

Lectins from *Eichornia crassipens* and *Lemna minor* may be involved in *Vibrio Cholerae* El Tor adhesion

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ABSTRACT

Vibrio cholerae (Vc) has been isolated from roots of aquatic plants during epidemic or interepidemic periods. It has been suggested that the lectins from the roots of aquatic plants play a role as reservoirs of Vc. In this paper, we evaluated the activity of lectins from *Lemna minor* and *Eichornia crassipens* plants as potential mediators of the Vc strain El Tor (Vct). We found that *Lemna minor* extract showed high specificity towards blood groups O and B. *Eichornia crassipens* extract showed high specificity towards blood group A and O. Sugar competition experiments demonstrated that *Lemna minor* extract showed a high recognition to Neu5Ac (acid N acetyl neuraminic or sialic acid) and GlcNAc (N-acetyl D glucoseamine) in group B; and GlcNAc in group O. *Eichornia*

crassipens, the recognition was that of GalNAc (N-acetyl-D-galactoseamine) and GlcNAc in group O; and Fuc (L-Fucose) and GlcNAc in group A.

Lemna minor and *Eichornia crassipens* protein extracts (p-ext) increased Vct proliferation and protected to the red cells group O against the hemolytic activity of Vct. Both p-exts did not show any statistical significance on agglutination to Vct when compared to the results from phosphate buffer. According to the results, lectins present in roots may be involved in the proliferation and survival of Vct.

KEYWORDS

Vibrio cholerae - *Lemna minor* - *Eichornia crassipens* - lectins

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INTRODUCTION

Bacteria such as *Vibrio cholerae* (Vc) are rarely found in the planktonic or free-swimming phase in aquatic environments, they are found in association with a solid surface, such as the exoskeletons of crustaceans or insects, which provide nutritional factors. Vc has been isolated from coastal and estuarine environmental samples, both as free-living and in association with phytoplankton, zooplankton, crustaceans, mollusks and algae [1-5].

Vc is capable to colonize surfaces where are present chitin, which may provide it nutrition directly, because this bacterium is able to metabolize chitin or mucin [6, 7]. Chitin, is one of the most abundant biopolymer of GlcNAc (N-acetyl D glucoseamine) in the aquatic environments, is an important substrate for Vc and has impact on its physiology, including the utilization of carbon and nitrogen source as well as an inductor of natural competence [6, 7].

The capacity of the Vc to respond to changes of salinity, temperature and the availability of nutrients, shows that this species can successfully occupy one or more ecological niches in a variety of aquatic habitats including the human intestine. Vc is the causal agent of severe secretory diarrhea disease named cholera, that means that the life cycle of clinically relevant Vc strains are in

transition between these two dissimilar habits: on other words in a natural inhabitant of the aquatic ecosystems and as a pathogen in the human gastrointestinal tract. The cholera is a reemergence diseases in several countries and in about 50 countries is epidemic [8], and it is transmitted by fecal-oral route through water. For this reason, it is necessary to understand its permanency on aquatic niches during interepidemic and the epidemic periods, because nature of cholera latency depends to keep in a viable form, but not necessarily cultivable, in ecological aquatic niches through factors that keep and protected it. A mechanism used by Vc is the formation of biofilms in natural aquatic medium, which favors its survival and transmission, also provide a stable environment and maintains diffusion vibrions large number of microorganisms [9, 10]. In most aquatic and physiologic environment, Vc forms biofilms on surfaces provided by plants, algae, zooplankton, crustaceans and insects, also several gram negative and gram positive pathogens bacteria have the ability to form matrix-enclosed surface associated communities and in particular those biofilms play an important role in the physiology, ecology and epidemiology of cholera [11, 13].

The matrix of the biofilms mainly consists in exopolysaccharides or extracellular polysaccharide (EPS), and other compounds like protein, nucleases (Dns and Xds), lipid

and extracellular DNA (eDNA). All these compounds participate in diverse processes including the development of typical biofilm architectures, nutrient acquisition, detachment, dispersion from the biofilms and the colonization fitness of biofilms after ingestion by host; in other words the biofilm enhancing colonization to new niches and facilitating transmission of the disease [11, 12]. There are strains of *Vc* characterized by being rugose, in that case, the biofilm formation is made in combination with the pili, the flagella and EPS, also are involved nucleases, lipid, proteins and eDNA [14, 15]. *Vc* produces a unique EPS, mainly of glucose, galactose, GlcNAc, mannose, xylose and deoxy D galactose, which confers resistance to chloride and promotes biofilm formation. EPS synthesis is controlled by virulence genes (VPS). It has been described two types of *Vc* strains which respond to different environmental factors: VPS dependent and VPS independent. In VPS dependents strains are necessary factors presents in freshwater; on the contrary, in the independent VPS strain those factors are in salad water [14–16]. The different factors environmental requirements for biofilm formation play different roles in these habitats, which participate in their pathogenicity, life style, survival [7, 10].

Lectins have high specificity for sugar recognition and are implicated in bacterial agglutination, adhesion, pathogenesis and in the biofilm formation by increasing their ability to persist under stress [17–19]. The bacterial fimbriae lectins facilitate cell-cell interactions and are involved in biofilm surface-adhesion. In addition, there are other matrix associated lectin-binding proteins that recognize and bind carbohydrate moieties that favored cell-matrix or cell-cell interactions within the biofilm [10].

The presence of lectins with high specificity for GlcNAc has been described in *Vc*, able to colonize chitin-containing surfaces [7]. Also it has been described a type of lectin from the serum and mucus of oysters with high specificity to agglutinate the toxigenic *Vc* [20].

Vc variant *El Tor* (*Vct*) has been isolated from the roots of aquatic macrophytes such as *Eichornia crassipens* and *Lemna minor*; this suggests that aquatic plants could be natural environmental reservoirs of the microbes through either a non-specific association or a commensal relationship [1–5]. Microbiological monitoring studies in the Actopan River in Veracruz, México demonstrated the presence of *Vibrio parahaemolyticus* and *Vibrio cholerae* in roots from *Lemna minor* and *Eichornia crassipens* plants [21]. Monitoring the same places after 2 years the results indicated that one of the isolation strains corresponded to *Vct* [22], their presence may be due to the explosive outbreaks of *Vct* responsible for the seven pandemics. Several years in the same places *Vct* is again isolated in a viable and cultivable form from *Lemna minor* and *Eichornia crassipens* roots suggesting that these aquatic plants behave as permanent reservoirs of *Vct* in freshwater [23]. In this work was assayed if the lectins from *Lemna minor* or *Eichornia crassipens* roots may be involved in maintaining the association commensal with *Vct*.

MATERIALS AND METHODS

Plants collection

Plants were collected in sterile bags from Ursulo Galvan river in Veracruz State, roots were separated and rinsed

twice with chloride solution to isolate and to identify *Vct* and also to obtain the p-ext from the roots. In addition, water sample was collected in 500 ml sterile flasks in order to detect free *Vct*.

Extraction of proteins

To 100 gr of each plant root from *Lemna minor* and *Eichornia crassipens* were added 200 ml of petroleum ether in order to remove lipids; this was incubated for 30 min at room temperature. After being delipidized, the sample was macerated in 250 ml of buffer 10 mM phosphate, pH 7 and 125 mM sodium chloride. The maceration obtained was filtered with a gauze; to the filtrate were added several drops of trichloroacetic acid (TCA) to 10% to precipitate proteins, then centrifuged for 15 min at 7000 rpm, each pellet was washed twice with water distilled to remove excess acid, soluble proteins such as albumin in water and soluble carbohydrates. Aliquots of 10 ml phosphate buffer were stored at 0 °C.

The protein concentration of the extracts (p-ext) was quantified by Lowry procedure, utilizing 100 µg/ml of bovine albumin as standard. The sugar concentration was determined by the Smith method, using 100 µg/ml of glucose as standard.

Vc detection

The roots from *Lemna minor* and *Eichornia crassipens* were rinsed and incubated directly in alkaline peptone pH 8.5 with 1% saline solution incubate at 45 °C, during 48 hrs to recuperate only *Vibrio cholerae*, *Vct* is thermoestable. The p-exts were incubated in the same way in alkaline peptone, and them sowing into thiosulphate citrate bile salts (TCBS) medium or directly the roots were collocated on the TCBS, the incubation was about 24 to 72 hrs in order to identify *Vc* colonies. The *Vibrio Cholerae sp* was donated and isolated from a patient with Cholerae of the Centro de Especialidades Médicas de Veracruz. The *Vct* was isolated from the roots of the *Lemna minor* and *Eichornia crassipens* and characterized as Inaba [23].

Erythrocytes from groups O, A and B obtaining

Erythrocytes from healthy human donors were collected in heparinized syringes and washed 3 times in PBS (phosphate buffer); finally, a suspension of 5% of red cells was prepared for hemagglutination test.

Hemagglutinating activity (HA)

HA of the extracts was evaluated by modified Coutiño procedure [18], using microplates with 50 µl of 5% red cell suspension from groups A, B and O, in 50 µl of phosphates buffer, followed were added 50 µl of p-ext using the double dilution procedure. HA titer obtained after 1 hour is expressed as the highest dilution showing detectable Hemagglutination. HA units are expressed as the minimum concentration of protein necessary to obtain HA.

Lectin hemagglutinating activity inhibition (HAI) by sugars

The procedure was similar to that for HA, except in this case 1mM of various sugars in double serial dilutions was mixed with a lectin solution at 1:8 titer and incubated for 30 min before the 5% suspension of red cells was added. Ten sugars were tested: GalNAc (N-acetyl-

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-D-galactoseamine), Gal (Galactose), Fuc (L-Fucose), GlcNAc (N-acetyl D glucoseamine), Man (Mannose), Glc (Glucose), Neu5Ac (acid n acetyl neuraminic or sialic acid), Xyl (Xylose), GalN (galactosamine) and GlcN (glucoseamine) (Sigma chemicals, St Louis, Missouri).

SDS-page

Gels at 10% were prepared with or without mercaptoethanol. 50 µg/ml of protein from extract of *Lemna minor* and *Eichornia crassipens* roots were added. To determine molecular weight, pre-stained standards (BioRad) and cross-linkage hemoglobin were used (Sigma chemicals), and after stained with coomassie. The resulting bands were analyzed with the Kodak 40 program Digital Sciences 1D.

Inhibition of Vc hemolytic activity by roots p-ext

Vc donate was previously typified [23] and then was cultivated in alkaline peptone water pH 8.5 and inoculated in a nonselective medium such as TSI. After 24 hrs at 37 °C, the bacterial suspension was centrifuged at 3000 rpm for 15 min, 50 µl of the supernatant were used as a source of hemolysin and mixed with 50 µl of 2.5% erythrocyte suspension prepared in an isotonic solution. Vc hemolysin was detect until a titer of 1 : 128. Several double dilutions from *Lemna minor* and *Eichornia crassipens* extracts were used to verify hemolytic activity inhibition from the Vc (at titer of 1 : 128), after 30 min of incubation at 37 °C.

Proteic extracts activity on Vct proliferation

SensiDiscs were made with different dilutions of p-ext from *Lemna minor* and *Eichornia crassipens*, and placed at the corners of the zig-zag proliferation pattern of Vct.

Vc agglutination by roots p-ext

The procedure was similar to that for HA, except that a 2% bacterial suspension of Vct replaced the erythrocytes and saline; polyvalent serum and phosphate buffer were used as positive and negative controls. The Vct aggregation was directly observed by microscope. Determinations also were done in microplates incubated for 30 min, stained with acridine orange and read at 560 nM, corroborated through microscopy analysis. As positive control polyvalent serum and phosphate buffer were used and isotonic solution as negative controls.

RESULTS

Plants obtaining and molecular weight characterization of p-ext

The plants *Eichornia crassipes* and *Lemna minor* were collected from Ursulo Galvan river in Veracruz State. Both plants were identified in the Institute of Ecology's herbarium as was reported [23] and in from their roots p-ext were obtained like is described in materials and methods. The amount of protein and carbohydrates in both extracts were analyzed and it was found that amount of proteins in both extracts was 177 µg/gr of root, and the carbohydrate concentration was 55 µg/gr in *Eichornia crassipens* and 44 µg/gr in *Lemna minor* (Table 1). Protein concentration in *Lemna minor* decreases with time.

To determine the protein pattern of the p-exts, SDS-page was performed. The results in the table 4 show that p-ext

from *Eichornia crassipens* remained the same three bands with and without mercaptoethanol; with molecular weight around 265 kD, 65 kD and 30 kD. Regarding *Lemna minor* some bands changed its electrophoretic mobility by using mercaptoethanol. Without mercaptoethanol only three bands 191 kD, 66 kD and 16kD were observed, with mercaptoethanol use, four bands: 186 kD, 66 kD, 56 kD and 15.9 kD were observed. The above means that the bands of 191 kD and 66 kD are made up of two polypeptides, one of them about 186 and 56 kD attached to another polypeptide that not detected in the gel probably by its low molecular weight.

Isolation and persistence of Vc

For the Vc isolation, the roots and the p-ext were sowing on TCBS. After two days, yellow and green colonies very homogeneous were observed also few big yellow brownish colonies were observed too. For the identification of strains of Vc, the size and coloration of colony were analyzed [21], based on this classification three strains were detected: *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio alginolyticus* both the roots and the p-ext (Table 2). In the water sample after centrifuge at 7,000 rpm in the pellet we didn't found any colonies, these results corroborate that *Vibrio cholerae* rarely found in the planktonic or free-swimming phase at least in fresh water and suggest that those plants are permanent reservoirs perhaps they lives in a commensal o symbiotic association [5, 24]. After keep at 4 °C for five years, the p-ext and roots from

Table 1. Characteristics of the p-exts extraction from roots of *Lemna minor* and *Eichornia crassipens*.

	<i>Eichornia crassipens</i>	<i>Lemna minor</i>
Weight of the sample (gr)	190	140
10% TCA volume (ml) to precipitate	23	160
Proteins (µg/gr of roots)	176.8	176.8
Carbohydrates (µg/gr of roots)	55	44
Proportion Carbohydrate/protein	0.311	0.25

Lemna minor and *Eichornia crassipens*, only *Vibrio* colonies were detected in *Eichornia crassipens* in both conditions, corroborating their permanency under stress conditions (see Table 2). It must mention that the roots from *Lemna minor* become dust and in *Eichornia crassipens* the roots were preserved intact.

It will be mention that when we added antibody poly-O and Inaba to roots to corroborate previous results [23], and incubation for about 5 days *Lemna minor*, unexpectedly the roots disappears, but before it forms a mucin, probably with the stress of the antibody *Vibrio* activated a protease activity such as mucinase.

Eichornia crassipens and *Lemna minor* show different activity of lectins

Lectin activity was determined by the HA test in A, B and O groups. *Eichornia crassipens* p-ext showed higher

Table 2. Morphology and abundance of Vibrions colonies in TCBS from *Eichornia crassipens* and *Lemna minor* p-ext

	<i>L. minor</i> roots p-exts	<i>E. crassipens</i> roots p-exts
Sizes	2-6 mm	2-6 mm 6-10mm
Form	Round point	Round point and some amoeboid
Edges	Entire margen	Entire margen and lobulated
Color	Flourescent yellow and green	Flourescent yellow and green some opaque yellow brownish
Abundance	<i>V cholerae</i> ++++ <i>V parahaemolyticus</i> +++	<i>V cholerae</i> +++ <i>V parahaemolyticus</i> +++ <i>V alginolyticus</i> ++
Abundance after keep 6 years	No colony grew and the root disappear	<i>V cholerae</i> ++ <i>V parahaemolyticus</i> ++ <i>V alginolyticus</i> +++

Presence of colonies ++++ very abundance, +++ abundance, ++ few abundance

Table 3. Lectin activity by the specificity of p-ext from *Lemna minor* and *Eichornia crassipens* in their HA activity on the healthy male blood groups (A, B and O)

Male blood group	<i>Eichornia crassipens</i>			<i>Lemna minor</i>		
	A	B	O	A	B	O
Specificity of Hemagglutination (HA) activity	52,717 (1:2048)	0	101 (1:4)	0	18,823 (1:32)	75,294 (1:128)

activity in group A, with 52,717 units HA/mgr of protein compared to 101 units HA/mgr protein in group O. *Lemna minor* shows more activity in group O, with 75,294 units HA/mgr and 18,823 units HA/mgr in group B. The results suggest that the p-ext have different lectin activity; and that *Eichornia crassipens* lectins detected a more specifically a sugar residue in red cells in group A, and *Lemna minor* detected a different sugar residue in red cells from group O (Table 3).

Sugar specificity of the lectin activity in proteic extracts from plant roots

To know which sugar could inhibit the hemmagglutination between the p-ext and the red cells, ten different sugars were tested: GalNAc, Gal, Fuc, GlcNAc, Man, Glc, Neu5Ac, Xyl, GalN and GlcN. The results from the HAI show that the *Eichornia crassipens* p-ext, in group A, was inhibited by Fuc and GlcNAc at 1 mM; and in group O, by GlcNAc, GalNAc at 500 µM; and Fuc, Neu5Ac, GlucN,

GalN and Gal at 1 mM. Regarding the analysis of HAI by *Lemna minor*, inhibitors were in group O all sugars tested at 1 mM except GalNAc 125 mM; and in group B, the inhibitors were GlcNAc at 125 µM, Neu5Ac at 25 µM, Fuc, GalN and Glc at 1 mM (Table 4).

Results shows lectin activity with recognition in O group with high specificity to GlcNAc, GalNAc in both *Eichornia crassipens* and *Lemna minor* p-ext and also highly specificity to Neu5Ac recognition in *Lemna minor* p-ext. The results indicate that both p-ext displayed a common GlcNAc/GalNAc and also a Neu5Ac/GalNAc lectin activity similar as other type lectins reported [19].

Effect of the p-exts on the hemolytic activity of Vc

Lemna minor and *Eichornia crassipens* p-ext protect the group O of the hemolytic activity of *Vibrio cholerae* at titer of 1:4 and 1 : 64, respectively, using the hemolysin of *Vibrio cholerae* at titer of 1 : 128 as the materials and methods (Table 5).

Table 4. Activity of p-ext from *Lemna minor* and *Eichornia crassipens* in HA inhibition by sugar titer.

Male blood group	<i>Eichornia crassipens</i>			<i>Lemna minor</i>		
	A	B	O	A	B	O
Sugar concentration used to inhibit the HA at the titer 1:4 and 1: 8	1mM GlcNAc Fuc	-	1mM Neu5Ac GalN GlcN Gal Fuc 500µM GlcNAc GalNAc	-	1mM GalN Glc 125µM GlcNAc 25µM Neu5Ac Fuc	1mM Fuc GlcNAc Neu5Ac Xyl Man GalN GlcN Gal Glc 125µM GalNAc

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Table 5. Effects of the p-ext from *Lemna minor* and *Eichornia crassipens* on some *Vct* activities such as agglutination, proliferation and hemolytic activity

	<i>Eichornia crassipens</i>	<i>Lemna minor</i>
Vc agglutination p-exts serial dilutions	Negative	Negative
Vc increased 2 cm around disc	Positive at titer 1:8	Positive at titer 1:4
Inhibition of the Vc hemolytic activity in group O at titer 1:128	100% at titer 1:64	100% at titer 1:4

Both p-exts contain lectins capable to recognize Fuc. Due to Fuc is involved in the hemolytic activity *Vct*, we consider that the p-exts may have lectins which recognized either Fuc, Glc or GlcNAc in glycosidic chains present in the hemolysin or its receptor in group O, and they are different of the domain of the antigenic O group since the HA activity in that group was 1 : 128 for *Lemna minor* and 1 : 4 for *Eichornia crassipens*. Whereas the protective effect against hemolytic activity was 1 : 4 and 1 : 64, respectively. This means that *Eichornia crassipens* p-ext has a protein with more specific anti-hemolytic activity and *Lemna minor* p-ext has more recognition to group O.

Effects of root p-ext on *Vc* proliferation y conservation

Lemna minor and *Eichornia crassipens* p-ext produced an effect on *Vct* density at 1 : 4 and 1 : 8 titer around the disk (see Table 5), both has the same effect on increase proliferation. The other tested dilutions seem to have a bacteriostatic effect. These results are different on the environmental habitats of *Vc* because the reservoir capacity for *Vc* and *parahaemolyticus* is higher in *Lemna minor* than in *Eichornia crassipens* root [21], however the results in Table 2 shows that *Vct* has similar growth in both roots but has a longer periods of survival in *Eichornia crassipens* than in *Lemna minor*, where the roots disappear.

Effect of root p-ext on agglutination of *Vct*

Graph 1 shows that when was using 214 µgr/ml to *Eichornia crassipens* and 175 µgr/ml to *Lemna minor* in double dilutions serials added to 2% of *Vc* suspension, in both p-ext was a significant reduction of the OD at 214 µgr/mL $p \leq 0.01$ and 10 µgr/mL $p \leq 0.05$ with the *Eichornia crassipens* and *Lemna minor* p-exts respectivaly. The Graph only shows *Eichornia crassipens* results, *Lemna minor* has similar results. OD reduction is related to agglutination, likewise to polyvalent serum compared to saline alone. However, we discovered that phosphate also reduced OD likewise to polyvalent serum, the OD significant reduction, probably belongs from phosphate agglutination or salt precipitation because at that concentration neither of both p-exts caused *Vc* agglutination (Figure 1).

DISCUSSION

The world has gone through several cholera pandemics. The cholera disease depends of behaves of the toxigenic vibriions, which can abruptly appear and disappear and letting the question behind: what happens to the *Vc* during those intervals? Most researches believed that *Vc* remains in interepidemics reservoirs.

The interepidemic reservoirs should be sites of survival and multiplication of pathogenic vibriions between epidemics that are not well known. During epidemics, toxigenic *Vc* can be isolated from local fresh water [25], but it disappears after the epidemic diminishes. Since vibriions normally inhabit oceans and estuaries during most of the year, there are no explanations of how the seasonality and endemicity of cholera are maintained, and how it can live in freshwater. Researchers have shown the association between vibriions and marine plants and plankton, and also are associated with freshwater plants cohabiting in the epidemic season [2, 5, 26, 27], In-vitro conditions *Vc* survive longer in association with *Lemna minor* that in freshwater.

Our results have demonstrated and corroborated the stability and permanency of *Vct* on roots of *Lemna minor*

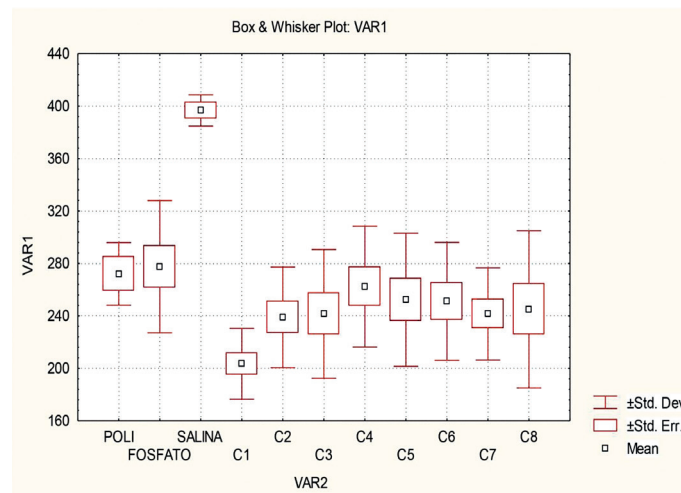


Figure 1. Effect of p-ext on agglutination of *Vct*

The agglutination of *Vct* was performed following the instructions for Sanofi polyvalent serum to *Vc*, the determinations were carried out in microplates, stained with acridine orange and read at DO 560nM. The agglutination was corroborated by microscopy analysis, as positive control Polyvalent serum and 10mM phosphate 150mM NaCl pH 7.4 solution were used, and isotonic solution as negative controls; the p-ext from *Eichornia crassipens* and *Lemna minor* were proved at initial concentration C-1 214 µgr/mL to *Eichornia crassipens* and 175 µgr/mL to *Lemna minor* double dilutions serials were done. Both initial concentrations affected the agglutination with significance statistical $p < 0.05$ but the graphic only shows the results from Ec; Lm has similar behave as Ec.

and *Eichornia crassipens* after almost ten years following the last outbreaks and from first isolation [23]. In those isolation was showed the presence of gram negative bacteria attached to a thin mucin layer on *Lemna minor* roots [21, 22]; this layer may contain lectins, where the lectins probably serve as an adhesive factor as well as nutriment, and may be Chi or Rip type; by its high recognition to GlcNAc, Neu5Ac and GalNAc, probably the lectins from plants recognized those sugars on the Vc membrane composition or in the EPS. Furthermore, Vc also may recognize a glycoprotein or GlcNAc derivatives on the roots plants which contained hemicelluloses and pectin polysaccharides [24, 25]. The specificity of Vc to GlcNAc derivatives used as source of energy is well known [6, 7], also the adhesion, survival, and proliferation of vibrios on the surface of small crustaceans and on aquatic plants. The vibrios species produce chitinase or mucinase responsible for the degradation of chitin or mucin into soluble oligosaccharides or monosaccharides, which become sources of carbon and nitrogen in this way the vibrios return to the ecosystem biologically useful forms from insoluble polysaccharides to soluble sugars this could be the reason to keep in commensal or symbiotic association with those plants.

Therefore, the study of interactions between vibrios and surfaces containing GlcNAc or mucin is important for their impact on human health and their ecological significance. The way how the same bacterial surface binding to compound (GlcNAc) may have a dual function in the *Vibrio cholerae* life cycle, mediating the attachment to inanimate or animate substrates in the environment and to cell membranes in the intestinal tract and after discovery the biofilm formation this association is more clear. During these interactions, changes from hydrophilic to hydrophobic conditions may occur, which it could be associated with their lectin activity also with an induction of some catalytic function involved in the association, invasion and virulence. It is reported that in a biofilm the development of gradients can create anoxic and acidic zones in the interior of biofilm clusters.

At these proteins concentration, the lectins from plants did not agglutinate Vc despite that this bacterium may have GlcNAc in its membrane as part of the antigen O1 in the LPS, similar to the O1 antigen from *Salmonella typhimurium* (galactose-glucose - Rhamnose-Mannose - abequose-OAc-GlcNAc), which shares with antigenic groups of Vc because cross immunity with the typhoid has been reported [28]. It is possible that those lectins may recognizing GlcNAc in EPS composition in order to attachment in them and help to the development the biofilm, or also probably, those lectins recognize GlcNAc in membrane *Vibrio cholerae* and allowed the adhesion. It is probably that after the association through either mechanism, 2 events may occur: one stimulates the mucinase, chitinase or perhaps pectinase *Vibrio cholerae* activity in order to obtain nutritional factors; and the second, the promotion of biofilm development to survival in extreme conditions, such as protection from oxidative stress and an acquired resistance to osmosis, including changes in pH, salinity, and oxidative environments and provides adaptive advantages for aquatic organisms.

The results of this work suggest that, perhaps the lectins present in roots of plants, could play an important role

in the balance of Vc in them by controlling the proliferation or death in order to obtain the extra-DNA (eDNA) required to maintain the biofilm matrix [11, 12] and thus keep the Vc viable and cultivable form such as in *Eichornia crassipens*. On the contrarily with the roots of *Lemna minor*, these results may be due to the loss of nutrient, protease activity or a toxic lectin inhibited its growth and in the case of *Eichornia crassipens* roots may favored the biofilm formation and keep in a viable and cultivable form the Vc. But it is also possible that Vc form part of the intracellular biota of the macrophytes to maintain the sources of carbon and nitrogen.

These events may be involved in the ability to act as permanent reservoirs and may also have a role in transforming toxigenic Vct, mainly because the filamentous phage that carrying genes toxigenic, is coexisting in those environments, and finally climatological factors such as high temperatures followed by rains trigger the destruction of the biofilm and the dissemination of Vc, the biofilms are involved in nutrition acquisition, detachment and dissemination of Vc, important factors in epidemiology of the cholera.

The plant-vibrio association may be involved in the Vc survival, pathogenesis, life cycle and the epidemiological periodicity in places where this is endemic mainly in about 50 development countries, because those plants are fecal contamination indicator into water. However the commensal association between those plants with Vc should be considered and studied extensively in ecological terms and not only from the perspective of health.

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Conflict of interest

The authors declare no conflict of interest.

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