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Ability of *Vibrio vulnificus* isolated from fish of the Lagoa dos Patos estuary in south Brazil to form biofilms after sublethal stress and bacterial resistance to antibiotics and sanitizers

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ABSTRACT

The present study aimed to investigate the presence of *Vibrio vulnificus* in fish captured at the Lagoa dos Patos estuary (RS, Brazil), to establish a correlation between bacterial biofilm formation and sublethal stress, and to assess the resistance of the isolates to antimicrobials and sanitizers. A total of 217 isolates characteristic of *Vibrio* sp. were analyzed. Isolates were identified and subsequently their ability to form biofilm, the impact of exposure to sublethal stress on their biofilm formation ability, and their resistance to antimicrobial and to sodium hypochlorite and chlorine dioxide sanitizers were evaluated. *V. vulnificus* was isolated from the fish *Paralichthys orbignyanus* and *Micropogonias furnieri*. The bacterial isolates examined were able to form biofilms. Biofilm formation ability of these strains was decreased or inhibited after being exposed to sublethal stress. The isolates were resistant to most antimicrobials. The sanitizer concentrations necessary to eliminate *V. vulnificus* were higher than those usually used in the fishing industry.

1. Introduction

The Lagoa dos Patos estuary is located in the extreme south of Brazil. It occupies a surface area of 963.8 km^2 which corresponds to 10% of the total area of the lagoon. This estuary receives water from the rivers located in the northern area and from Lagoa Mirim southwards through the São Gonçalo canal (Calliari, 1998). This estuary is an area of major importance for a number of species of fish and crustaceans of great commercial value. It is considered an artisanal fishing center which importance is highlighted by the supply of fish in the southern region of Brazil (Reis, 1999). To ensure the quality of these food products, it is necessary to ensure safe and proper handling during capture, processing, storage, and commercialization of fish.

According to Baldisserotto (2009), the Lagoa dos Patos estuary is the area in the State of Rio Grande do Sul, south Brazil, with greater representation by a significant number of artisanal fishermen working in the area that are undocumented and are poorly educated (Garcez and Sánchez-Botero, 2011). These factors may contribute to informal commerce and negligence regarding sanitary and hygienic measures during fish handling and processing.

Fish may become contaminated by microorganisms in the ocean. They may remain as such after being captured and during transport, handling, contact with ice, surfaces, equipment, processing, during storage and in the commerce environment (Huss, 1997; Ogawa and Maia, 1999; Cardoso et al., 2003). Industrial establishments of meat and its derivatives should meet specific sanitary hygienic requirements regarding structure and production. These facilities aim to maintain favorable conditions in order to avoid bacterial contamination that may cause damage to the product or pose risk to consumer health (Santos et al., 2001).

Foodborne diseases are usually toxinfections or food poisoning caused by bacteria, viruses, parasites, or chemical substances that are transmitted to humans through the ingestion of contaminated food or water. Some foodborne pathogens cause severe diarrhea or debilitating and life-threatening infections including meningitis and septicemia. Foodborne illnesses can result in long-term disability and may be fatal. Examples of unsafe food products include undercooked products of animal origin and vegetables contaminated with feces (Who, 2015).

Bacteria of the genus *Vibrio* are Gram-negative motile bacteria that usually have a single polar flagellum. These bacterial pathogens are typically found in marine and estuarine environments and therefore may be isolated from fish and crustaceans. *Vibrio* spp. is also able to

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multiply outside their hosts in marine waters (CDC, 2016). Vibriosis are underdiagnosed as specific tests for the identification of this microorganism are not routinely included in the comprehensive panel used in laboratories for the detection of foodborne pathogens. *Vibrio vulnificus, Vibrio parahaemolyticus, Vibrio cholerae,* and *Vibrio mimicus* are the main species of *Vibrio* reported as causes of infection in humans. *V. vulnificus* is considered the most pathogenic species of *Vibrio* causing septicemia and ultimately death (Silveira et al., 2016).

Colony morphology of *V. vulnificus* and *V. parahaemolyticus* are similar to one another in TCBS Agar. They can be differentiated between each other by several biochemical reactions including β-galactosidase activity and growth in different saline concentrations. *V. vulnificus* may cause septicemia and death after seafood ingestion or infection of wounds originated in the marine environment. PCR assays have been developed to detect and identify this pathogen (Kaysner and de Paola Jr, 2004).

V. vulnificus is the most lethal food pathogen in the United States, and possibly globally, with a mortality rate of approximately 50%, representing 95% of all deaths related to fish consumption in the country. Such mortality rates exceed those of other foodborne pathogens such as *Salmonella*, *Escherichia coli*, and *Clostridium botulinum*. Although the infective dose of *Vibrio* is unknown, it is estimated that 100 cells or less causes disease in predisposed (immunocompromised) individuals (Oliver, 2015). Several studies carried out in South America report that additional research on the detection of *V. vulnificus* in fish is warranted. The presence of this microorganism has been recorded in the waters along the South American Atlantic coast. Despite its importance as a foodborne pathogen, there is limited research on *Vibrio* as a fish contaminant (Givens et al., 2014).

In Brazil, reports of *V. vulnificus* toxi-infection have been mainly associated with oyster consumption. Cases of diarrhea and sepsis in humans have been reported in the states of São Paulo, Bahia, and Ceará (Araujo et al., 2007). *V. vulnificus* was isolated from 1 fish of the 21 fish sampled in a public market in the State of Bahia, northeast Brazil. This finding demonstrates its ability to contaminate fish in the Brazilian coast and to remain viable in the retail market. The authors attributed the dissemination of the bacterium to infrastructure problens and inadequate cooling (Evangelista-Barreto et al., 2017).

Recently, *V. parahaemolyticus* which is a pathogenic species that has been associated with the consumption of contaminated fish was isolated from fish captured at the Lagoa dos Patos estuary in south Brazil. Such finding shows that this bacterium occurs in the region and that fish contamination by bacteria is possible in this environment. This microorganism was isolated from a number of fish species including the *Micropogonias furnieri* (“corvina” - whitemouth croaker), *Mugil platanus* (“tainha” - mullet), *Paralichthys orbignyanus* (“linguado” - flounder), and *Farfantepenaeus paulensis* (“camarão-rosa” - shrimp) (Rosa et al., 2016; Milan et al., 2016). Since *V. parahaemolyticus* and *V. vulnificus* have many similar characteristics and inhabit the same environment, the presence of both species may occur in the same area (Blackwell and Oliver, 2008).

Biofilm is a community of sessile microorganisms characterized by bacterial cells that adhere to a surface. These bacteria are embedded in an extracellular matrix formed by exopolysaccharides (Donlan and Costerton, 2002). Biofilm-related infections are highly prevalent in human populations consuming food products of animal origin. Bacteria within biofilms are more resistant to antibiotics and reach sufficient numbers that corresponds to a potential infective dose (Davies, 2003). In the food industry, biofilm formation may occur in manufacturing plants and equipment (Gámez et al., 2004). According to the microorganism characteristics, biofilms may form on a variety of substrates including stainless steel, glass, and rubber (Parizzi et al., 2004).

Information on the occurrence of *V. vulnificus* in seafood from both the Lagoa dos Patos Estuary and the Brazilian coast is lacking. *Vibrio* is an important etiological agent of food poisoning. *Vibrio* infection has a high mortality rate in humans in the countries where this bacterial pathogen is screened for as part of a comprehensive panel of tests during a diagnostic workup in a laboratory. Therefore, research is needed in order to gain knowledge on the occurrence of *Vibrio* in fish in this geographic area as well as to learn about the characteristics of these isolates. This data may contribute to the effective control of this bacterial pathogen in processing plants.

The general objectives of the present study are to investigate the occurrence of *V. vulnificus* in seafood originated from the Lagoa dos Patos estuary in south Brazil, assess the ability of this microorganism to form biofilms, and evaluate the resistance of this bacterial pathogen to antibiotics and sanitizers. The specific objectives of this survey are to screen for *V. vulnificus* in fish captured at the Lagoa dos Patos estuary, south Brazil, assess the ability of the bacterial isolates to form biofilms, expose these isolates to different types of stress and evaluate the potential effect of such stressful events on biofilm formation, evaluate the resistance of isolates to antimicrobial agents, and assess the sensitivity of these isolates to the sanitizers sodium hypochlorite and chlorine dioxide.

2. Materials and methods

2.1. Sample collection

217 isolates characteristic of *Vibrio* sp. were analyzed. These isolates originated from the studies carried out by Milan et al. (2016), Rosa et al. (2016), and Rosa et al. (2017), i.e. 318 samples from fish captured at the Lagoa dos Patos estuary, south Brazil between years 2012 and 2016 (Table 1).

2.2. Bacterial recovery

Isolates were maintained at −20 °C in Alkaline Peptone Water (APA, Himedia, Mumbai, India) with 1% sodium chloride (NaCl) APA 1% and 20% glycerol. These isolates were recovered in Plate Count Agar (PCA, Kasvi, Roseti Degli Abruzzi, Italy), 1% NaCl for 24 h at 37 °C, in 2 consecutive cultures to ensure that cultures were in exponential growth phase whenever necessary.

2.3. DNA extraction

DNA was extracted from isolates according to the protocol published by Sambrook and Russel (2001). In short, the pellet obtained by centrifugation of 1 mL of culture was resuspended in 100 μL of buffer STES [Tris-HCl 0.2 M, 0.5 M NaCl, 0.1% SDS (*w/v*), EDTA 0.01 M, pH 7.6]; 50 μL of glass beads and 100 μL of phenol/chloroform were added to it. After homogenization for 1 min, the mixture was centrifuged at 13,000*g* for 5 min. The supernatant was collected and precipitated in 2 volumes of absolute ethanol and 0.1 volume of 5 M NaCl solution at 20 °C for 30 min. The supernatant was centrifuged again at 13,000*g* for 20 min. Then the supernatant was discarded and the pellet washed with ethanol at 70%. After elution in 40 μL of Elution Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4), 1 μL of RNAse was added (10 μg/μL) to it. The extracted DNA was stored at −20 °C.

2.4. V. vulnificus identification

For the identification of *V. vulnificus*, the isolates of *Vibrio* were analyzed by polymerase chain reaction (PCR) screening for the *cth* gene with primers recommended by Kaysner and de Paola Jr (2004) (Table 2). According *to* Hill et al. (1991), the *cth* gene specifically encodes cytolysin in *V. vulnificus.* Each reaction had a final volume of 25 μL, i.e. 12.5 μL of the Master Mix, 1.25 μL of each primer, 2 μL of DNA, and 8 μL of water to complete the volume of the reaction. The amplification was performed using a TC-3000 thermocycler. The PCR-thermocycler program consisted of the following steps: a stage of initial denaturation at 94 °C for 10 min followed by 30 cycles of denaturation

Table 1
Isolates characteristic of *Vibrio* sp. in the medium Thiosulfate Citrate Bile Sucrose (TCBS, Himedia, Bombay, India) obtained from fish samples captured at the Lagoa dos Patos estuary in south Brazil used in the present study.

Species	References	Number of isolates	Number of samples
Mugil platanus	Milan et al. (2016)	11	10
("tainha" - mullet)	Rosa et al. (2016)	38	30
	Rosa et al. (2017)	21	50
	Total	70	90
Micropogonias furnieri	Milan et al. (2016)	9	10
("corvina" - whitemouth croaker)	Rosa et al. (2016)	56	60
	Rosa et al. (2017)	8	35
	Total	73	105
Paralichthys orbignyanus	Milan et al. (2016)	12	20
("linguado" - flounder)	Rosa et al. (2016)	42	40
	Rosa et al. (2017)	2	35
	Total	66	95
Netuna barba	Milan et al. (2016)	0	3
("bagre" - cat fish)	Total	0	3
Farfantepenaeus paulensis ("camarão-rosa" - pink shrimp)	Milan et al. (2016)	16	13
	Rosa et al. (2017)	2	12
	Total	2	12

Table 2
Primers used in the identification of *V. vulnificus* by PCR.

Primer	Sequence (5′ to 3′)	Amplification size (pb)	Reference
Vvh-785F	CCGCGGTACAGGTTGGCGCA	519	Kaysner and de Paola (2004)
Vvh-1303R	CGCCACCCACTTTCGGGCC		

at 94 °C for 1 min 45 s, primer annealing at 67 °C for 2 min, extension at 72 °C for 2 min, and a final extension stage at 72 °C for 7 min. PCR products were stained with GelRed (Uniscience, São Paulo, Brazil), and the electrophoresis was performed in agarose gel at 1.8%. A strain of *V. vulnificus* (ATCC 27562) was used as the positive control.

2.5. Assessment of biofilm formation ability

The isolates were evaluated in triplicate regarding their biofilm formation ability in microtiter plates (Nunclon, Nune, Roskilde, Denmark) according to the technique described by Janssens et al. (2008). Modifications were made to the original technique in order to adjust this method for the *V. vulnificus* microtiter plate assay; 100 µL of APA 1% NaCl were placed into each well of a microtiter plate and added 100 µL of overnight cultures in PCA 1% NaCl of each isolate which was standardized in a spectrophotometer at 600 nm to 0.5 value of optical density (OD). Wells with 200 µL of broth APA 1% NaCl without bacterial culture were used as a control. The plate was covered with a lid and incubated for 48 h at 37 °C without stirring. A cover was placed over the plate which was incubated for 48 h at 37 °C without stirring. During the incubation, biofilms formed on the surface of the cover villi. After the incubation, the lids were rinsed with 200 µL of phosphate buffered saline (PBS, 0.1 M, pH 7.0). The material that remained on the lid was stained with 200 µL of crystal violet 0.1% (m/v) for 30 min and washed with 200 µL of sterile distilled water. This lid was allowed to dry at room temperature for 30 min. Remnants of dye were removed from the biofilm with 200 µL of glacial acetic acid 30% and put in a new plate. In order to quantify biofilm formation (i.e. quantification of biofilm production), the OD_{570} of each well was measured using a microtiter plate reader. Each isolate was categorized as non-biofilm former, weak biofilm former, moderate biofilm former, and strong biofilm former according to the classification scheme proposed by Stepanovic et al. (2000). The cut-off point (ODc) was defined as three standard deviations above the mean of the controls. The classification was established as follows.

$OD \leq ODc = non - biofilm\ former$

$ODc < OD \leq 2 \times ODc = weak\ biofilm\ former$

$ODc < OD \leq 4 \times ODc = moderate\ biofilm\ former$

$4 \times ODc = strong\ biofilm\ former$

2.6. Biofilm formation after sublethal stress exposure

In addition to the tests to assess biofilm formation ability on microtiter plates, cultures of different isolates were tested in triplicate after being subjected to single sublethal stress. For the preparation of the bacterial cells to heat shock and cold shock, overnight cultures in PCA 1% NaCl were kept in water bath at 42 °C for 45 min and then at 20 °C for 4 h according to the methods proposed by Chang et al. (2004) and Lin et al. (2004), respectively. Bacterial cells were also exposed to a single sublethal stress at 4 °C for 4 h. The procedures described by Wong et al. (1998) were used to expose bacterial cells to an acidic environment. Overnight cultures in PCA 1% NaCl had their pH adjusted to 5.0 with HCl 6 N and were then incubated at 37 °C for 30 min. In order to run the test in different saline concentrations, overnight cultures in PCA 1% NaCl were subjected to concentrations of 0%, 2.5%, and 5% NaCl and incubated at 37 °C for 30 min. These saline concentrations were determined by Rosa et al. (2017) at the Lagoa dos Patos estuary in south Brazil which is the site of origin of the fish used in our study. After these steps had been completed, assessment of biofilm formation ability of each isolate was carried out as already described in item 2.5 of the present manuscript.

2.7. Resistance to antimicrobials

The disk diffusion technique with modifications was used for the determination of antimicrobial resistance and sensitivity, Cultures in PCA 1% NaCl were standardized using a spectrophotometer at 600 nm for a value of 0.5. With the aid of sterile swabs, samples were uniformly plated on Mueller Hinton agar (Kasvi, Roseti Degli Abruzzi, Italy) with 1% NaCl (agar MH 1% NaCl). With the aid of flambed and cooled tweezers, the disks impregnated with antimicrobials were placed on the surface of the inoculated. Plates were incubated at 37 °C for 24 h according to the guidelines provided by the National Committee for Clinical Laboratory Standards (2008).

For the antibiotic sensitivity testing, antimicrobials from previous studies published by other authors elsewhere were used and a few additional ones were selected as follows: chloramphenicol 30 µg (Han et al., 2007; Ottaviani et al., 2001; Sudha et al., 2014), tetracycline 30 µg (Han et al., 2007; Sudha et al., 2014), gentamicin 10 µg (Han

et al., 2007; Shaw et al., 2014; Sudha et al., 2014), ampicillin 10 μg (Han et al., 2007; Sudha et al., 2014), ciprofloxacin 5 μg (Han et al., 2007; Baker-Austin et al., 2009), norfloxacin 10 μg (Okoh and Igbinosa, 2010), neomycin 30 μg, penicillin G 10 μg, enrofloxacin 5 μg, and amikacin 30 μg.

The diameters of the inhibition zones were interpreted according to the recommendations published by the National Committee for Clinical Laboratory Standards (2008).

The tests were run in triplicate.

2.8. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

For the determination of the MIC and MBC, the techniques described in the National Committee for Clinical Laboratory Standards publication (2008) were used with modifications. The effect of the sanitizers 1% sodium hypochlorite and 7% sodium dioxide were tested on each isolate.

For the CIM test, the isolates were plated on PCA with 2% of NaCl (PCA 2% NaCl) and incubated at 37 °C for 24 h. The Agar PCA and the saline concentration of 2% was considered ideal for bacterial culture using this medium after tests with different concentrations were run (data not shown). The inoculum was prepared using the PCA colonies obtained in 2% NaCl, which were diluted in saline solution 1% and standardized in a spectrophotometer at 600 nm to 0.5 of the value DO that corresponds to the concentration of 10^8 CFU/mL. After adjustments, 0.5 mL of the solution was inoculated into 4.5 mL of Mueller Hinton Broth (MH, Kasvi, Roseti Degli Abruzzi, Italy) with 1% NaCl (MH broth 1% NaCl); 100 μL of broth MH 1% NaCl were inoculated into 96 wells of microtiter plates and 100 μL of sanitizer tested were added in the first column. Then 100 μL were inoculated successively into the remaining wells up to 10 serial dilutions; 100 μL of the last well of each series were discarded. This procedure was repeated using APA 1% NaCl in order to determine if there was any difference between each of these methods. Wells containing 1% NaCl MH broth or APA 1% NaCl were used as negative controls. Sterile water replaced the sanitizer added to the culture inocula. Plates were incubated at 37 °C for 24 h. Plate reading was performed on a spectrophotometer considering the presence or absence of turbidity in the culture medium.

CBM was performed according the method described by Santurio et al. (2007); 10 μL were removed from the wells with no growth, plated on PCA 2% NaCl, and incubated at 37 °C for 24 h. The reading was performed considering the presence or absence of bacterial growth.

The experiment was carried out in triplicate.

3. Results and discussion

3.1. Occurrence of V. vulnificus

Of the 217 strains analyzed which originated from 318 fish, 3 (1%) were identified as *V. vulnificus*. These isolates were recovered from 2 specimens of *P. orbignyanus* and 1 specimen of *M. furnieri*. Whole carcasses of fish (i.e. uneviscerated fish) contaminated with bacteria were analyzed. Standard methods were used for sample collection. These fish were captured between October 2013 and November 2013. This is the first report of *V. vulnificus* in *P. orbignyanus* and *M. furnieri* and also the first isolation of this bacterial pathogen of fish captured at the Lagoa dos Patos estuary in south Brazil.

DePaola et al. (1994) quantified *V. vulnificus* in fish, oysters, crabs, sediment and waters of the coast of Gulf of Mexico. These researchers found low levels of *V. vulnificus* during the winter. All the insulations occurred during the spring, summer, or fall. In our study, there was no insulation during the winter. All isolates were obtained from fish captured during the spring.

In the study carried out by DePaola et al. (1994), *V. vulnificus* was most frequently isolated from the fish *Archosargus probatocephalus* ("sargo-de-dentes" - sheepshead) than from sediment and sea water. The densities of *V. vulnificus* were higher (2 to 5 logs) in estuarine fish than in the surrounding waters, sediment, oysters, and shellfish. These findings from a previous study should not be directly related to the findings from our survey. In our study, bacterial solation was carried whereas in the study conducted by DePaola et al. (1994) bacterial count was performed. In addition, these authors did not provide any information on environmental conditions such as temperature and salinity at the time of sampling. However, in the present study, *V. vulnificus* was found only in fish. No isolates were recovered from the crustacean *F. paulensis* which corroborates the finding published by DePaola et al. (1994).

Fish from the genus *Paralichthys* are classified as benthic fish (de Astarloa and Munroe, 1998), i.e., fish that inhabit the bottom waters. These fish have the ability to swim, and are in constant contact with the substrate that precipitate at bottom waters. According to a study carried out by DePaola et al. (1994), *V. vulnificus* was most frequently found in the intestinal contents of fish with counts above other sources analyzed. Similarly, in our study 2 of the 3 isolates of *V. vulnificus* were found in *P. orbignyanus*.

According to Denadai et al. (2015), in southeast Brazil *M. furnieri* feeds basically on crustaceans and polychaetas. Feeding on mollusks is considered an opportunistic behavior due to the large number and extensive presence of these invertebrates in the area of study which demonstrates that *M. furnieri* may be considered a carnivore with a preference for benthic organisms. The study conducted by Denadai et al. (2015) is the closest to the southern region of Brazil. The results presented by these authors confirms that *M. furnieri* has feeding habits of demersal fish and is in constant contact with the substrate and possibly with the *V. vulnificus* at bottom waters.

Lack of adequate information precludes the distinction between pathogenic and non-pathogenic strains of *V. vulnificus*. Therefore, all strains of *V. vulnificus* are considered as potentially pathogenic species (Stelma Junior et al., 1992). Despite the low occurrence of this pathogen, the possibility of this microorganism being present in fish in a commerce setting would pose a risk to health of consumers, *V. vulnificus* is a bacterial pathogen that causes disease in humans with high mortality rate. It is worth noting that *Vibrio* infection is more prevalent in raw and undercooked seafood. However, cross-contamination between raw and ready-to-eat foods and utensils (fomites) is also an effective route to spread this foodborne pathogen (Froelich and Noble, 2016; Malcolm et al., 2018).

3.2. Biofilm formation on microtiter plates and after sublethal stress

Additional tests for molecular identification were performed on *Vibrio* isolates recovered from the flounder *P. orbignyanus*. These isolates were identified herein as "Isolate 37" and "Isolate 38".

Both isolates were able to form biofilm when not subjected to sublethal stress. One isolate - Isolate 37 - was considered a weak biofilm former whereas the other - Isolate 38 - was categorized aa a strong biofilm former (Stepanovic et al., 2000). In the present study, Isolate 37 did not form biofilm when subjected to (exposed to) different stress conditions and levels.

In contrast, Isolate 38, when subjected to a temperature of 4 °C became a weak biofilm former. Similarly, this isolate failed to form biofilm when subjected to a concentration of 0% NaCl. Regarding the other stress conditions, Isolate 38 did not form biofilm.

Jefferson (2004) states that bacteria form of the biofilm as a defense mechanism in response to stress. However, in the present study biofilm formation was observed under optimal conditions to grow.

This is the first study in which wild isolates of *V. vulnificus* were usedv to assess the effect of stress factors on biofilm formation. However, a similar study carried out by Rosa et al. (2017) evaluated biofilm formation by *V. parahaemolyticus* subjected to the same stress factors used in our study. The majority of the isolates maintained their

ability to form biofilm after stress exposure and 25% of these isolates increased their ability to form biofilms. Our findings are not accordance with those of Rosa et al. (2017) as in our study biofilm formation ability of *Vibrio* decreased or was inhibited after stress exposure.

Similar studies assessed biofilm formation ability of other microorganisms. Galvão et al. (2012) assessed the biofilm formation ability of *Listeria monocytogenes* in different inoculum concentrations, nutrient availability, NaCl concentrations, incubation time, and pH, and found no correlation between stressors and an increase in adhesion. In contrast, Lianou and Koutsoumanis (2012) evaluated *Salmonella enterica* strains isolated from humans and cattle and noted an increase in biofilm formation in a pH, temperature, and NaCl concentration less favorable to the development of this microorganism. Variation in results between studies show that each species of bacteria has certain behaviors when exposed hostile environments and therefore may or may not form biofilms.

3.3. Antimicrobial resistance

Isolate 37 expressed intermediate sensitivity to chloramphenicol. Isolate 38 expressed intermediate sensitivity to ciprofloxacin and norfloxacin (National Committee for Clinical Laboratory Standards, 2003). Both isolates were resistant to all the other antimicrobials tested.

According to a survey conducted by Elmahdi et al. (2016) in several countries, *V. vulnificus* and *V. parahaemolyticus* have similar profiles of antimicrobial resistance. There is greater resistance to the antimicrobials ampicillin, penicillin and tetracycline regardless of the country where these bacteria were isolated. Our results are in agreement with those of Elmahdi et al. (2016) since the two isolates of *V. vulnificus* obtained from fish captured at the Lagoa dos Patos Estuary in south Brazil were resistant to these antimicrobials with inhibition halos of 0 mm in diameter in the disc-diffusion antibiotic testing. The authors speculate that the resistance developed by bacteria to similar antimicrobials both environmental and clinical is probably not due to the erroneous use and/or injudicious use of antimicrobials. This ability that bacteria has to resist antibiotic therapy is probably related to a characteristic of some species.

Tetracycline was considered the antibiotic of choice for the treatment of cases of *Vibrio* infection (Morris and Tenney, 1985). However, recent studies have suggested that both *V. parahaemolyticus* and *V. vulnificus* have developed resistance to tetracycline (Labella et al., 2013; Pan et al., 2013). Our findings corroborate those of previous studies conducted by other authors. The present study shows that both isolates are resistant to tetracycline (Fig. 1). Rebouças et al. (2011) analyzed 31 isolates of *Vibrio* from shrimp (*Litopenaeus vannamei*) farms in Brazil and tested the sensitivity of these isolates to 11 antimicrobial agents using the disk diffusion method. The authors demonstrated that the many isolates were resistant to ampicillin (45.2% of the isolates) and to tetracycline (38.7% of the isolates). In our study, both isolates were resistant to ampicillin, and these isolates did not form an inhibition halo in the culture media.

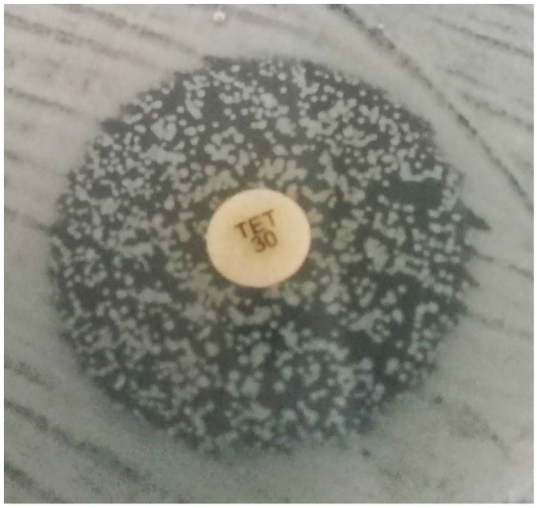

Fig. 1. Disk-diffusion test with *V. vulnificus* resistant to tetracycline 30 µg.

Han et al. (2007) used MIC to test the antibiotic sensitivity of 319 strains of *V. vulnificus* and *V. parahaemolyticus* to chloramphenicol, tetracycline, gentamycin, ciprofloxacin, and ampicillin. These authors found that the *V. vulnificus* isolates were susceptible to these antimicrobials. These results show that first line drugs are effective against *V. vulnificus*. In our study, different results were obtained as both isolates were resistant to tetracycline, streptomycin, and ampicillin. Isolate 38 showed antibiotic resistance. Isolate 37 had intermediate resistance to chloramphenicol. Isolate 37 expressed resistance to ciprofloxacin where Isolate 38 expressed intermediate resistance to ciprofloxacin (Fig. 1). These different results may have occurred due to differences between the strains or variations in method and concentrations of antimicrobials used.

Sudha et al. (2014) evaluated antimicrobial susceptibility of isolates of *V. vulnificus* obtained from shrimp, crab, and shellfish in India to many. These researchers showed that, unlike our isolates, their isolates expressed sensitivity to ampicillin, tetracycline and chloramphenicol, gentamicin. In the present study, Isolate 38 expressed intermediate resistance to ciprofloxacin and resistance to chloramphenicol and gentamicin. Other studies previously published elsewhere by other researchers emphasize that *V. vulnificus* express the sensitivity to chloramphenicol (Ottaviani et al., 2001), gentamicin (Shaw et al., 2014), ciprofloxacin (Baker-Austin et al., 2009), and norfloxacin (Okoh and Igbinosa, 2010) unlike our study, demonstrating that there may be variations in the antibiotic sensitivity of *V. vulnificus* strains.

To our knowledge, there are no published reports in the literature regarding the action of penicillin G, neomycin, enrofloxacin and amikacin. However, in the present study we demonstrated that these antimicrobials are not adequate for the treatment of *V. vulnificus* infections.

3.4. Resistance to sanitizers

MIC was determined according to the last dilution with no turbidity in three replicates whereas MBC was determined after streaking the last three dilutions which no turbidity in the of MIC test in PCA with 1% NaCl (Table 3).

According to the results obtained in the MIC and MBC methods, we noted that the isolates grew most rapidly in MH broth 1% NaCl than the APA 1% NaCl which is commonly used for this method. Higher concentrations of sanitizers were necessary to kill the pathogen when this medium was used. MH 1% NaCl is therefore the most appropriate medium to run MIC and MBC for *V. vulnificus* isolates.

The concentration of sodium hypochlorite solution for water supply recommended by the Brazilian legislation is 2 ppm of Cl_2 (Brasil, 2017).

Table 3
Minimum Inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC) in ppm of sanitizers hypochlorite of sodium and chlorine dioxide to *V. vulnificus*.

Strain	Sodium hypochlorite				Chlorine dioxide			
	MH 1% NaCl[a]		APA 1% NaCl[b]		MH 1% NaCl		APA 1% NaCl	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
37	1250	2500	2500	2500	2187	8750	1093	2187
38	1250	5000	1250	1250	1093	8750	2187	2187

[a] Mueller Hinton (MH) broth with 1% NaCl;
[b] Alkaline Peptone Water with 1% NaCl.

The concentrations commonly used in the fish industry to wash fillets, the fish external aspect and the surface for disinfection are 2, 5, and 20 ppm of Cl_2, respectively. In our study. None of these was efficient in killing or decreasing the numbers of *V. vulnificus.* These results highlight the importance of this review for manuals in good practices in the fish industry and establishments of adequate control measure to prevent contamination of fish with *V. vulnificus*.

Some manuals such as the Community Centers of production for Fish Warehouse (Eletrobras, 2015) recommend sanitizing and disinfection with sodium hypochlorite which may be performed by contact - flow, immersion and/or spraying of the parties - using a solution of 100 ppm (immersion) or 200 ppm (spraying). The material should remain in contact with the solution for 15 min and this should be done after rinsing it with water. The goal is to reduce significantly the number of deteriorating microorganisms and eliminate the pathogen. However, our finding show that this concentration is not sufficient for the inhibition of *V. vulnificus* but possibly for the microorganism elimination.

4. Conclusions

V. vulnificus occurs in *P. orbignyanus* and *M. furnieri* captured at the Lagoa dos Patos Estuary in south Brazil. The isolates tested, although with different intensity, are able to form biofilm in favorable conditions for the microbial multiplication. However, this ability is decreased or inhibited after the isolates are exposed to sublethal stress.

V. vulnificus is resistant to most antimicrobials tested. This finding confirm the difficulty in finding antimicrobial agents able to fight off infections caused by these microorganisms. The concentrations of sodium hypochlorite and chlorine dioxide that are necessary to eliminate *V. vulnificus* are higher than those commonly used in the fish industry.

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