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## ENZYMATIC CHARACTERIZATION OF *Vibrio alginolyticus* STRAINS ISOLATED FROM BIVALVES HARVESTED AT VENICE LAGOON (ITALY) AND GUANABARA BAY (BRAZIL)

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### SUMMARY

The aquatic ecosystem is the natural *habitat* of microorganisms including *Vibrio* and *Aeromonas* genus which are pathogenic to human and animals. In the present investigation the frequency of these bacteria and the enzymatic characteristics of 34 *Vibrio alginolyticus* strains isolated from bivalves harvested in Venice Lagoon (Italy) and Guanabara Bay (Brazil) were carried out from November 2003 to February 2004. The mussels' samples were submitted to enrichment in Alkaline Peptone Water (APW) added with 1% of sodium chloride (NaCl) and APW plus 3% NaCl incubated at 37 °C for 18-24h. Following the samples were streaked onto TCBS Agar (Thiosulfate Citrate Bile Sucrose Agar) and the suspected colonies were submitted to biochemical characterization. Also, the *Vibrio alginolyticus* strains were evaluated to collagenase, elastase and chondroitinase production. The results showed the isolation of 127 microorganisms distributed as follows: 105 *Vibrio* strains such as *V. alginolyticus* (32.4%), *V. harveyi* (19%) and *V. parahaemolyticus* (7.6%), 20 *Aeromonas* strains and two *Plesiomonas shigelloides* were the main pathogens isolated. We observed the production of the three enzymes from *V. alginolyticus* strains considered as the main virulence factors of the bacteria, especially in cases of human dermatological infection.

**KEYWORDS:** *Vibrio* spp.; *Vibrio alginolyticus*; Enzymatic Activities; Pathogenicity.

### INTRODUCTION

The marine bivalve's consumption is an increased alimentary habit at coastal regions around the world. Therefore, mussels consumed without cooking or after an insufficient process of cooking may be at risk for humans because these animals are capable to retain pollutants and pathogenic bacteria in their organisms<sup>5,7</sup>.

The aquatic ecosystem is the natural habitat of microorganisms belonging to the *Vibrio* genus and the most known is *Vibrio cholerae*, the causative agent of cholera. Otherwise, many species are able to transmit intestinal or extra intestinal diseases to human. Amongst these last ones *V. vulnificus*, *V. carchariae* and *V. alginolyticus* are widely spread in the marine environment; both in closed seas, like the Mediterranean, and in oceans<sup>5,10,11</sup>.

These bacteria have been found in water samples from marine environment, mussels, fish and their products. Since the end of the 80s, the presence of these microorganisms was correlated to human infections. The infection sites (the medium ear, conjunctive membrane, open wounds) were hosting a complex microbial flora. Infections caused by *Vibrio alginolyticus* can present themselves as septicemic related to the ingestion of contaminated seawater or seafood products specially

in patients as elders, children, carriers of *diabetes mellitus* or chronic hepatic pathologies<sup>1,3</sup>.

The cutaneous infections can be caused when wounds were in the presence of water where the bacterium is autochthonous. Often patients with dermatitis can develop septicemia from primary lesion (wounds infections). The symptomatology of dermatitis is the same to all *Vibrio* dermatopathogens and includes edema, erythema, and blisters with serum-hemorrhagic content, ulcers and necrosis of the affected area. In some cases, the lesion becomes deeper with sections of necrotizing fasciitis or osteomyelitis. The pathogenesis of these symptoms is not total elucidated, but diverse factors are included as the action of isoenzymes such as elastases, collagenases, DNAses, chondroitinases, gelatinases, lechitinases and keratinases. These enzymes have different functions: the collagenase enzyme is able to degrade conjunctive tissue and basal epithelial membrane is the key to cause extra intestinal pathology and dissemination to blood stream. The inability of some species, like the *V. fluvialis*, to degrade the components of the conjunctive tissue seems to limit its capacity to cause extra intestinal pathology. The concomitant lack of the keratinases in the majority of *Vibrio* species, and the ability to degrade the conjunctive tissue is consistent with their condition of opportunists' bacteria which need a primary lesion to invade the derma and hypodermic layers of the skin<sup>3,5,10,11</sup>.

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The decision to compare the bacteria isolated from mussels harvested in two distant geographical areas, Venice and Rio de Janeiro, was based on the same characteristics of these cities. In both we observed anthropogenic problems as chemical and microbiological pollution and the progressive increase of water temperature recorded in last years in consequence of global climate changes. Otherwise, we observed the increase of development activities associated to mollusk cultures and seafood consumption, particularly of the species: *Mytilus galloprovincialis* and *Ruditapes semidecussatus* in Venice Lagoon and *Perna perna* in Guanabara Bay<sup>4</sup>.

The aim of this research was evaluate the frequency of *Vibrio alginolyticus* isolated from bivalves mollusks harvested in Italy and Brazil and their enzymes (collagenases, elastases and chondroitinases).

## MATERIAL AND METHODS

A total of 30 samples of bivalve mollusks were collected in Venice Lagoon between September and November 2003. Dipartimento di Sanità Pubblica, Patologia Comparata ed Igiene Veterinaria Agripolis, Università di Padova (Italia). The samples were composed by 200 adult mussels presenting their valves closed and commercial size (larger than 6 cm). The mussels were carried out at 6-10 °C and sent to Dipartimento di Sanità Pubblica, Patologia Comparata ed Igiene Veterinaria Agripolis, Università di Padova (Italia). From these *in natura* samples some strains of bacteria belonging to the *Vibrio* kind have been isolated according to the American Food and Drug Administration (FDA) guidelines (Bacteriological Analytical Manual - BAM). The samples were submitted to enrichment at Alkaline Peptone Water (APW) added of 1% of sodium chloride (NaCl) and APW plus 3% NaCl (37 °C/18-24 h). Then, samples were streaked onto TCBS Agar (Thiosulfate Citrate Bile Sucrose Agar). The classification of bacteria isolated has been carried out using the API 20 E (Bio Mérieux) kits. The bacteria identified have been subsequently sent to the Laboratório de Referência Nacional de Cólera e outras Enteroinfecções Bacterianas (LRNCEB), Instituto Oswaldo Cruz (IOC), FIOCRUZ in Rio de Janeiro, to confirm the biochemical characterization of the species and to carry out the virulence tests<sup>2</sup>.

In Brazil, a total of 20 samples of *Perna perna* were collected, between January and February 2004, in Guanabara Bay (Rio de Janeiro) and analyzed at LRNCEB. Also, these *in natura* samples were submitted to the same enrichment described above and streaked onto TCBS Agar. The preliminary confirmation strains were carried out according to colonies behavior in LIA (Lysine Iron Agar) and TSI (Triple Sugar Iron Agar) allowing the *Vibrio* spp. presumptive differentiation. After, the cytochrome oxidase positive colonies were submitted to biochemical tests including resistance to O/129 vibriostatic agent (2,4 diaminopiperidine), ONPG production (ortho-nitrophenyl- $\beta$ -D galactopyranoside), VP (Voges-Proskauer), lysine/ornithine decarboxylase and arginine dihydrolase and halophilic characteristics (0 to 10% of sodium chloride)<sup>2</sup>.

The dermatopathogenicity of all strains of *V. alginolyticus* was analyzed for the presence of virulence factors represented by the three enzymes collagenolytic: elastases, collagenases and chondroitinases. The *Vibrio alginolyticus* strains were growth in BHI (Brain Heart Infusion) broth (37 °C/18-24h) and then streaked onto Agar BHI added

with 1% of collagen. Agar BHI added with 1% of elastin and Agar BHI added with 0.04% chondroitin sulfate and 1% bovine serum (37 °C/18-24h). The interpretative test was carried out in seven days considering positive colonies result as a clearing zone (translucent and luminous halo) under and around the spots inoculums<sup>2</sup>.

## RESULTS

The results showed a total of 127 bacteria strains distributed in 15 different species of *Vibrio* (82.6%), five *Aeromonas* species (15.74%) and two *Plesiomonas shigelloides* (1.57%). The main *Vibrio* isolated from mussels samples were *Vibrio alginolyticus* (32.4%), *V. harveyi* (19%), *V. parahaemolyticus* (7.6%) and *Vibrio* spp. (18%). Other species as *V. carchariae*, *V. logei*, *V. mediterranei*, *V. damsela* and *V. anguillarum* were also isolated as shown in Table 1. Some species were isolated in low frequency (< 1%) as *V. cholerae* non O1, *V. campbelli*, *V. fluvialis*, *V. gazogenes*, *V. metschnikovii*, *V. fisheri* and *V. aestuarinus*.

**Table 1**  
Distribution of Vibrios isolated from bivalve mollusks in Venice Lagoon (Italy) and Guanabara Bay (Brazil)

Microorganisms	Venice Lagoon		Guanabara Bay		Total	
	n	(%)	n	(%)	n	(%)
<i>Vibrio cholerae</i> non O1	1	0.95	-	-	1	0.95
<i>Vibrio campbelli</i>	-	-	1	0.95	1	0.95
<i>Vibrio fluvialis</i>	-	-	1	0.95	1	0.95
<i>Vibrio gazogenes</i>	-	-	1	0.95	1	0.95
<i>Vibrio metschnikovii</i>	-	-	1	0.95	1	0.95
<i>Vibrio fisheri</i>	-	-	1	0.95	1	0.95
<i>Vibrio aestuarinus</i>	-	-	1	0.95	1	0.95
<i>Vibrio anguillarum</i>	-	-	2	1.9	2	1.9
<i>Vibrio damsela</i>	-	-	2	1.9	2	1.9
<i>Vibrio mediterranei</i>	-	-	3	2.8	3	2.8
<i>Vibrio logei</i>	-	-	4	3.8	4	3.8
<i>Vibrio carchariae</i>	-	-	6	5.7	6	5.7
<i>Vibrio parahaemolyticus</i>	2	1.9	6	5.7	8	7.6
<i>Vibrio harveyi</i>	-	-	20	19	20	19
<i>Vibrio alginolyticus</i>	6	5.7	28	26.7	34	32.4
<i>Vibrio</i> spp.	3	2.8	16	15.3	19	18
<b>Total</b>	<b>12</b>	<b>11.5</b>	<b>93</b>	<b>88.5</b>	<b>105</b>	<b>100</b>

Otherwise, the main *Aeromonas* isolated were *Aeromonas sobria* (18.2%), *A. veronii* biogroup *veronii* (18.2%), *A. veronii* biogroup *sobria* (18.2%), *A. caviae* (13.6%), *A. hydrophila* (13.6%) and *Aeromonas* spp. (9%) (Table 2).

A total of six *Vibrio alginolyticus* strains coming from the Venice Lagoon and 28 coming from the Guanabara Bay have been analyzed. Among the Italian strains we observed that four strains (11.8%) produced all three enzymes: elastases (ELA), collagenases (COL) and chondroitinases (CHO); the other two (2.9% each) produced only one enzyme: one elastases, the other one chondroitinases. Amongst the Brazilian strains we observed that 14 strains (41%) produced the three enzymes altogether; 11 produced two enzymes (ELA-COL, ELA-CHO and CHO-COL). Amid these, four (11.8%) produced elastases and

**Table 2**

Distribution of *Aeromonas* and *Plesiomonas shigelloides* isolated from bivalve mollusks in Guanabara Bay (Brazil)

Microorganisms	n	(%)
<i>Aeromonas sobria</i>	4	18.2
<i>Aeromonas caviae</i>	3	13.6
<i>Aeromonas veronii</i> biogroup <i>veronii</i>	4	18.2
<i>Aeromonas veronii</i> biogroup <i>sobria</i>	4	18.2
<i>Aeromonas hydrophila</i>	3	13.6
<i>Aeromonas</i> spp.	2	9
<i>Plesiomonas shigelloides</i>	2	9
<b>Total</b>	<b>22</b>	<b>100</b>

**Table 3**

Frequency of virulence factors production (enzymes) from *V. alginolyticus* strains isolated in Italy and Brazil

Enzymes Profiles	<i>Vibrio alginolyticus</i> strains					
	Italy		Brazil		Total	
	n	(%)	n	(%)	n	(%)
ELA-COL-CHO	4	11.8	14	41	18	53
ELA-COL	-	-	4	11.8	4	11.8
ELA-CHL	-	-	4	11.8	4	11.8
CHO-COL	-	-	3	8.8	3	8.8
COL	-	-	1	2.9	1	2.9
ELA	1	2.9	1	2.9	2	5.8
CHO	1	2.9	-	-	1	2.9
No enzyme production	-	-	1	2.9	1	2.9
<b>Total</b>	<b>6</b>	<b>17.6</b>	<b>28</b>	<b>82.3</b>	<b>34</b>	<b>100</b>

ELA = elastases, COL = collagenases and CHO = chondroitinases.

collagenases and just as many elastases and chondroitinases, three produced CHO-COL (8.8%). A strain did not produce any of the three enzymes (Table 3).

## DISCUSSION

In this study the presence of Vibrionaceae and Aeromonadaceae families isolated from bivalves mollusks harvested in Italy and Brazil was, not surprisingly also present and considered as an important group of autochthonous pathogens inhabitant of marine environment and distributed worldwide<sup>7</sup>.

There was observed the frequency of certain *Vibrio* (*V. parahaemolyticus* and *V. cholerae* non O1) and *Aeromonas* species (*A. hydrophila* and *A. sobria*) related to foodborne outbreaks after the consuming of *in natura* or undercooked seafoods. Also, we observed the isolation of *Plesiomonas shigelloides* an emergent pathogen isolated from aquatic ecosystem including fresh and estuarine water. These pathogens may cause different pathologies especially to immunocompromised patients that are more susceptible to infections<sup>5,7</sup>.

*Vibrio alginolyticus* was the major specie isolated from the mussels

analyzed showing the relevance to marine ecosystem particularly when associated to the risks for human health (handlers, fisheries, swimmers and seafood consumers) after exposure to aquatic environment. Moreover, the high incidence of this pathogen has been related to gastrointestinal and extraintestinal infections as otitis and cutaneous lesions in humans and animals. This epidemiological aspect is relevant specially to food chain and aquaculture professionals<sup>1,7</sup>.

Both in Venice Lagoon and Guanabara Bay, the majority of strains of *Vibrio alginolyticus* point to increasing virulence characteristics expressed by the presence of three enzymes at the same time while strains with low or null infectivity were minimal or absent in these cities<sup>5</sup>.

There are several works dealing on extra cellular enzymes in waterborne (both salt- and fresh-water) bacteria. They include the investigation developed by RODRIGUES *et al.*, (1992), who studied the pathogenicity of *V. vulnificus* from seawater and oyster from the state of Rio de Janeiro; RIBEIRO (1994), who analyzes enzymatic patterns in species of the *Vibrio* genus (*V. vulnificus*, *V. fluvialis*, *V. mimicus*) isolated from seawater from the coast of the state of Rio de Janeiro; and PAVLOV *et al.*, (2003), who studied several potentially pathogenic features of heterotrophic plate count bacteria (HPC) isolated from drinking water in South Africa. No studies have been found on pathogenicity of *V. alginolyticus* in bivalve molluscs from Rio de Janeiro, nor from the Lagoon of Venice<sup>6,8,9</sup>.

The results of these similar researches found non pathogenic *V. vulnificus* (absence of elastase, collagenase and chondroitinase enzymes) as the most common specie isolated but they did not describe the presence of elastase, collagenase and chondroitinase enzymes production at the same time in any *V. vulnificus* strain (the most common profile among our *V. alginolyticus* strains)<sup>8,9</sup>.

These studies described the frequency of elastase, collagenase and chondroitinase among the strains analyzed. RIBEIRO (1994) found that the most common enzyme among *Vibrio* spp. strains was chondroitinase, present in 68.1% of analyzed strains. Others found the most common enzyme among *V. vulnificus* and HPC was elastase (50% of *V. vulnificus*, 33% of HPC strains)<sup>6,9</sup>. The latter results are in accordance with those we found in *V. alginolyticus*, although our bacteria produced elastase much more frequently: it is found in 23 of all Brazilian strains. Collagenase (22 strain productions) and chondroitinase (21 strain productions) are also more frequent among the bacteria we analyzed, than among those studied by others. These results show how *V. alginolyticus*, although often considered to be a low pathogenic or no-pathogenic species does have a considerable pathogenic potential, because of its capacity to produce pathogenic enzymes and because of its frequency, in both the Lagoon of Venice and Guanabara Bay.

However there may be several reasons why reported cases of dermatitis due to this bacteria are so scarce, e.g. the specificity of symptoms, which may lead to misidentification of pathogen causing dermatitis; little knowledge on the part of doctors about *Vibrio* dermatitis; the susceptibility of this bacteria to a great number of antibiotics, so often prescribed when any kind of infection occurs; and the low resistance of bacteria when exposed to air and sun. Further

studies will be carried out to study these bacteria, analyzing the role of polluted environments on bacterial resistance to the most common antibiotics and to heavy metals<sup>6,8,9</sup>.

Molecular studies showed that *Vibrio alginolyticus* strains could acquire virulence factor genes by transduction between other marine bacteria. Then, *V. alginolyticus* can be a reservoir for virulence genes present in the aquatic environment. In spite of these premises, the clinical cases of reported human cutaneous vibriosis are insufficient both in the Venetian area and in Rio de Janeiro. Doctors' inadequate knowledge of this bacterium, the specificity of the symptoms correlated to it and its susceptibility to many antibiotics surely participate to the scarcity of cases of dermatitis reported<sup>3,10,11</sup>.

The occurrence of potential pathogens with or without virulence factors isolated in our investigation alert to their importance in Public Health and suggests the need to implant changes in hygiene and sanitary fields, particularly in practices involving handlers in order to prevent or control the multiplication of these pathogens in seafood or aquaculture systems.

## RESUMO

### Caracterização enzimática de cepas de *Vibrio alginolyticus* isoladas de bivalves coletados na Lagoa de Veneza (Itália) e Baía de Guanabara (Brasil)

O ecossistema aquático é o *habitat* natural de microrganismos incluindo aqueles dos gêneros *Vibrio* e *Aeromonas* os quais são patogênicos para o homem e animais. Na presente investigação foi avaliada a frequência destas bactérias e a característica enzimática de 34 cepas de *Vibrio alginolyticus* isoladas de bivalves coletados na Lagoa de Veneza (Itália) e Baía de Guanabara (Brasil) durante o período de Novembro-2003 a Fevereiro-2004. As amostras de mexilhões foram submetidas a enriquecimento em Água Peptonada Alcalina (APA) adicionada de 1% de Cloreto de Sódio (NaCl) e APA com 3% de NaCl (37 °C/18-24h). Em seguida as amostras foram semeadas em Agar TCBS (Agar Tiosulfato Citrato Bile Sacarose) e as colônias suspeitas foram submetidas à caracterização bioquímica. As cepas de *Vibrio alginolyticus* foram avaliadas quanto à produção das enzimas colagenase, elastase e condroitinase. Os resultados demonstraram o isolamento de 127 microrganismos assim distribuídos: 105 cepas de *Vibrio* das quais *V. alginolyticus* (32,4%), *V. harveyi* (19%) e *V. parahaemolyticus* (7,6%), 20 cepas de *Aeromonas* e 2 *Plesiomonas shigelloides* foram os principais patógenos isolados. Observou-se a

produção das três enzimas a partir de *V. alginolyticus*, consideradas principais fatores de virulência da bactéria, em especial em casos de infecção dermatológica humana.

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