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ARTÍCULO DE INVESTIGACIÓN

Adherence and enzymatic activity of *Vibrio alginolyticus* isolated from shellfish for human consumption

Adherencia y actividad enzimática de *Vibrio alginolyticus* aislados de mariscos para consumo humano

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ABSTRACT: *Vibrio alginolyticus* is an emerging pathogen of seafood-borne diseases. **Aims:** to determinate adherence capacity and enzymatic activity of *V. alginolyticus* isolated from shellfish. **Materials and Methods:** In this study, the adherence capacity and enzymatic activity (DNAse, lipase, and gelatinase) were determined in 50 *V. alginolyticus* strains isolated from shellfish for human consumption. **Results and discussion:** 98% of the strains were able to adhere to HEp-2 cells. In this regard, 24 (48%) and 5 (10%) of *V. alginolyticus* isolates were classified as having a medium and strong adherence ability, respectively. On the other hand, production of DNAse, lipase and gelatinase enzymes were confirmed in all strains. **Conclusions:** Our results suggest a virulence potential of *V. alginolyticus* isolated from shellfish, which could contribute to infection associated to seafood consumed raw or undercooking.

KEYWORDS: ***Vibrio alginolyticus*, shellfish, adherence.**

INTRODUCTION

Vibrio alginolyticus is a Gram-negative bacterium inhabitant of estuarine and marine environments. It is described as the most halophilic species of the genus, growing in NaCl concentrations of 3 to 10% (Yin *et al.*, 2022). This species has been isolated from different marine organisms, being part of saprobic microbiota. However, different studies highlight the pathogenic role of this bacterium in several species of marine animals and human beings (Gómez-León *et al.,* 2005; Li *et al.,* 2009). Human infection is related to the consumption of raw or undercooking seafood (i.e., shellfish and fish), mainly causing acute diarrhea. In lesser extent, extra-intestinal infections, such as otitis, infected injuries and septicaemia have been reported (Balebona *et al.,* 1998; Snoussi *et al.,* 2008a; Zhou *et al.,* 2021).

Adherence capacity is the first event in bacterial pathogenesis, and this process benefits saprobic and pathogenic bacteria. In saprobic bacteria, an increase in metabolic activity has been demonstrated; in pathogenic bacteria, it would be the starting point of an infection in a susceptible host where it would subsequently invade and/or produce extra-cellular products that could damage tissues (Carbone *et al.,* 2003; Snoussi *et al.*, 2008b). Moreover, several virulence factors have been identified in *V. alginolyticus* isolated from oysters, such as a hemolysin similar to the thermostable direct hemolysin (TDH)-related hemolysin of *V. parahaemolyticus* (González-Escalona *et al.,* 2006). On the other hand, the presence of *toxR*, *toxT* and *toxS* virulence genes in this species has been demonstrated (Snoussi *et al.*, 2008a), which have been previously detected in *V. cholerae*, alongside a type III secretion system and several extra-cellular enzymes such as DNAse, amylase, elastase, collagenase, lipase, gelatinase and β lactamase production (Lafisca *et al.,* 2008; Zhao *et al.,* 2011; Cheng *et al.,* 2021).

The consumption of shellfish is the main transmission route of this pathogen, since inadequate cooking can cause an intestinal infection (Schets *et al.*, 2010). Hence, the aim of this study

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was to evaluate the adherence ability and enzymatic activity of *V. alginolyticus* isolates recovered from shellfish destined for human consumption in Southern Chile.

MATERIAL AND METHODS

Samples

Fifty-three shellfish samples were analyzed. Each sample consisted in two to three shellfish corresponding to ribbed mussels (*Aulacomya ater*), razor clams (*Tagelus dombeii*), clams (*Venus antiqua*), mussels (*Mytilus chilensis*) and giant mussels (*Choromytilus chorus*). The samples were obtained from the fish market in Valdivia, Southern Chile (lat –39.8126, long –3.248), and immediately taken to the laboratory in order to be processed. The samples were placed in hermetic bags and homogenized. Then, 90 mL of alkaline peptone water (APA, pH 8.6) with 3% NaCl was added to each bag and incubated at 30 ºC for 20 h. After incubation time, a 100 µL APA aliquot was seeded in a TCBS plate (Difco) and incubated at 37 ºC for 24 h. From each positive plate yellow suspicious colonies were subcultured in sheep blood agar plates and incubated under the same conditions described above. The strains obtained, were preserved in brain-heart infusion broth (Merck), supplemented with 20% glycerol at –80 ºC until its identification.

IDENTIFICATION

For the identification of *V. alginolyticus,* a species-specific PCR was used, utilizing primers VA-F and VA-R (Di pinto *et al.,* 2005). The extraction of DNA from the strains was carried out by the boiling point method (Houf *et al.,* 2002). For the PCR reaction a total volume of 25 µL was used which contained the following components: 2.5 µL PCR Buffer 10X, 0.75 µL $MgCl_2$ (50 mM), 0.5 µL dNTPs (10 mM each), 1.25 µL from each primer, 0.5 µL of Taq polymerase (5 U/ µL), 17.75 µL nuclease free water, and 0.5 µL genomic DNA from the strains to be investigated. As a positive control, a *V. alginolyticus* NCTC 12160 strain was used. The PCR conditions were the following: initial denaturation of 95 ºC during 15 min, followed by 35 denaturation cycles at 94 ºC for 30 s, alignment at 57 ºC for 30 s and an extension at 72 ºC during 1 min, finishing with a final extension at 72 ºC for 5 min. The separation of the PCR amplification products was carried out in agarose gel at 1% at a constant 100 W voltage during 50 min using a tri-acetate-EDTA (TAE) buffer at 1X. To visualize the stripes, 2 µL of MaestroSafe® was added to the agarose gel, and after finishing the electrophoretic run, the gel was visualized under the UV light. A 100 pb weight standard was used (Fermentas).

ADHERENCE ASSAYS

Adherence assays were performed using HEp-2 epithelial cells monolayers prepared on coverslips with a concentration of 1.5 x 10^5 of cells using RPMI culture medium (Sigma-Aldrich), supplemented with a 10% bovine fetal calf serum (Sigma-Aldrich), and incubated at 37 °C under a 5% CO2 atmosphere. HEp-2 cells monolayers were infected with 67 µL (1x10^7 bacteria approximately), of a 0.5 McFarland suspension of *V. alginolyticus*. To allow bacterial adherence, the cell culture trays were incubated at 37 ºC for 3 h in a 5% CO_2 atmosphere. After the incubation, monolayers were washed ten times with a sterile Phosphate Buffered Saline and then, fixed with methanol at 40% for 15 min, and dyed with May Grünwald-Giemsa. As a positive control, an enteropathogenic *Escherichia coli* adherent proven strain of was used. To establish the adherence capacity of the strains of *V. alginolyticus,* 100 cells per coverslip were counted, and the number of adhered bacteria per cell was recorded. Strains were classified as non-adherent [NA] (0-10 bacteria/cell), weak adherence [WA] (10-20 bacteria/cell), medium adherence [MA] (20-50 bacteria/cell), and strong adherence [SA] (50-100 bacteria/cell), as previously described (Snoussi *et al.*, 2008a).

ENZYMATIC ACTIVITY

In order to assess the activity of DNAse (Difco), gelatinase (Difco) and lipase (Merck) enzymes, the respective culture medium was prepared, following the instructions from the manufacturer. Suspensions of the strains under study were prepared with turbidity equal to the 0.5 McFarland and then, using a Steers inoculator, the strains were inoculated in the mediums. The plates were incubated for 24 h, at 37 ºC in aerobic conditions. A strain of *Staphylococcus aureus* ATCC 25923 and a strain of *Escherichia coli* ATCC 25922 were used as a positive and negative controls, respectively (Kelly *et al.,* 1989).

RESULTS AND DISCUSSION

Vibrio alginolyticus was isolated in 94.3% (50/53) of the samples, being the only *Vibrio* species isolated. This high prevalence agrees with previous reports of *V. alginolyticus* from seawater collected near aquaculture areas and seafood product in Italy, and from Dutch shellfish (Baffone *et al.,* 2000; Schets *et al.*, 2010).

In relation to adherence, 49 (98%) strains were able to adhere to Hep-2 cells, having found heterogeneous results related to adherence classification, 24 strains (48%) presented medium adherence, 5 (10%) showed strong adherence, 20 (40%) of them presented weak adherence and one strain was non-adherent Figure 1.

This correlates with a previous study conducted in Italy, which determined the adherence capacity of 24 strains of *V. alginolyticus*, isolated from seawater and seafoods collected from the central coast of the Adriatic Sea, demonstrating that 21 (98.5%) strains were able to adhere to Hep-2 cells (Baffone *et al.*, 2005). However, with regard to adherence intensity, their results were different since 62.5% and 21.4% of their strains presented weak adherence and medium adherence, respectively. None of their showed strong adherence whereas 10% of our strains showed this type of adherence. On the other hand, *V. alginolyticus* strains isolated from Mediterranean seawater (Bay of Khenis, Tunisia) were less adherent then our strains being 57.1% non-adherent, 10.7% showed weak adherence, 3.5% showed medium adherence and 21.4% presented weak adherence (Snoussi *et al.,* 2008a). In accordance to the previously mentioned authors, the origin of the strains could be an important factor related to the adherence capacity, since the most adherent strains were isolated from marine animals (clams, squids, mussels, fish) while the strains isolated from water presented a less strong adherence (Baffone *et al.*, 2005).

Despite *V. alginolyticus* is not recognized as the main *Vibrio* species pathogenic for humans, being considered of scarce virulence and low invasion ability, infection incidences due to these bacteria have increased and several clinical manifestations, mainly intestinal, have been reported, mainly related to the consumption of raw or undercooked seafood (Mustapha *et al.*, 2013).

All the *V. alginolyticus* strains studied presented DNAse, gelatinase and lipase activity, which has been also observed in *V. alginolyticus* strains isolated in Indonesia and from Mediterranean seawater (Molitoris *et al.,* 1985; Snoussi *et al.*, 2008a).

Our results indicate that the infection risk due to *V. alginolyticus* could be higher due the adherence capacity found as well as their hydrolytic enzymes production. Both, adherence and hydrolytic enzymes activity could contribute to their virulence. This is the reason why the routine culture for *V. alginolyticus* should be implemented in cases of diarrhea suspected to be acquired through marine food consumption. The Chilean Health System recommends

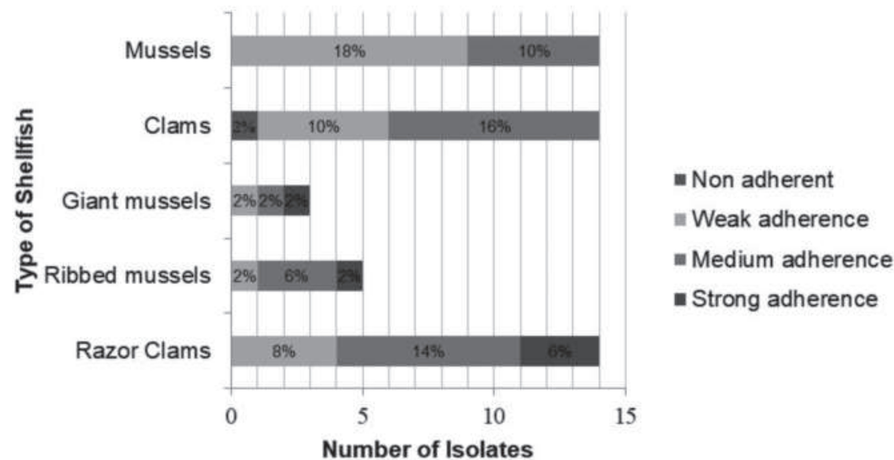

Figure 1. Number and percentage of strains according to type of adhesion expressed and type of sample origin.

epidemiological surveillance in cases of acute diarrhea incorporating to the coproculture protocol an appropriate culture media (TCBS) in 1 out of 5 cases of diarrhea in people under the age of 18, and 1 out of 10 cases in people aged 18 and above (Minsal, 2011). Despite this practice, it could be possible that *V. alginolyticus,* should be sub-diagnosed because this is the most frequently isolated *Vibrio* species in this region (southern Chile) (data not shown). This bacteriological finding proves that these bacteria isolated mainly in tropical areas, can also be isolated in colder regions (Snoussi *et al.*, 2008a).

CONCLUSIONS

In summary, our results confirm in our region the presence of *V. alginolyticus* in shellfish destined for human consumption, suggesting that their adherence capacity and enzymatic activity could contribute to the development of infection associated to seafood consumption.

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RESUMEN: *Vibrio alginolyticus* es un patógeno emergente relacionado con alimentos marinos. **Objetivo:** determinar la capacidad de adherencia y actividad enzimática de *V. alginolyticus* aislados de mariscos. **Material y método:** En este estudio se determinó la capacidad de adherencia y actividad enzimática de 50 cepas de *V. alginolyticus* aisladas de mariscos para consumo humano. **Resultado y discusión:** 98% de las cepas lograron adherirse a las células HEp-2. En este sentido, 24 (48%) y 5 (10%) de los aislados de *V. alginolyticus* se clasificaron como de capacidad de adherencia media y fuerte, respectivamente. Por otro lado, en todas las cepas se confirmó la producción de las enzimas ADNasa, lipasa y gelatinasa. **Conclusiones:** Nuestros resultados sugieren un potencial de virulencia de *V. alginolyticus* aislado de mariscos, lo que podría contribuir a la infección asociada a mariscos consumidos crudos o poco cocidos.

PALABRAS CLAVES: *Vibrio alginolyticus*, mariscos, adherencia.

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