in predicting outbreaks and epidemics involving *V. cholerae* as

pathogenic agent.

Conflict of Interests

The authors have not declared any conflict of interests.

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