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IDENTIFICATION OF VIBRIO CHOLERAE IN THE PORT OF THE CITY OF RIO DE JANEIRO: A CONTRIBUTION TO THE PROBLEM OF BALLAST WATER AND THE POSSIBLE INTERACTION WITH HUMAN HEALTH

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ABSTRACT

Cholera, caused by *Vibrio cholerae* O1 and O139, has high incidences in regions with neglected populations, especially in terms of sanitation. *V. cholerae* can be transported by ship ballast water which makes it a D2 standard evaluation bioindicator for the International Convention for the Control and Management of Ship´s Ballast Water and Sediments. The objective of this is to identify the presence of the organism in the port area of the City of Rio de Janeiro (Brazil), in samples of surface water and ballast tanks, contributing to the analysis of the ballasting process and its interactivity with human health. In superficial and vessel sampling campaigns, the non-toxic form of the organism was detected. The conclusive facts suggest the vulnerability of the area, methodological elaboration for the selection of ships to be sampled, incentives for installation, inspection of the D2 standard and constant monitoring of port waters and vessels, aiming to prevent possible cholera outbreaks caused by the ballast of the modal maritime in region.

Keyword: Navigation; Public Health; Cholera.

INTRODUCTION

Vibrio cholera is characterized by being autochthonous in aquatic environments if presented in forms such as free, parasitic and benthic biofilm-forming, having as ecological function the chitin degradation (Kokashvili et al. 2015, Chowdhury et al. 2017). It has a wide range of environmental survival, with ideal conditions of salinity between 0.5 and 20, pH between 7.0 and 9.0 and temperature between 20 °C and 30 °C (Kokashvili et al. 2015, Chowdhury et al. 2017).

Serogroups O1 and O139 of *V. cholerae* cause cholera, a serious enteric disease characterized by a large loss of water and electrolytes due to intense diarrhea (CDC 2018a). Treatment is rapid hydration and secondarily the administration of antibiotics (Ramamurthy et al. 2019, WHO 2019). However, the pathogen has high resistance to the main lines of antimicrobials used (Ghosh & Ramamurthy 2011), a fact that mainly arises from the horizontal gene transfer process (Das et al. 2020). This process may also be associated with the acquisition of genes linked to cholera toxin production by non-toxigenic samples (Chiang & Mekalonos 1999).

The environmental isolates of *V. cholerae* have a lower frequency of genes associated with virulence, which suggests that the origin of toxigenic strains can be influenced, evolutionarily, by transduction or the insertion of genetic material of other species of the genus Vibrio (Motta et al. 2020), as well of accessory virulence factors such as hemolysins and hemagglutinins, associated with gastrointestinal diseases, septicemia and extraintestinal infections, which warns of the potential emergence of isolates of epidemiological importance in the environment (Chen et al. 2015).

Cholera is one of the biggest public health problems in developing countries, especially in areas with populations in neglected situations by social and infrastructure problems (Somboonwit et al. 2017, Nadri et al. 2018) and without basic sanitation (Silva et al. 2019). Between 2000 and 2018, approximately 5.3 billion cases of the disease were recorded worldwide, with approximately 76 thousand deaths, of which 83% occurred in countries on the African and Asian continents (WHO 2020).

It is well documented that *V. cholerae* is a potentially invasive species, mainly due to the process of ballasting of vessels (Brasil 2009, Rivera et al. 2013, Silva et al. 2015, Silva et al. 2019). Thousands of invasive marine species can be transported by ballast water, as long as they are sufficiently small to pass through the intake pump entry and among them are the pathogenic organisms to human health as the bacteria (Satir 2014). These microorganisms represent a major concern, due to their size, reproductive rate and high population density (Starliper et al. 2015) in addition to the high resilience in confined environments such as ballast water tanks (Ramaiah et al. 2005).

Ballast water is a portion of water captured into specific tanks on vessels, in order to maintain its stability during navigation without an onboard cargo (Silva et al. 2015). It is discharged as the cargo is introduced into the vessels, according to their stability curves. Thus, the discharge of ballast water can contribute to the species transfer process between marine environments (Brasil, 2009). Ballast water has been considered, worldwide, as the main means of transferring aquatic, animal and / or vegetable species between coastal environments, since ship hulls are not in principle vectors due to the use of anti-fouling paints specifically formulated to reduce the bio-encrustation process (Fernandez & Pinheiro 2007). It is estimated that, each year, about 10 billion cubic tons of ballast water are transported worldwide, with about 7 thousand species carried daily through oceans (Zhang et al. 2017).

With an approximate handling of 600 million tons / year of cargo and about 74 thousand vessels registered in open sea, navigation currently accounts for about 90% of all world trade (UNCTAD 2019), thus establishing countless maritime commercial routes around the world (Kaluza et al. 2010), a fact that ends up further aggravating this issue.

The International Maritime Organization (IMO) adopted, in 2004, the International Convention for the Control and Management of Ship´s Ballast Water and Sediments: BWM 2004 (IMO, 2004). This convention indicates measures to prevent and safeguard human and environmental health, in an attempt to avoid, minimize and, if possible, eliminate the risks of introducing harmful aquatic organisms and pathogens into ports through ballast water (IMO, 2016).

The regulation D2 of BWM 2004 is concerned with the quality of the disposal with regard to the eliminated species, using bioindicators (IMO, 2004). In order to guarantee the efficiency of ballast water treatment methodologies, the following organisms were standardized as bioindicators: (i) *V. cholerae* (serogroups O1 and O139) with < 1 CFU/100 mL or < 1 CFU/g (wet weight) of zooplankton samples; (ii) *Escherichia coli* with < 250 CFU/100 mL and (iii) Enterococci of intestinal origin with < 100 CFU/100 mL (IMO, 2004).

Port regions present a scenario of progressive degradation aggravated by social factors, mainly by the reception of urban effluents (Silva et al. 2015), causing a situation of environmental vulnerability with possible establishment of pathogens, mainly in developing countries. Many studies have already identified the presence of toxigenic *V. cholerae* in port waters (Rivera et al. 2013), as well as in ballast tank samples (Kaluza et al. 2010, Starliper et al. 2015) and others related outbreaks of the disease to the ballast discharge process (Ng et al, 2018).

Thus, in order to contribute to studies related to the introduction of *V. cholerae* through the ballast process in port waters, this work aimed to identify the presence of this bacterium in surface waters in the port region of the city of Rio de Janeiro (Brazil) and in samples of ballast tanks from ships berthed at the site.

MATERIAL AND METHODS

The methodology was based on the collection of samples of surface water and ballast tanks from vessels anchored in the port area of the City of Rio de Janeiro (Brazil) and subsequent microbiological analysis for the identification and biochemical characterization of the presumptives found of *V. cholerae*.

(i) Surface Sample Collection (SSC). Surface sampling of port waters was carried out with the aim of identifying the presence of *V. cholerae* and characterizing the physical-chemical conditions present at the site, observing whether the standards previously established at the study site, by Silva et al. (2015) and Silva et al. (2019), remained. Therefore, based on the aforementioned studies, an annual period which presents more favorable conditions for the organism's survival in the environment was selected to carry out the sampling campaign, as well as the proximity of some collection points used in the aforementioned studies, thus obtaining a temporal comparison of the results.

The sampling took place on January 21st of 2019 (from 6 am to 11 am) with the following environmental conditions: temperature (minimum 27 °C / maximum 32 °C), relative humidity of 86%, sunny weather, tidal fluctuation from 1.0 to 0.5 m during collection and Northwest wind with a speed of 5 km/h, with nine collection points (figure 1): The P1 “Canal do Cunha” (22 ° 51′54.55′′ / 43 ° 12′33.70 ′′W) and P3 “Canal do Mangue” (22 ° 53′49.04′′S / 43 ° 12′36.04′′W) were selected because they are places of discharge of urban effluents; P2 “Cais São Cristóvão” (22 ° 52′49.91′′S / 43 ° 12′16.83′′W), P4 “Cais Gamboa” (22 ° 53′20.84′′S / 43 ° 11′37.90′′W) and P6 “Cais Caju” (22 ° 53′14.53′′S / 43 ° 12′44.80′′W) were selected as representatives of each section of the port because they are part of the area for maneuvering vessels and receiving a wash contribution of the mooring berths and the P5 “Museu do Amanhã” (22 ° 53′26.05′′S / 43 ° 10′40.90′′W) for presenting a tourist construction that caused a grounding into the bay influencing the movement of water in the local. To check the physical-chemical parameters, Niskin bottles were used at a depth of one meter from the surface, followed by the determination of pH and temperature (multi-processed pH Meter AT-315 ALFAKIT® Brazil) and salinity (Conductivity Meter 8306 AZ® Brazil).

In the collection of the aliquot for microbiological analysis, 1000 mL of superficial water were collected, 20 cm from the surface, in borosilicate glass flasks with a screw cap duly identified and previously sterilized by autoclaving (121 ºC / 15 min). At the time of collection, the surface of the flasks and the operator's gloves were disinfected with hydrated ethyl alcohol 70 ° INPM and the practice of “double rinsing” (collection and disposal) was performed to homogenize the container as recommended by Huq et al., (2012). Immediately, the material was placed in an isothermal box (4 °C to 10 °C) with a start time of microbiological analysis not exceeding 6 hours of sampling (CDC 2018).

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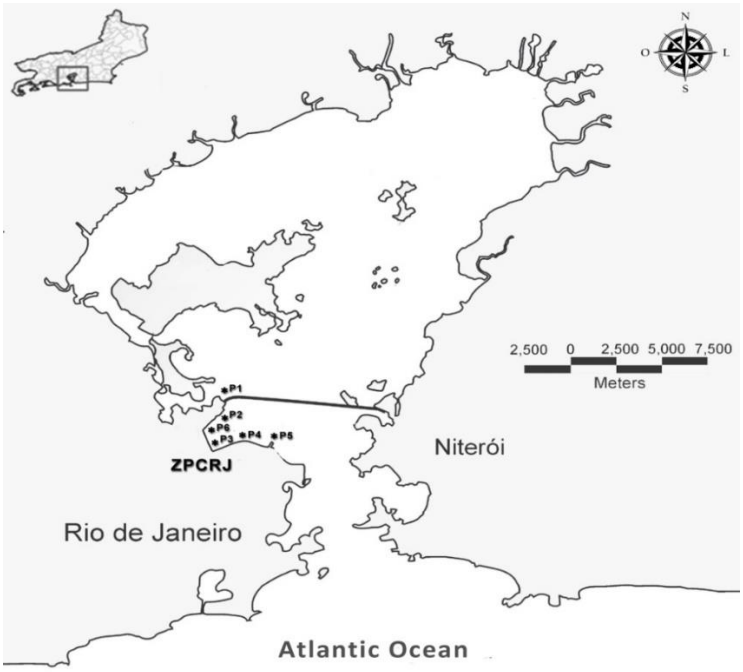
Figure 1. Points (P1 to P6) selected in the port area of the City of Rio de Janeiro (ZPCRJ) for surface sampling.



Font: This figure is a derivative of "Location of monitoring points: Guanabara Bay (Rio de Janeiro, Brazil)" by Silva *et al*. (2019).

(ii) Ballast Tank Sample Collection (BTSC). The great difficulty in carrying out sampling in ballast tanks hangs over the existing sovereignty of international vessels in different world ports, which ends up generating a bureaucratic methodological process and articulated with the competent bodies for this purpose, which makes the procedure a challenge for researchers in the field. At the study site, the responsible body that made the campaigns possible was the Port Authority of the City of Rio de Janeiro (Brazil), an entity affiliated with the Brazilian Navy.

Therefore, two vessels were sampled, monitored with the help of the virtual tool *Marine Traffic* (https://www.marinetraffic.com/), between the period of July to December 2019: (N1) Vessel *Roll On - Roll Off* vehicles carrier with Panama flag and keel stroke in 2006. 179.99 m of total length by 32.2 m of breadth, with 14 ballast tanks (total volume of 75,843 m^3) and without installed ballast water treatment on board. It had cargo initially shipped in Mexico and distributed to several countries on the West Coast of the American Continent and Brazilian ports before reaching the port of Rio de Janeiro from shipping between Brazilian national ports and (N2) Container vessel, from Singapore, registered on the flag of Malta and with a keel strike in 2017. 299.92 m of total length by 48.33 of breadth, with 26 ballast tanks (total volume of 31,746 m^3), being equipped with a treatment system of filtration and ultraviolet radiation, in line with the D2 standard.

The choice of the vessels to be sampled had as main question a possible introduction of the organism through international waters or the detection of its presence in Brazilian waters, therefore the N1 sampling occurred on November 18th 2019, with water being collected in a loaded tank close to the Brazilian coast (02 ° 01'07 "N / 44 ° 40'07" W). Although Brazil did not have cholera occurring during the selected period, this tank was sampled for presenting better logistical conditions as determined by the vessel's captain. As this vessel did not have a sampling point, the collection was carried out directly in the ballast tank with the help of a manual water intake pump, previously cleaned with 70 ° alcohol, also carrying out the double rinsing process, thus collecting 1000 ml of water sample. The N2 sampling took place on December 4th 2019 in a loaded tank at the Port of Singapore, according to the ballast water register of maneuvers. This vessel had a sampling point connected to the outlet of the treatment system.

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The material and procedures were the same as described in the SSC process, as well as the volume of 1000 mL collected. The measurement of physical-chemical parameters was performed with samples collected in two flasks of borosilicate glass previously sterilized by autoclaving and measured with them. For both N1 and N2 sampling, the maximum time to start microbiological processing of the sample was 6 hours.

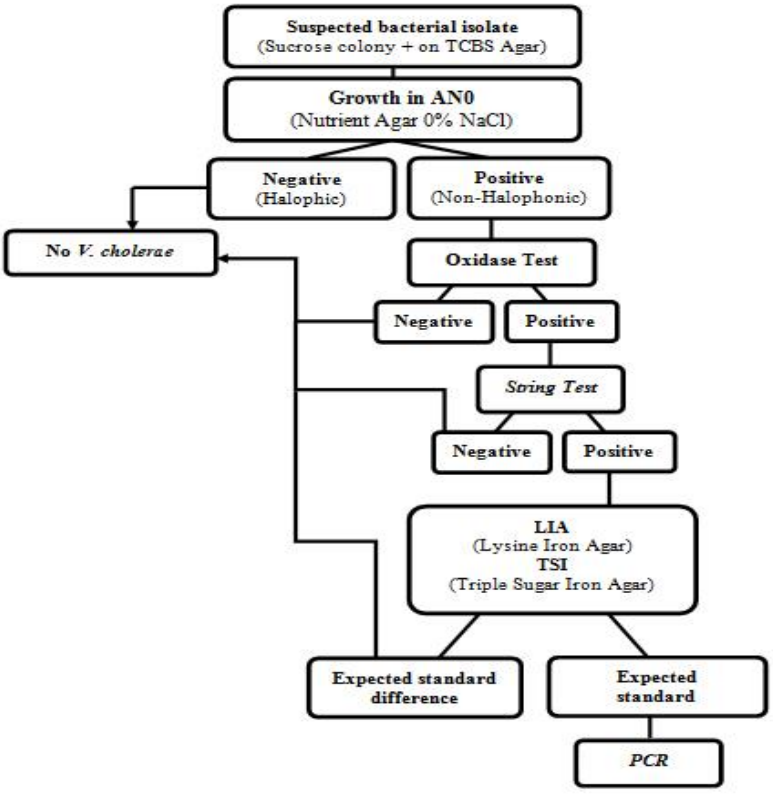
(iii) Sample processing for microbiological analysis.

(iii.1) Filtration and filtrate enrichment. The samples were homogenized by inversion of the flasks (10x) and filtered using a manifold system using polycarbonate cups and filtering membranes of cellulose esters mixture, with a diameter of 47 mm and pores of 0.45 µm. After filtration, the membranes were placed in a solution of alkaline peptone water[47] and incubated at 35 ºC at 37 °C for 8 hours (Huq *et al.* 2012, CDC 2018b).

(iii.2) Samples isolation and storage of presumptive isolates. 100 µL of the surface portion of the enrichment medium were transferred to a TCBS Agar plate and seeded by the sewage seeding technique and incubated in an inverted position at 35 ºC for 18 to 24 hours (CDC 2018a). Presumptive colonies of *V. cholerae* (2-4 mm in diameter, circular, smooth, slightly flattened and a typically yellow color that extends to the surrounding culture medium) as suggested by the CDC (CDC 2018a, CDC 2018b), were peaked onto plates of Tryptic Soy Agar (TSA) and incubated at 35 ºC for 16 to 18 hours (Menezes *et al.* 2014). The isolates were conditioned to "fast working stock" on Mueller Hinton Agar and frozen BHI-glycerol (Saeki *et al.* 2015).

(iii.3) Routines for the identification and characterization of bacterial isolates. In order to minimize the number of presumptives directed to the routine of molecular characterization by Polymerase Chain Reaction (PCR), screening procedures and biochemical tests of the isolates on TSA Agar were performed: (i) Growth Tests on Nutrient Agar 0% of NaCl (AN0), Oxidase test, string test, biochemical tests on Lysine Iron Agar (LIA) and Triple Sugar Iron Agar (TSI) (CDC 2018a, CDC 2018b) as shown in Figure 2.

Figure 2. Flowchart of decision of Screening Tests and Biochemical Evidence for the identification of bacterial isolates.



Note: Expected standard for LIA: K / K * and TSI: A / A ** tests
* K / K = alkaline / alkaline; ** A / A = acid / acid.

(iii.4) Characterization and molecular determination by simple PCR of bacterial isolates. Colonies developed in the TSA were added with 1000 µL of sterile ultrapure water in microtubes (1.5 mL). This suspension was agitated on a mechanical vortex mixer, centrifuged (7,000 rpm for 10 min) and the pellet obtained resuspended in 300 µL of sterile ultrapure and water heated (10 minutes) in boiling water for cell lysis. Cell debris was removed by centrifugation (7,000 rpm for 10 min) and the supernatant was transferred to microtubes (500 µL). For the amplification reactions of the gene fragments, the *Taq DNA Polymerase 2x Master Mix RED* reagent (Ampliqon A / S, Denmark) was used. This reagent is composed of Tris-HCl (pH 8.5), $(NH_4)_2SO_4$, 0.2% of Tween® 20.2 U / µL of Taq DNA polymerase, 0.4 mM of each dNTP, 4.0 mM $MgCl_2$, stabilizer and red inert dye. The reactions were designed for a final concentration of 25 µL with 12.5 µL of the *Taq DNA polymerase reagent 2x Master Mix RED*, 2 µL of the primer oligonucleotide mixture (1 µM), 1 µL of bacterial DNA and 9.5 µL of sterile ultrapure water and subjected to amplification in *a LifePro thermocycler (Hangzhou Bioer Technology Co., China)* with an initial denaturation cycle at 94 ºC (5 minutes), 30 cycles of denaturation at 94 ºC (30s) and annealing (1 minute) at the temperatures of each primer oligonucleotide specified in Table 1 (72 ºC for 1 minute and final cycle at 72 ºC for 5 minutes).

The analysis of the amplified products was performed using electrophoresis in a standard low-electroendosmosis agarose gel *(Agargen, Hispanagar SA, España)* at 1.5% in tris-borate-EDTA (TBE) buffer 0.5 X (TBE 1 X = 0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0*) (Tris base, boric acid, EDTA - Hexapur® Amsterdam, Netherlands)* subjected to an electrical current of 100 V (30 to 45 minutes). The amplified products were observed by a blue LED light transilluminator, and the size of the amplified products was estimated by comparison with a DNA size marker from 100 to 1500 bp *(DNA Ladder, Invitrogen ™ - Life Technologies*, Canada). The identification of the isolates as belonging to the species *V. cholerae* was carried out with the use of species-specific primers described by NANDI *et al.*, (2000), for the detection of sequences of the *ompW* gene. The isolates whose DNA showed expected amplified products (588 bp) were identified as belonging to the species *V. cholerae*. For the determination of serogroups O1 and O139, oligonucleotides primers were used, O1-*rfb* and O139-*rfb* (Hoshino *et al.* 1998). When there was no amplification reaction with the DNA of the isolates, they were classified as "not O1 / not O139" ("not O1 / O139"). The relation of the oligonucleotides primers, annealing temperatures and amplified products in the PCR reactions used are described in Table 1.

Table 1. Primers oligonucleotides utilized and its annealing temperatures, amplified products in simple PCR reactions.

Target Gene	Primer oligonucleotide (5' 3')	TA*	*Amplicon*	Reference
ompW	F= CACCAAGAAGGTGACTTTATTGTG R= GAACTTATAACCACCCGCG	64 ºC	588 pb	Nandi *et al.* (2000)
O1-*rfb*	F= GTTTCACTGAACAGATGGG R= GGTCATCTGTAAGTACAAC	55 ºC	192 pb	Hoshino *et al.* (1998)
O139-*rfb*	F= AGCCTCTTTATTACGGGTGG R= GTCAAACCCGATCGTAAAGG	55 ºC	449 pb	Hoshino *et al.* (1998)
16S rRNA	F= CCTGGTAGTCCACGCCGTAA R= CGAATTAAACCACATGCTCCA	60 ºC	168 pb	Wei *et al.* (2014)

Note: (*) TA: Annealing temperature (there may be variation in the annealing temperature values) in relation to those mentioned in the references, as they used the multiplex PCR technique).

(iii.5) Positive and negative controls. Throughout the microbiological analysis procedure, negative and positive controls were used to verify the functionality of the test mediums. The negative controls were selected from isolated bacterial samples, from the surface water samples, which showed a negative result for

the use of sucrose in Thiosulfate Citrate Bile Salt Sucrose Agar - TCBS Agar. The positive controls of *V. cholerae* of serogroup O1 were obtained from the Collection of Reference Microorganisms in Health Surveillance (CMRVS) of the National Institute for Quality Control in Health (ATCC 9458,0709156, INCQS FIOCRUZ BRASIL), and for serogroup O139 were obtained at the Nucleus of Collection of Microorganisms of the Adolfo Lutz Institute (M045, VC 0139, IAL 2013). In the PCR routine, in addition to the controls mentioned above, the bacterial isolates were tested for the presence of DNA using a primer (0.5 µM) of the *16S rRNA* gene (Wei *et al.* 2014).

RESULTS AND DISCUSSION

In case of superficial sampling of the study region, the values of the physical-chemical data collected presented itself in accordance with the past work of Silva *et al.*, (2015) and Silva *et al.*, (2019), thus showing a continuity of the patterns of these factors in the region. Such values, if compared with some specific literature (Kokashvili *et al.* 2015, Chowdhury 2017), show a favorable framework for the survival of the organism, in which salinity presents itself as the only possible limiting factor, a fact that was previously verified by Silva *et al.*, (2019). It is worth mentioning that studies (Ramaiah *et al.* 2005, Rivera *et al.* 2013, Ng *et al.* 2018, Silva *et al.*2019) demonstrated the adaptation of *V. cholerae* to these parameters.

During the study period there were monitored 563 visits by vessels to the port of the City of Rio de Janeiro and, in the two vessels sampled, the values of the physical-chemical parameters measured, at the time of sampling, were also favorable to the survival of the organism according to the abovementioned literature. *V. cholerae* was isolated at all six surface points in the study area as well as on the N2 vessel. In N1, no specimen was detected. However, of these, none were diagnosed as toxigenic (O1 or O139) according to the PCR methodology used, confirming the results found by Silva *et al.*, (2015). The number of strains that passed at the biochemical screening and went for molecular analysis by PCR, as well as the results obtained and the values of the physical-chemical factors detected at the time of sampling are shown in Table 2.

Table 2. Result of the microbiological analysis, with the number of presumptives, and detected values of pH, temperature and salinity, at the time of collection, of all points in the port area (P1 to P6) and of the vessels (S1 and S2) sampled, showing that although, the presence of the organism was detected in all study areas and ballast of one of the vessels (S2), none if known toxigenic (serogroup O1 or O139).

Place	Collect Place	Number of presumptive samples after biochemical screening	Physical-chemical patterns detected in sampling			Result of PCR		
			pH	Temperature	Salinity	V.C	O1	O139
P1	Canal do Cunha	7	8,25	27,80	25,40	+	-	-
P2	São Cristovão Pier	3	8,15	27,00	25,90	+	-	-
P3	Canal do Mangue	2	8,39	27,20	21,60	+	-	-
P4	Gamboa Pier	1	8,25	27,30	25,30	+	-	-
P5	Museu do Amanhã	1	8,28	27,20	25,90	+	-	-
P6	Cajú Pier	2	8,32	27,20	21,60	+	-	-
S1	Ship 1*	0	7,70	28,10	27,90	-	-	-
S2	Ship 2	8	8,30	28,20	28,60	+	-	-

Note: (*) No isolate from this vessel has passed biochemical screening. However, they were all submitted to the PCR routine and were negative for the three examined primes.

CONCLUSIONS

The problem of introducing exotic species, mainly pathogenic organisms, such as *V. cholerae*, with regard to the BWM 2004 D2 standard (IMO 2004), requires actions that range from the elaboration of ballast treatment methodologies to the continuous monitoring of its effectiveness. In the meantime, it is necessary to build systematic measures for the selection of ships to be sampled taking into account important factors such as: origin of the ballast, loading or unloading maneuver, type of vessel, presence of ballast treatment methodology, among others. This is an important point due to the large number of maritime visits between ports in the world, making it practically impossible to carry out the sampling process on all visiting vessels, whether due to the number of people involved and / or the financial investment of the entire collection process and sample processing and analysis.

Another important point is the elaboration of microbiological analysis processes that are able to determine the presence, or not, of the pathogenic agent in ballast samples more quickly, since generally the ships stay a few hours moored in the berths of the port areas. In addition, it is important to ponder over possible changes or environmental adaptations of the organism, which end up directly influencing the isolation methodologies, since the vibrio was detected, in this work, under salinity conditions above the ideal range described in the consulted literature for its growth (Kokashvili *et al.* 2015, Silva *et al.* 2015, Chowdhury *et al.* 2017, Silva *et al.* 2019), suggesting a possible halophilic behavior.

The non-occurrence of the organism of interest in one of the sampled vessels (which had a treatment methodology on board) suggests the importance of implementing the D2 standard in ships that do not yet have it, in order to reduce the risk of unwanted introductions of pathogenic species between ports, thus mitigating possible afflictions of diseases caused by these agents worldwide.

Although the work did not detect any *V. cholerae* in its toxigenic form in ballast water samples, Rivera *et al.*, (2013) identified the presence of *V. cholerae* serogroup O1, in free life and / or parasitizing phytoplanktonic organisms, in vessels anchored in national ports such as Belém, Fortaleza, Recife, Santos, Ubu and Paranaguá (Brazil), a fact that indicates the need for constant monitoring and validation of treatment methodologies on board as to their effectiveness, as well as the imposition, soon as possible, of the installation of these on vessels that do not have them.

In addition to the constant monitoring of *V. cholerae* serogroup O1 and O139 in ballast waters, it is necessary to deepen the knowledge of the genotype of these isolates, due to the high pathogenic potential that they have as a result of genetic transfer between bacteria of the same species, and of other microbial genera that share the habitat. Therefore, in future studies, the molecular characterization of these isolates will be carried out, by amplifying the genes associated with toxigenic characters and involved in the pathogenesis. This will increase the knowledge of the *V. cholerae* genotypes that circulate, as well as provide relevant information to assess the epidemiological risk profile they present at the study site.

Although the work used a small “n” sample, the need for constant monitoring of port waters, the construction of methodologies for selecting vessels to be sampled, as well as the inspection of the effectiveness of the D2 standard, more specifically with regard to the occurrence of *V. cholerae* toxigenic, are shown as essential measures in the prevention of possible outbreaks of diseases whose introduction vehicle is the transport of ballast water worldwide.

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