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Phytoplankton-linked viable non-culturable *Vibrio cholerae* O1 (VNC) from rivers in Tucumán, Argentina

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Plankton-linked Vibrio cholerae has been detected in aquatic environments since the first decades of the 20th century. Several mechanisms have been proposed to explain the survival of V. cholerae during inter-epidemic periods, one of which is through existence in its viable non-culturable (VNC) form in biological reservoirs. This latent form of V. cholerae (VNC) would explain the seasonal outbreaks and the way the pathogen survives in the environment. The objectives of this study were to assay V. cholerae O1 in its viable non-culturable form in two rivers in Tucumán, Argentina, as well as its possible association to phytoplankton and its relation to four environmental variables (pH, temperature, conductivity and dissolved oxygen), using direct immunofluorescence. Analysis of phytoplankton revealed that diatoms were predominant with percentages between 85 and 100%. Nitzschia palea was the only species found at all three sample sites with percentages between 0 and 38%. Of the 54 samples obtained during the 18 sampling periods, V. cholerae VNC was detected through direct immunofluorescence in 39% of the cases and at all three sampling sites. Positive samples were analysed for association of VNC with phytoplankton and between 1 and 10 bacteria were found adhered to a single algal cell. This confirms for the first time in northwestern Argentina adherence of this microorganism to the genera Stigeoclonium and Nitzschia as environmental reservoirs. No correlation could be found between the latent form of Vibrio and the environmental variables assayed.

INTRODUCTION

Vibrio is a normal inhabitant of the aquatic environment (Sakazaki, 1992). *Vibrio cholerae* O1, the etiological cholera agent, is responsible for the morbidity and mortality in numerous areas in Asia, Africa and Latin America (Swerdlow *et al.*, 1992; Siddique *et al.*, 1992; Weil and Merche, 1992; Lipp *et al.*, 2003; Sack *et al.*, 2003). The endemic and seasonal character of cholera depends on the survival and the viable, but not necessarily culturable, state of the bacterium in ecological niches in aquatic environments. Singleton *et al.* (Singleton *et al.*, 1982) and Miller *et al.* (Miller *et al.*, 1984) support the hypothesis that this microorganism is a native of the microbial community of brackish aquatic environments.

Studies carried out in several countries have demonstrated adherence of *V. cholerae* to plankton in aquatic environments. Environmental factors such pH, temperature, salinity and nutrient concentration are important

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for *Vibrio* survival. The microorganism is more active when the conditions resemble those in natural environments (Borroto, 1997; Pascual *et al.*, 2000; Gonçalves *et al.*, 2004). However, it has been observed that *Vibrio* sometimes requires NaCl and even grows in high saline aquatic environments. An adequate concentration of nutrients in fresh water may meet its salinity requirements. Furthermore, it is facultatively anaerobic, highly sensitive to acidity and has little resistance to solar radiation (Borroto, 1997, 1998).

Several mechanisms have been proposed to explain survival of the bacillus during inter-epidemic periods. Existence of viable non-culturable (VNC) forms of *V. cholerae* in environmental reservoirs is the most consistent one. In response to environmental stress situations in aquatic environments such as low nutrient availability and/or low temperatures *V. cholerae* O1 converts to a latent state which allows it to maintain metabolic functions but it cannot be cultured *in vitro*. If conditions become favourable again it can revert to the culturable state (Xu *et al.*, 1982; Rollins and Colwell, 1986; Roszak and Colwell, 1987; Nilsson *et al.*, 1991; Borroto, 1997; Louis *et al.*, 2003). It is assumed that these states would explain the seasonal outbreaks of cholera and that *V. cholerae* persists in the environment during these periods while adhered to plankton (Colwell, 1996). Islam *et al.* (Islam *et al.*, 1989) observed that *V. cholerae* O1 tox^+, biotype El Tor, showed a higher tendency to adhere to *Rhizoclonium fontanum* Kützing Cholophyta than to other fresh water species and proposed that prolonged survival of *V. cholerae* O1 is due to its capacity to utilize extracellular products released by the algal species. Similarly, using fluorescent monoclonal antibodies, *V. cholerae* O1 was found in Bangladesh in water reservoirs adhered to *Volvox* species thus indicating that in this way *Vibrio* endured environmental changes (Huq *et al.*, 1990).

Numerous studies indicate that although *V. cholerae* is part of the normal aquatic flora and is generally isolated from environmental reservoirs, its presence does not only depend on human faecal contamination (Hood and Ness, 1982).

Probably, the resurgence of the disease in Latin America has been associated with the existence of VNC forms of *V. cholerae* O1 in the Pacific Ocean, which reverted to viable culturable (VC), pathogenic and transmissible forms due to climatic changes owing to the El Niño current (Colwell, 1996). The threat exists that cholera may become endemic, because in Ecuador, Peru and some Central-American countries the disease has become seasonal (OPS, 1994). In Argentina, the first cholera case was detected in 1991 and until 1998 there were seven epidemic outbreaks caused by *V. cholerae* O1 with 4833 cases and 72 deaths (Weil and Merche, 1992; OPS, 2003).

In Tucumán, a province in the Northwest of Argentina, 40 cholera cases were caused by *V. cholerae* O1 between 1993 and 1999: 25 affected inhabitants of Banda de Río Salí, 7 of the Lules region, 4 of the Capital of Tucumán and 4 of La Cocha in the South. Most of the cases were detected between November and January. These results were atypical compared to the rest of Argentina, because most of the cases affected children under 5 years of age (Trejo, personal communication). Furthermore, the cholera-toxin negative *V. cholerae* O1 strains detected in Tucumán were carriers of possible new virulence factors (Binsztein *et al.*, 2004).

Ninety percent of the cholera cases in Tucumán occurred in areas of the Lules River, Banda de Río Salí and Tucumán city. Therefore, studies of the reservoirs in the province were thought to be necessary, especially in those areas with the highest number of cases. So far there has been no proof in Tucumán that shows a possible association between *V. cholerae* O1 and fresh water phytoplankton species.

Due to the fact that during previous studies (Mirande *et al.*, 2007) isolation of *V. cholerae* O1 was impossible with conventional bacteriological techniques, the objectives of the current study were to assay viable non-culturable *V. cholerae* O1 in two rivers in Tucumán applying direct immunofluorescence as well as investigating the possible association of the pathogen to phytoplankton and its relation with four environmental variables.

METHODS

Three sampling sites were selected in Tucumán province based on the explanation given above. Two in the Salí River [Canal Norte (CN) (26°47′,26″S, 65°09′,40″W) and Banda (B) (26°50′,39″S, 65°10′,73″W)] and one in the Lules River (LR) (26°55′,15″S, 65°19′,46″W) (Fig. 1). Eighteen sampling periods, six each year, were carried out between 2003 and 2005.

Phytoplankton samples were obtained using a net with a 20 μm mesh and samples were divided for analysis of presence of *V. cholerae* O1 (VNC) and phytoplankton.

Physicochemical variables

The physicochemical variables pH, water temperature and conductivity were determined *in situ*, whereas dissolved oxygen (DO) was measured in the laboratory according to recommendations of APHA (APHA, 1992).

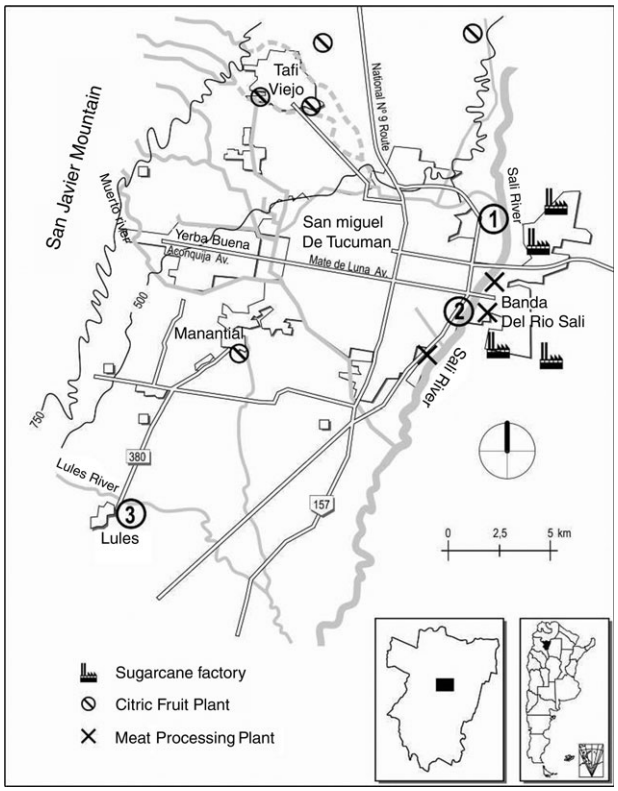

Fig. 1. Sampling sites: 1: Salí River (CN, Canal Norte), 2: Salí River (B, Banda), 3: Lules River (LR).

Detection of *V. cholerae* O1 (VNC) using immunofluorescence

One hundred microlitre of YNA (0.025% yeast extract and 0.002% nalidixic acid) was added to 1 mL of phytoplankton sample. The mixture was incubated at 37°C for 6–8 h and then fixed with 4% formol. Detection of *V. cholerae* O1 (VNC) was carried out through direct immunofluorescence (DFA-DVC). The sample was homogenized and 5 μL were placed on a slide, air-dried and fixed with 5 μL of 98% ethanol. Eight microlitre of monoclonal anti-O1 antibodies conjugated to fluorescein isothiocyanate were added to the dried droplet and the slide was incubated in a humid chamber for 30 min at 37°C. Afterwards, the slide was rinsed with phosphate buffer (PBS, pH 7.2), dried with tissue paper and mounting medium was added to the slant. One hundred fields were observed under a fluorescence trinocular microscope (1000×) (Axiostar plus, Zeiss, with a photo camera) at 490 (maximum excitation) and 520 nm (maximum emission) with a blue filter and the number of fluorescent bacilli was counted. All procedures were carried out in the dark with positive and negative controls in each case. Readings were carried out within 24 h after preparation of the samples.

Phytoplankton analysis

Samples for analysis of phytoplankton were fixed with 4% formaldehyde in the laboratory. Qualitative analysis was carried out using a binocular microscope (Leitz SM Lux, with a drawing device). Taxonomic determinations were made according to Bourrelly (Bourrelly, 1972, 1985), Komárek and Anagnostidis (Komárek and Anagnostidis, 1999, 2005), Komárek and Fott (Komárek and Fott, 1983), Krammer and Lange-Bertalot (Krammer and Lange-Bertalot, 1986, 1988, 1991, 2004), Krieger (Krieger, 1937) and Uherkovich (Uherkovich, 1966), among others. The relative composition of the phytoplankton was determined with optical microscopy (400×), counting 20 randomly selected fields from 5 preparations.

Detection of *V. cholerae* O1 (VNC) associated with phytoplankton

In order to determine association of *V. cholerae* O1 (VNC) with phytoplankton, aliquots of positive DFA-DVC samples were washed with sterile distilled water to eliminate organic and inorganic residual material until a pool of approximately 5 algal species

was obtained. These pools were then studied with direct immunofluorescence (IF).

Analysis of the correlation between *V. cholerae* O1 (VNC), phytoplankton species and physicochemical variables

Pearson correlation analysis ($P < 0.05 = *$ and $< 0.01 = **$) was carried out with the statistical SPSS (version 10) programme for Windows in order to examine the relation between *V. cholerae* O1 (VNC), the four physicochemical variables and phytoplankton (relative frequency).

RESULTS

Physicochemical variables

Water temperature fluctuated between 8 and 26°C, DO between 0 and 9 mg L^{-1}, conductivity between 339 and 2060 μS cm^{-1} and pH between 4.6 and 9. Anoxic periods were only observed in the Salí River and this river also presented highest conductivity values (Table I).

DFA-DVC

Vibrio cholerae O1 VNC was detected in 21 of the 54 samples (39%) obtained during the 18 sampling periods. It was found at all three sampling sites in January 2004 and January and February 2005, whereas the microorganism was not found in November and December 2003 and March, September and November 2004 in either river. Table II shows the presence of *V. cholerae* O1 VNC in phytoplankton samples for each of the sites examined using DFA-DVC.

VNC was detected in water with pH > 6, except for September 2003, when pH was 4.72. Regarding dissolved oxygen (DO) and conductivity *V. cholerae* O1 VNC was found both during anoxic periods and periods with DO between 2.8 and 9 mg L^{-1} and a conductivity between 850 and 2060 μS cm^{-1} (Fig. 2).

Phytoplankton analysis

The total number of phytoplankton species found at B, CN and LR was 87, 67 and 72, respectively. Diatoms were predominant and abundant and the relative frequency at each sample site was between 85 and 100%. In the Salí River 33 to 39 Bacillariophyceae species were identified, whereas the number in the Lules River was 49. *Aulacoseira granulata* (Ehrenberg) Simonsen, *Cyclotella meneghiniana* Kützing, *Cymbella affinis* Kützing, *C. amphicephala* Nägeli, *Cocconeis* sp., *Diatoma vulgare* Bory, *Gomphonema parvulum* (Kützing) Kützing, *Melosira varians* Agardh, *Navicula* sp. 2, *Nitzschia palea* (Kützing) W. Smith, *N.* sp. 1 and *N.* sp. 2, *Pleurosira laevis* (Ehrenberg) Compère, *Rhoicosphenia abbreviata* (Agardh) Lange-Bertalot, *Surirella ovalis* Brébisson, *Ulnaria ulna* (Kützing) Compère, *Lyngbya* sp., *Closterium lanceolatum*

Table I: Physicochemical variables [Temp: temperature (°C), DO: dissolved oxygen (mg L^{-1}), Cond: conductivity (μS cm^{-1}) and pH] for each sample period at the following sampling sites: Salí River (CN), Salí River (B) and Lules River (LR)

	Salí River (CN)				Salí River (B)				Lules River (LR)			
Samples	Temp.	pH	DO	Cond.	Temp.	pH	DO	Cond.	Temp.	pH	DO	Cond.
Jul-03	19.2	7.13	0	890	25	6.93	0	1374	16.6	7.86	9	612
Aug-03	14.6	5.14	0	870	22	6.66	0	1312	10.3	8.04	9	622
Sep-03	11.5	4.72	4.9	902	20	6.65	0	1329	8	8.38	9	650
Oct-03	20.5	8.12	9	559	21	7.7	2.35	1215	25	8.68	9	619
Nov-03	24	8.34	9	924	23	8	7.5	1124	25	7.7	7.3	649
Dec-03	26	8.48	9	938	25	8.14	9	1093	25	8.46	8.7	658
Jan-04	23	7.68	5.7	872	25	7.5	4.1	1504	26	8.5	8.8	460
Mar-04	25	7.78	4.3	858	26	7.6	6.4	1430	26	8.44	8.9	426
May-04	15	7.44	1.7	736	17	7.8	8.4	1000	12	8.1	9	400
Jul-04	19	5.6	0.48	1135	20	6.93	0	1410	14.2	8.16	9	574
Sep-04	15	4.6	4.9	1080	26	4.7	0	1280	11.5	8.38	9	630
Nov-04	19	4.52	7.8	778	19	4.47	7.2	1040	20	7.7	9	516
Jan-05	25	8.2	9	850	22	8	8.6	1153	26	8.4	8.4	741
Feb-05	25	8.01	3.7	1013	23	7.93	6	1318	22	9.06	8.1	403
Mar-05	22	7.62	3.3	1162	25	7.7	4.5	2060	21.5	8.32	8.7	349
Apr-05	20.5	7.04	0	1311	21.5	7.69	0.4	1634	19.5	8.19	9	339
May-05	14	7.78	0.7	988	15	8.08	4.2	1318	14.5	8.7	8	441
Jun-05	16	6.59	2.8	1059	16	7.9	0	1364	14	6.59	9	549

Table II: Results of direct immunofluorescence (DFA-DVC) for Vibrio cholerae O1 VNC in 54 phytoplankton samples for each sampling period in the Salí River (CN and B) and the Lules River (LR)

	Salí River [CN]	Salí River [B]	Lules River [LR]
2003			
July	+	−	−
August	−	+	−
September	+	−	−
October	−	−	+
November	−	−	−
December	−	−	−
2004			
January	+	+	+
March	−	−	−
May	−	+	+
July	−	+	−
September	−	−	−
November	−	−	−
2005			
January	+	+	+
February	+	+	+
March	−	+	−
April	−	−	+
May	−	+	−
June	+	−	+
Percentages of positive VNC samples using DFA-DVC	33.3% ($n = 6$)	44% ($n = 8$)	38% ($n = 7$)

Kützing and *Stigeoclonium* sp. were found at highest relative frequency in the positive DFA-DVC samples. Furthermore, it was found that *Nitzschia palea* was the only species present at all sites with highest percentage (38%) at CN (Salí River) (Table III).

Detection of the association between *Vibrio cholerae* O1 (VNC) and phytoplankton

Analysis of each pool of washed algae after DFA-DVC showed the presence of *V. cholerae* O1 (VNC) adhered to *Stigeoclonium* sp. and *Nitzschia palea*, with 1 to 10 bacteria per algal cell (Figs 3 and 4).

Pearson correlation

Pearson statistical analysis did not reveal any correlation between immunofluorescence results and environmental variables. However, during the months with elevated temperature (January to March), *V. cholerae* O1 VNC was detected at all three sample sites. The phytoplankton species *Stigeoclonium* sp. (0.54**), *Phormidium breve* (Kützing ex Gomont) Anagnostidis & Komárek (0.52**) and *Closterium lanceolatum* Kützing (0.34*) showed positive correlation with VNC.

DISCUSSION

Vibrio cholerae is an aquatic microorganism, and several reports have shown that there are environmental reservoirs of this pathogen in different geographical areas of the world. In Argentina, as in the rest of Latin America, cholera has occurred since 1991 in an epidemic pattern. It has been suggested that *V. cholerae* O1 remains in the environment during inter-epidemic periods in a viable but non-culturable state. Prior to this study, many cruises had been conducted in the Río de la Plata and on the continental shelf of Argentina in a search for culturable forms of *V. cholerae* in which traditional culture methods were used, and only non-O1 strains were recovered (Binsztein *et al.*, 2004). These authors observed for the first time *V. cholerae* O1 VNC in estuarine and marine waters in the Río de la Plata estuary and the continental shelf of Argentina in the southwest of the Atlantic Ocean. This is the first study in the northwest of Argentina that proves that *V. cholerae* O1 VNC is present in freshwater environments. Therefore, the present contribution is the first report on the existence of an environmental reservoir of this pathogen in this geographical area.

The presence of *Vibrio* in its VNC form is subject to a variety of abiotic factors (e.g. low pH) that would lead to a seasonal relationship between VNC and environmental variables (Borroto, 1997; Pascual *et al.*, 2000; Gonçalves *et al.*, 2004). Our results did not reveal such a relationship, because even under adverse conditions the VNC form could be observed all year round. In previous studies, our research group examined the presence of *V. cholerae* both in its viable and viable non-culturable form in water samples using conventional culture methods and DFA-DVC. No relationship between occurrence and physicochemical variables could be found in the rivers studied (Aulet *et al.*, 2007). Islam *et al.* (Islam *et al.*, 1989), Roszak *et al.* (Roszak *et al.* 1984), Roszak and Colwell (Roszak and Colwell, 1987) and Xu *et al.* (Xu *et al.*, 1982) maintain that the presence of *V. cholerae* O1 is not just limited to saline environments (estuaries), and that the bacterium can also be found in fresh water where it is associated with flora and fauna, which act as a reservoir for the microorganism. Under environmental stress that accompanies decreases in nutrient availability and salinity, toxigenic *V. cholerae* 01 assumes a viable state that allows it to metabolize and to form colonies without being culturable. In this viable but non-culturable state, concentrations of the microorganism adhere to the surface of various species of aquatic macrophytes, phytoplankton and zooplankton. Through such ecological associations, *V. cholerae* can survive between epidemic periods without sacrificing its

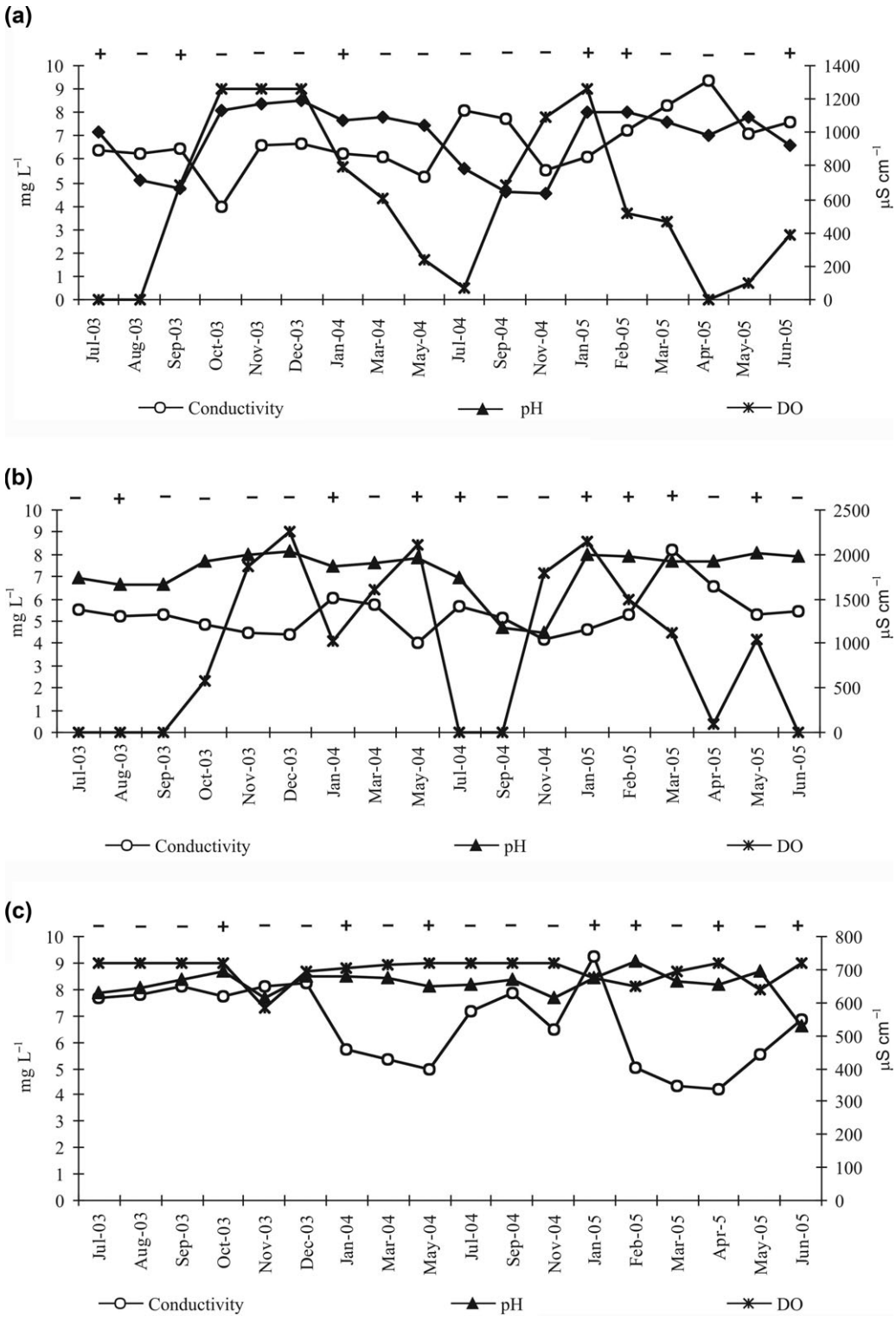

Fig. 2. Detection of *V. cholerae* O1 VNC in relation to the following abiotic factors: pH, conductivity and dissolved oxygen (DO). (**a**) Sali River (CN), (**b**) Sali River (B), (**c**) Lules River (LR), "+": positive DFA-DVC, "−": negative DFA-DVC.

Table III: Relative frequencies of species selected in positive immunofluorescence (IF) samples from the Salí River (CN and B) and Lules River (LR) from July 2003 (07/2003) to July 2005 (07/2005)

	Salí River (CN)						
Species	08/2003	01/2004	07/2004	01/2005	02/2005	03/2005	05/2005
Leptolyngbya sp.	2	0	0	1	0	1	38
Aulacoseira granulata	44	3	47	35	1	1	1
Cyclotella meneghiniana	0	14	2	1	2	0	1
Cymbella amphicephala	2	14	3	1	8	10	11
Nitzschia palea	11	7	9	10	16	17	11
Pleurosira laevis	2	16	2	12	6	21	3
Surirella ovalis	2	8	0	1	13	1	2
Ulnaria ulna	8	2	5	11	6	7	5
	Salí River (B)						
	07/2003	09/2003	01/2004	01/2005	02/2005	07/2005	
Aulacoseira granulata	6	7	4	23	0	0	
Cymbella affinis	0	68	00	0	0	0	
Cymbella amphicephala	1	4	1	3	22	8	
Gomphonema parvulum	1	2	15	19	2	0	
Nitzschia palea	38	0	22	30	24	38	
Ulnaria ulna	40	7	4	4	0	8	
Closterium lanceolatum	1	0	5	0	11	0	
Stigeoclonium sp.	0	0	17	4	2	0	
	Lules River (LR)						
	10/2003	01/2004	05/2004	01/2005	02/2005	03/2005	05/2005
Lyngbya sp.	0	0	0	0	62	7	0
Cocconeis sp.	0	0	0	11	0	7	0
Cymbella affinis	16	1	0	0	0	0	3
Diatoma vulgare	1	1	20	9	3	2	55
Gomphonema parvulum	15	3	0	6	3	3	0
Melosita varians	0	2	33	0	0	0	1
Navicula sp. 2	0	17	4	1	4	0	3
Nitzschia palea	3	1	26	4	6	1	1
Nitzschia sp 1	8	38	0	1	0	0	0
Nitzschia sp. 2	7	2	0	1	0	0	0
Nitzschia sp. 3	0	0	0	0	0	0	15
Pleurosira laevis	0	0	0	19	5	14	0
Rhoicosphenia abbreviata	13	0	0	2	0	0	0
Ulnaria ulna	6	16	4	21	5	42	3

toxigenicity (Islam *et al.*, 1989), even in freshwater ecosystems. This phenomenon contributes to the endemic nature of cholera.

Pollution of the rivers in Tucumán is a serious problem and the concurrence of industrial activities such as sugarcane and paper processing and the dry winter season aggravate these effects (González and Domínguez, 1994; Seeligmann, 1999; Mirande *et al.*, 2000; Mirande and Tracanna, 2003, 2004; among others). Of the rivers studied, the Salí River is the most affected one (Seeligmann, 1999), especially between June and October, which is mainly due to anthropogenic activities that generate suitable conditions for the microorganism to persist in its viable non-culturable form. However, the fact that *V. cholerae* O1 was detected all year round with DFA-DVC would indicate the importance of the association between *Vibrio* and phytoplankton. The influence of other factors such as the start of the rainy season (November–December) and summer rainfall should also be taken into account. The first rains could wash away and reduce possible *V. cholerae* reservoirs, whereas the second factor could cause, among other issues, new cholera outbreaks at different sites of the area studied, as can be inferred from the results obtained. However, the study on the interaction of *V. cholerae* and phytoplankton would present a series of difficulties taking into account the multiplicity and complexity of factors that can be involved. Global climatic changes that affect plankton and macrophyte growth or modifications that have an influence on the presence of organic and inorganic matter like the amount of nutrients, light intensity, ultraviolet radiation, temperature and water currents could alter the growth of the microorganism.

Association between *V. cholerae* and plankton has been observed in different countries such as Brazil (Gonçalves *et al.*, 2004) and Peru (Tamplin and Carrillo, 1991) and also Bangladesh (Tamplin *et al.*, 1990). It has been found in association with a wide range of aquatic life, including cyanobacteria (*Anabaena variabilis*) (Islam *et al.*, 1989), diatoms (*Skeletonema costatum*) (Martin and Bianchi, 1980) and freshwater filamentous green algae

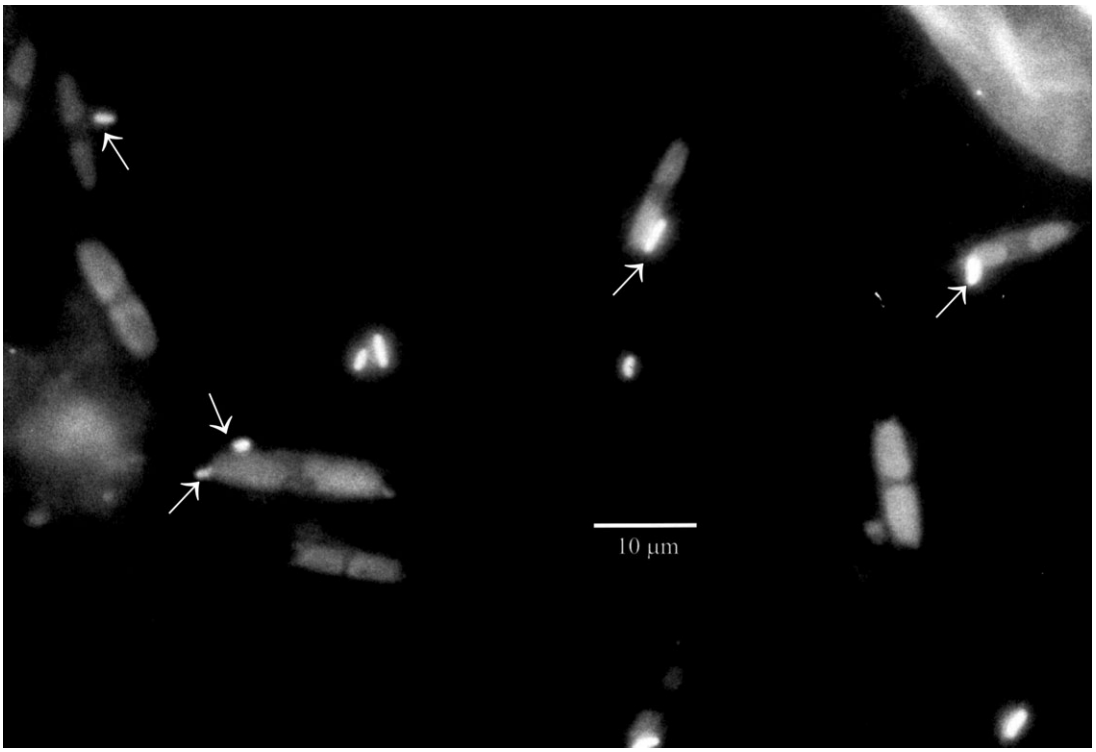

Fig. 3. Detection of *Vibrio cholerae* O1 VNC adhered to *Nitzschia palea* using direct immunofluorescence. The arrows point out the bacterium location above the algae.

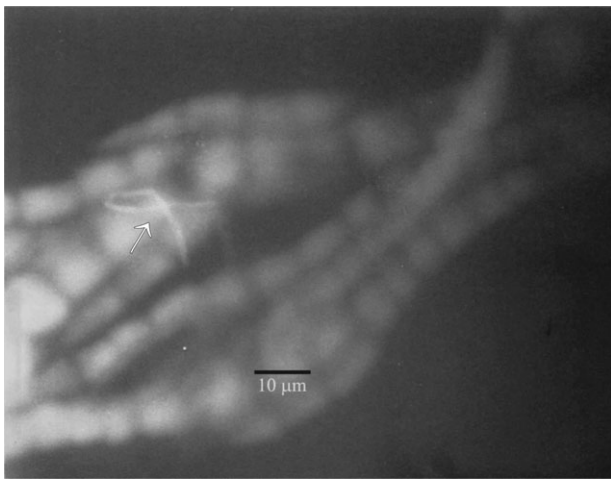

Fig. 4. Detection of *Vibrio cholerae* O1 VNC adhered to *Stigeoclonium* sp. using direct immunofluorescence. The arrow points out the bacterium location above the algae.

(*Rhizoclonium fontanum*) (Islam *et al.*, 1989). Binsztein *et al.* (Binsztein *et al.*, 2004) found an association with estuarine diatoms like *Thalassionema nitzschioides*, *Pleurosigma* cf. *normanii, Coscinodiscus* sp. and dinoflagellates such as *Ceratium furca* and *Noctiluca scitillans*. In our study, 1 to 10 VNC were found adhered to *Stigeoclonium* sp. and *Nitzschia palea*. One feasible explanation for the attachment of *V. cholerae* to aquatic organisms is its capacity to produce enzymes such as chitinases and mucinases (Epstein *et al.*, 1993), which would allow it to adhere for example to the surface of copepods (Huq *et al.*, 1983; Tamplin *et al.*, 1990) and *Volvox* sp., a type of mucilaginous phytoplankton colony (Colwell *et al.*, 1990). Another possible explanation for the prolonged survival of toxigenic *V. cholerae* when attached to *Rhizoclanium fontanum*, for example, could be its ability to derive nutrients from the extracellular products released by this species (Borroto, 1997). Islam *et al.* (Islam *et al.*, 1989) hypothesized that *V. cholerae* 01 uses extracellular products released from *A. variabilis* for nutrition and that the availability of salts on the mucilaginous surface of this blue–green alga enables *V. cholerae* 01 to survive for prolonged periods in freshwater environments. These authors also proposed that photosynthesis by *A. variabilis* supplies oxygen for the aerobic respiration of *V. cholerae* 01 and that the microorganism, in turn, is the source of carbon dioxide for *A. variabilis*. They concluded that *V. cholerae* 01 in a viable but non-culturable state is associated with *A. variabilis* and, possibly, with other species of blue–green algae with a mucilaginous surface, which could serve as ecological niches in estuaries and

freshwater ecosystems (Islam *et al.*, 1989; Borroto, 1997). The phytoplankton collected during our study demonstrated great species richness with clear predominance of diatoms, especially pennates, many of them with a wide range of tolerance to different factors (Lange-Bertalot, 1979; Gómez and Licursi, 2001; Lobo *et al.*, 2004). The dominance of this group of diatoms is typical of low-order lothic systems (Margalef, 1980; Oemke and Burton, 1986) and coincides with observations in mountain rivers in Córdoba (Corigliano *et al.*, 1994; Luque *et al.*, 1994), Salta (Moraña, 1998; Salusso, 1998) and Tucumán (Tracanna and Martínez De Marco, 1997; Seeligmann, 1998), provinces in Argentina. In contrast, in larger Argentine rivers centric diatoms are cited as the most important (Guarrera, 1950, Schiaffino, 1977; García de Emiliani, 1981; Luchini, 1981; Anselmi de Manavella and García de Emiliani, 1995).

Our results have shown that DFA-DVC is a useful technique for detection of *V. cholerae* O1 adhered to plankton, which agrees with findings by other authors (Xu *et al.*, 1984; Huq *et al.*, 1990; Tamplin *et al.*, 1990; Martins *et al.*, 1993). As this microorganism was almost constantly present in rivers in Tucumán in its viable non-culturable form, continuation of monitoring would be necessary (field and laboratory assaying). This would allow determination of the conditions that favour survival of *V. cholerae* and define other possible environmental reservoirs. This is the first time that adhesion of *V. cholerae* VNC to algal species has been detected in Tucumán and our study reports two new reservoirs not cited before in the literature.

Given the endemic nature of cholera in some Latin American countries, field and laboratory studies are necessary to define and identify the aquatic reservoirs that favour survival of toxigenic *V. cholerae* 01 between epidemic periods. Research is needed to determine whether its association with phytoplankton, zooplankton and macrophytes in aquatic ecosystems of this hemisphere is indiscriminate or whether, on the contrary, it exhibits a preference for certain species of blue–green algae or other organisms. It is also necessary to determine both the geographic location of waters that harbour reservoirs of toxigenic *V. cholerae* 01 and the communities that use this water for consumption, bathing, fishing and other activities. Factors that have not been considered in the present study, but should be included in future studies are migration and socio-cultural conditions of the affected areas because they can play important roles in the spread of the disease. Programs for prevention of cholera in South America should be not abandoned because under certain climate conditions *V. cholerae* O1 VNC could revert to a transmissible state.

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