Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Environment and virulence factors of *Vibrio cholerae* strains isolated in Argentina

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Keywords
antimicrobial resistance, Argentina, environment, plankton, *Vibrio cholerae,* virulence factors.

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2006/1433: received 12 October 2006, revised 23 May 2007 and accepted 25 May 2007

doi:10.1111/j.1365-2672.2007.03468.x

Abstract

Aims: To determine the presence of *Vibrio cholerae* in different areas of Argentina in three sample types, to determine the composition of planktonic communities in areas at which this pathogen was detected and to characterize the virulence properties and antimicrobial resistance of the recovered environmental isolates.

Methods and Results: Water and plankton samples were collected in marine, brackish and freshwater environments. *Vibrio cholerae* non-O1, non-O139 was isolated in 36·1% of the samples analysed. The micro-organism was detected in freshwater but not in marine or brackish samples. No relationship was found between isolation of *V. cholerae* and presence of any species of plankton. All the isolates presented very similar virulence profiles by PCR, lacking *ctxA* and *tcpA* El Tor and containing *hlyA* (98·7%), *rtxA* (99·0%), *toxR* (98·7%) and *stn-sto* (1·9%). Resistance to ampicillin was found in both Tucumán (21%) and Buenos Aires isolates (45%).

Conclusions: We identified two geographic areas in Argentina where *V. cholerae* was present: freshwaters of the rivers from Tucumán and the Río de la Plata.

Significance and Impact of the Study: The identification of *V. cholerae* strains in the environment, carrying both virulence factors and resistance to antimicrobial agents, highlight the need for a continuous and active surveillance of this pathogen.

Introduction

Re-emergence of *Vibrio cholerae* worldwide has posed a challenge for microbiology and epidemiological surveillance of cholera. Over the last few years, many studies that increased the understanding of *V. cholerae*'s ecology, pathogenesis and epidemiological behaviour have been conducted (Dalsgaard *et al.* 1995; Faruque *et al.* 1998; Sharma *et al.* 1998; Chakraborty *et al.*, 2000; Colwell 2004).

Vibrio cholerae is a natural inhabitant of freshwater, estuarine and seawater environments. Therefore, the aquatic environment acts as a reservoir and source of its transmission (Colwell 2004). It has been shown that in inter-epidemic periods, the micro-organism persists in association with phytoplankton and zooplankton. The interaction with plankton plays an important role in the ecology of the micro-organism, facilitating persistence, mainly in response to low temperature and reduced nutrient concentration (Huq *et al.* 1984; Lobitz *et al.* 2000; Louis *et al.* 2003; Eiler *et al.* 2006).

To date, only serogroups O1 and O139 have been associated with epidemic cholera. However, numerous outbreaks of diarrhoea caused by non-O1, non-O139

serogroups of *V. cholerae* have been reported (Bhattacharya *et al.* 1993; Rivas *et al.* 1996; Sharma *et al.* 1998). This pathogen also has been associated with sporadic cases of gastroenteritis, septicaemia and extraintestinal infections (Morris 1994; Rivas *et al.* 1996; Bhattacharya *et al.* 1998; Sharma *et al.* 1998; Dhar *et al.* 2004).

On the contrary, horizontal transfer of virulence genes by phages and other mobile elements has been demonstrated for *V. cholerae*. In fact, non-O1 and non-O139 strains may act as reservoirs of virulence genes in the environment which could lead to the emergence of new epidemic or virulent variants by 'mixing and matching' of genes in the environment or the human intestine (Chakraborty *et al.* 2000).

Up to now, there are few reports of the frequency of isolation of *V. cholerae* non-O1 in Latin America. In Cuba, *V. cholerae* non-O1 was found in 8·5% of 250 children under 5 years old with diarrhoea (Bravo *et al.* 1998). In addition, in Perú, a diarrhoea outbreak caused by CT-, NAG-ST – *V. cholerae* non-O1 (involving seven serogroups) was reported (Dalsgaard *et al.* 1995).

In Argentina, an active surveillance programme of cholera was installed in 1991 after the re-emergence of the disease in Latin America. As a result of this surveillance, which included the creation of a laboratory network, both *V. cholerae* O1 and non-O1 strains were recovered from clinical samples and from the environment in different regions of Argentina (Rivas *et al.* 1996; Costagliola *et al.* 2000). *Vibrio cholerae* O1 was only isolated during epidemic periods, mainly during the summer, from 1992 to 1998. Notwithstanding, isolation of *V. cholerae* non-O1, non-O139 followed a different pattern, with strains recovered both from the environment (751 isolates) and as causative agents of diarrhoea (794 isolates), not only mainly in summer and spring but also during winter and fall. The majority of the environmental strains were recovered in the warmer regions of the north and centre of the country from different water sources. Most of the clinical isolates were associated with sporadic cases of moderate to severe diarrhoea but three of them were isolated from septicaemia. During January–February 2000, in the Northern Province of Salta, an outbreak of diarrhoea that affected children and adults was reported, and *V. cholerae* non-O1 was isolated in ten patients (unpublished data).

Regarding the pathogenicity mechanisms of *V. cholerae* non-O1, non-O139, different virulence factors have been suggested to be involved in the disease caused by this pathogen (Morris 1994). Diarrhoea, although generally watery, is sometimes characterized by the presence of blood and mucous. This characteristic altogether with vomiting indicates a different process of pathogenicity than that of *V. cholerae* O1. Although some of these strains produce the cholera toxin (CT) or a cholera-like toxin, most of them lack the CTXΦ phage and the toxin coregulated factor (TCP) and even the complete *Vibrio* Pathogenicity Island (VPI) (Kurazono *et al.* 1995). Even if different studies on *V. cholerae* O1 and O139 indicate that CT, TCP and ToxR (the regulator of expression and secretion of CT and TCP) are necessary to cause diarrhoea, there are studies in which nontoxigenic environmental isolates of *V. cholerae* show a secretory response in intestinal tissue that may be caused by other virulence factors (Datta-Roy *et al.* 1986). On the contrary, *V. cholerae* non-O1, non-O139 produce other extracellular products such as the heat-stable toxin NAG-ST (coded by the gene *stn/sto)*, the hemolysin HlyA and the pore-forming toxin RtxA (Arita *et al.* 1986; Kurazono *et al.* 1995; Chow *et al.* 2001) that may be responsible for the pathogenesis of these strains.

The aims of this study were to test for the presence of *V. cholerae* in four different areas of Argentina and in three different sample types: water, microplankton and mesozooplankton. Moreover, we aimed to characterize the virulence properties and resistance to several antimicrobial agents of the recovered environmental *V. cholerae* isolates and to analyse the planktonic community present in areas at which the pathogen was detected.

Materials and methods

Sampling sites

A total of 360 samples were collected between January 2003 and June 2005, including 121 from water and 239 from plankton obtained from four different aquatic environments in Argentina (Fig. 1) as follows: (i) Marine – 20 sampling cruises were conducted monthly from May 2003 to March 2005 at a fixed station, and five cruises were conducted at Atlantic shelf waters during July–August 2003, October–November 2003, June 2004; October–November 2004 and December 2004; (ii) Brackish waters of Río de la Plata – five sampling cruises were conducted during July–August 2003, October–November 2003, June 2004, October–November 2004 and December 2004; (iii) Freshwaters of Río de la Plata – five sampling cruises were performed in January, February, March 2003 and January, February 2005 at four fixed stations on a transect parallel to the coast and (iv) Freshwaters of Tucumán rivers – 18 surveys were conducted at two different sites of the Salí River (Banda and Canal Norte) and one site at the Lules River. These samples were collected monthly from July 2003 to December 2003 and from January 2005 to June 2005. During 2004 samples were collected bimonthly.

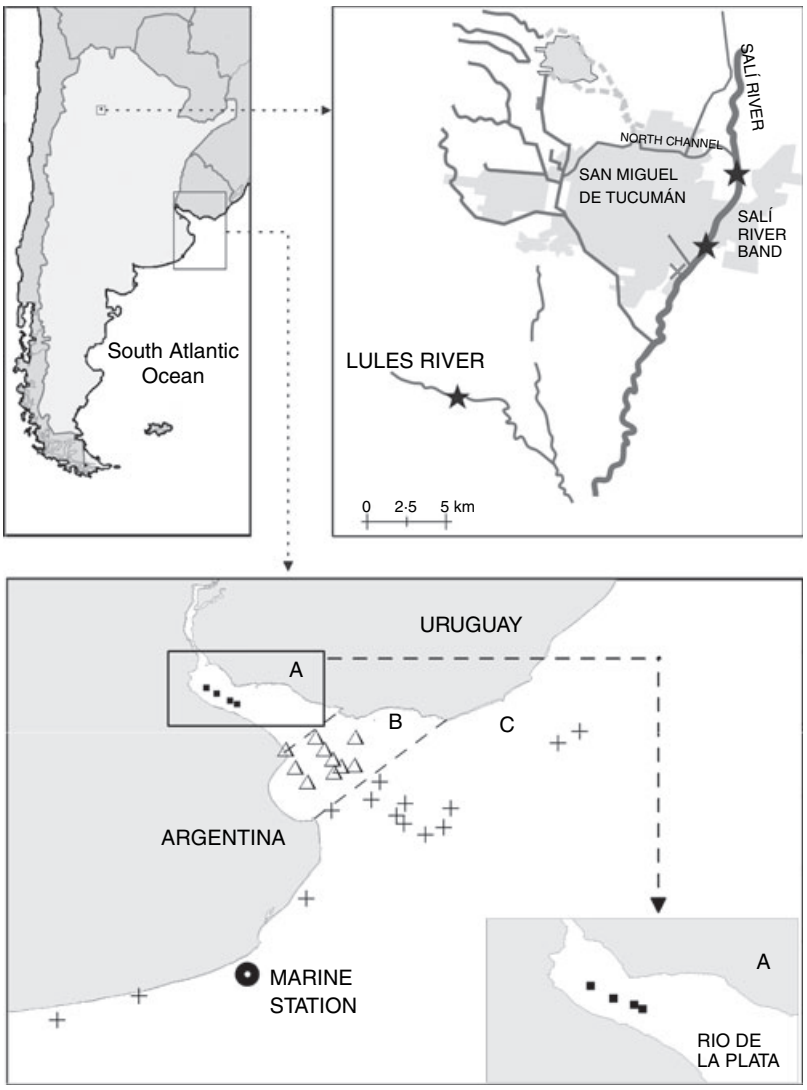

Figure 1 Map of Argentina showing the sampling sites, indicated as follows: ● Marine fixed station; ✚ Atlantic shelf waters; + Freshwaters of Río de la Plata; △ Brackish waters of Río de la Plata and ★ Freshwaters of Tucumán rivers.

Sample collection and processing

Water samples (W) were collected in sterile bottles and 2–3 l were filtered through 0·22-μm pore size polycarbonate membrane filters. At marine and brackish environments, plankton samples were collected using 25 and 200 μm mesh nets to analyse microplankton and mesozooplankton species, respectively. Plankton was collected by towing for 10 min (*c.* 500 l) for mesozooplankton and by vertical hauls from 1 m over the bottom up to surface for microplankton (*c.* 500 l). At freshwater areas, plankton samples were collected using 20- and 50-μm mesh nets to analyse microplankton and mesozooplankton species, respectively, by filtering 20 min through the nets (*c.* 500 l). Plankton was recovered in a final volume of *c.* 200 ml. For the identification of microplankton and mesozooplankton species, the sample was preserved with formaldehyde at a final concentration of 0·4% and 4%, respectively. Physical and chemical parameters, including temperature, conductivity, salinity, pH and dissolved oxygen (DO), were measured *in situ*.

Isolation and identification of *Vibrio cholerae*

All membrane filters from each water sample and 10 ml of each plankton sample (representing *c.* 20–30 l of the material before filtering) were incubated into an enrichment medium consisting of 50 ml of alkaline peptone water (Oxoid Ltd, Basingstoke, England) [1% (wt/vol) peptone, 1% (wt/vol) NaCl; pH 8·6] 6–8 h at 37°C. Two loopfuls of the culture broth, taken from the top layer of the alkaline peptone water, were streaked onto two thiosulfate citrate bile salt sucrose (TCBS) agar plates (Oxoid Ltd) and incubated 24 h at 37º. Approximately five flat,

1- to 3-mm-diameter sucrose positive colonies were picked from each sample and stabbed in T_1N_1 soft agar (1% triptone, 1% NaCl, 0·75% agar). These colonies were characterized using biochemical tests for *V. cholerae*, as described by Dodin and Fournier (1992). *Vibrio cholerae* isolates were serotyped by slide agglutination using polyclonal antisera (anti-O139, O1, Ogawa and Inaba) provided by the Instituto Nacional de Producción de Biológicos-ANLIS "Carlos G. Malbrán".

Polymerase chain reaction

Polymerase chain reaction (PCR) was used to confirm biochemical and serological results and for detection of virulence genes *ctxA* (that codes for the subunit A of CT), *tcpA* El Tor (that codes for the structural unit of TCP, allele El Tor), *toxR*, *hlyA*, *stn/sto* and *rtxA*, based on published protocols (Chun *et al.* 1999; Chow *et al.* 2001; Rivera *et al.* 2001, 2003). Protocols were slightly modified and multiplex PCR assays were implemented for: (i) *V. cholerae*, *ctxA* and *tcpA* El Tor and (ii) *stn/sto* and *rtxA*, as shown in Table 1. All primers (Operon Biotechnologies, Cologne, Germany) are listed in Table 1. Final concentration of $MgCl_2$ and primers were 1·5 mmol l^{-1} and 0·6 μmol l^{-1}, respectively. DNA templates were prepared by the boiling method and 2 μl was used for PCR amplification (final volume 25 μl). Three strains were used as controls for the PCRs: RC138 (*V. cholerae* O139, *ctxA*+, *tcpA* El Tor+, *hlyA* El Tor+, *toxR*+, *stn/sto*-, *rtxA*+) and RC2 (*V. cholerae* O1, *ctxA*+, *tcpA* El Tor-, *hlyA* Classic+, *toxR*+, *stn/sto*-, *rtxA*+) kindly provided by Dr Rita Colwell, Center of Marine Biotechnology, University of Maryland, MD, USA; and strain 83-7771 (*V. cholerae* non-O1, non-O139, *ctx*-, *tcpA* El Tor-, *hlyA* El Tor+, *toxR*+, *stn/sto*+, *rtxA*+) kindly provided by Dr Clifford Clark, National Microbiology Laboratory, Canada. As a negative control (reagents control), 2 μl of water was added to the PCR mix. The amplification programmes began with a denaturation step at 94°C of 5 min, followed by 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at variable temperature, variable extension time (Table 1) at 72°C, and finally an extension at 72°C for 10 min.

Antimicrobial susceptibility patterns

Antimicrobial susceptibility testing was performed by Kirby–Bauer diffusion method (Bauer *et al.* 1966) using commercial antibiotic disks (BBL™, Sparks, MD): trimethoprim-sulfamethoxazol (SXT, 1 : 25 μg), ampicillin (AMP, 10 μg), chloramphenicol (CHL, 30 μg), tetracycline (TET, 30 μg) and nitrofurantoin (NIT, 300 μg). The Control Laboratory Standard Institute (CLSI) has established

Table 1 PCR cycling conditions and amplicon sizes

PCR	Primers	Amplicon size (bp)	Annealing (°C)	Extension (min)	Reference
Multiplex A (*V. cholerae/ctxA/tcpA*)					
V. cholerae	pVC-F2 pVCm-R1	300	60	1	Chun *et al.* 1999
ctxA	CT 94F CT 614R	564			Rivera *et al.* 2001
tcpA El Tor	TCP 72F TCP 477R	461			Rivera *et al.* 2001
Multiplex B (O1/O139)					
O1	VCO1-F2 VCO1-R2	647	55	1.5	Rivera *et al.* 2003
O139	VCO139-F2 VCO139-R2	741			
Multiplex C (*stn/sto/rtxA*)					
stn/sto	*stn/o* 67F *stn/o* 194F	172	55	1	Rivera *et al.* 2001
rtxA	*rtxA*-F *rtxA*-R	417			Chow *et al.* 2001
Multiplex D (*hlyA*/Classic and El Tor)					
hlyA	*hlyA* 489-F *hlyA* 744-F *hlyA* 1184-R	737* 480†	60	1	Rivera *et al.* 2001
toxR	*toxR* 101F *toxR* 837R	779	60	1	Rivera *et al.* 2001

*Positive for biotypes Classic and El Tor.
†Specific for El Tor strains.

interpretative criteria for *V. cholerae* for SXT, AMP, CHL and TET (Control Laboratory Standard Institute 2005). For NIT, the CLSI criteria for enterobacteriaceae were used. A control strain of *Escherichia coli* (ATCC 25922) was used in this study.

Statistics

The log-likelihood ratio *G*-test (Sokal and Rohlf 1979) was used to evaluate the significance of different factors on *V. cholerae* non-O1, non-O139 (VC) occurrence. The factors analysed were sampling site (SS), sample type (ST) and month (M) (Table 2). Differences were considered significant at $P \leq 0{\cdot}05$. The dependent variable *VC* was divided into two categories: presence (value 1) or absence (value 0).

The proportions of *V. cholerae* presence for each SS, ST and each M were compared in pairs by using an *a posteriori* Tukey-type test for multiple comparisons (Zar 1996). Data were transformed using the formula:

$$P' = \arcsin\sqrt{\frac{x+3/8}{n+3/4}},$$

where x is the number of positive samples and n is the total number of samples. Data analyses were performed with STATISTICA version 5·1 (Statsoft Inc., Tusla, OK, USA).

Results

Isolation of *Vibrio cholerae* and environmental conditions

From a total of 360 samples, 130 (36·1%) were found positive for *V. cholerae*: 21 at the freshwater area of the Río de la Plata and 109 at freshwaters of Tucumán rivers. In these 130 samples, 311 isolates were identified as *V. cholerae* non-O1, non-O139. No isolates of *V. cholerae* O1 were recovered. No positive samples were detected neither at the brackish area of the Río de la Plata nor in the marine waters.

Marine area

Vibrio cholerae was not found among 383 sucrose positive isolates selected from 77 samples studied during all seasons at the marine fixed station. Salinity values at this station had a variation range of 31·5–33·9 psu and temporal distribution of temperature values showed sea surface values ranging from 10·2 to 21·5°C. At the Atlantic shelf waters, salinity values were from 28·9 to 33·4 psu and temperature from 15 to 17°C. Likewise, no *V. cholerae* isolates were identified from 150 sucrose positive colonies recovered from 32 samples.

Table 2 Percentage of samples positive for *V. cholerae* non-O1, non-O139 according to reservoirs, sampling site and date from the freshwater sector of Río de la Plata River (Buenos Aires) and Tucumán

Geographic area	Factor	Level	*V. cholerae* non-O1, non-O139 (%)
Buenos Aires	Sample type	Water	65·00
		Microplankton	20·00
		Mesozooplankton	20·00
	Sampling site	Station 1	46·67
		Station 2	20·00
		Station 3	33·33
		Station 4	40·00
	Sampling date	January 2003	25·00
		February 2003	58·33
		March 2003	16·67
		January 2005	58·33
		February 2005	16·67
Tucumán	Sample type	Water	64·81
		Microplankton	66·67
		Mesozooplankton	70·37
	Sampling site	LR	42·59
		SRB	85·19
		SRCN	74·07
	Sampling date	July 2003	33·33
		August 2003	44·44
		September 2003	55·56
		October 2003	55·56
		November 2003	100·00
		December 2003	100·00
		January 2004	100·00
		March 2004	88·89
		May 2004	66·67
		July 2004	44·44
		September 2004	22·22
		November 2004	100·00
		January 2005	77·78
		February 2005	77·78
		March 2005	88·89
		April 2005	55·56
		May 2005	66·67
		July 2005	33·33

Information from brackish and marine sampling sites was not included in this table because *V. cholerae* was not isolated from these sites.
LR, Lules River; SRB, Salí River (Banda); SRCN, Salí River (Canal Norte).

Brackish waters of Río de la Plata

Salinity ranged from 1·49 to 27·24 psu and temperature from 11·2 to 23·3°C. A total of 205 sucrose positive isolates from the TCBS plates were obtained from 46 studied samples. None of them were identified as *V. cholerae*. There were no sampling cruises conducted in this region in summer and autumn.

Freshwaters of Río de la Plata

Twenty-one of 60 (35%) samples studied were positive for *V. cholerae* non-O1, non-O139 and 68 isolates were recovered from 422 sucrose positive colonies. *Vibrio cholerae* was detected predominantly in water samples ($P = 0{\cdot}0028$) with no significant differences detected among the freshwater sampling sites ($P = 0{\cdot}4454$) (Table 2). The conductivity values in this area were of 127–250 μS cm^{-1} and the temperature ranged from 20·9 to 26·0°C.

Freshwaters of Tucumán rivers

A high proportion of samples (67·3%) from this area were positive for *V. cholerae* non-O1, non-O139 (Table 2). Among 612 sucrose positive colonies, 243 were identified as *V. cholerae*. The percentage of the samples where *V. cholerae* was detected did not differ significantly between the three sample types ($P = 0{\cdot}82$) (Table 2). The frequency of isolation was higher in the samples collected from Salí River at Banda and Canal Norte than those from Lules River ($P < 0{\cdot}0001$). A higher rate of isolation of *V. cholerae* was observed during the warmer months, being highly significant during 2003 and 2004 ($P = 0{\cdot}0037$ and $P = 0{\cdot}0001$, respectively).

The water temperature varied between 8 and 26·0°C in the sampling sites of Tucumán, with average temperatures of 20·7°C in Salí River and 18·7°C in Lules River. The lowest temperatures were found from June to September ($\bar{x} = 16{\cdot}6$°C)and the highest temperatures were found from November to March ($\bar{x} = 23{\cdot}7$°C).The pH fluctuated between 4·5 and 8·7 in the Salí River ($\bar{x} = 71$)and between 4·6 and 9·1 in the Lules River ($\bar{x} = 7{\cdot}8$).The electric conductivity varied from 559 to 2060 μS cm^{-1} in the Salí River ($\bar{x} = 1136$ μS cm^{-1})and from 339 to 741 μS cm^{-1} in the Lules River ($\bar{x} = 535$ μS cm^{-1}).The lowest values of both conductivity and pH for each river were found during August, September and October. The oxygen values in the Salí River oscillated between 0 and 9 mg l^{-1} throughout the study and in the Lules River between 3·7 and 9 mg l^{-1}. Abundant organic detritus and anoxia (DO of 0–2·8 mg l^{-1}) was found in the Salí River, especially between June and September, also coinciding with the higher industrial activity.

Characterization of plankton samples

Plankton composition was analysed in samples positive for *V. cholerae*. The microplankton samples from the freshwater area of the Río de la Plata showed a clear dominance of freshwater species, whereas marine species rarely appeared. Freshwater species of common phytoplanktonic groups included the cyanobacteria *Microcystis aeruginosa* and *Oscillatoria* sp., diatoms of the genus *Aulacoseira*, chlorophyceans of the genera *Coelastrum*, *Scenedesmus* and *Cosmarium*, and euglenophycean species. The few microplankters of marine origin were the dinoflagellate *Ceratium tripos*, a ciliate of the genus *Tintinnopsis* and colonies of the prymnesiophycean *Phaeocystis*. Copepoda crustaceans mainly composed mesozooplankton samples. Specimens of families Diaptomidae (*Argyrodiaptomus* sp.) and Pseudodiaptomidae (*Pseudodiaptomus* sp.) were the main Calanoidea present at the samples. In a lesser measure, Cyclopoidea of the family *Cyclopoidea* were also found. The mentioned groups are representatives of freshwater environments in Argentina.

The microplankton samples from the Salí and Lules Rivers were mainly composed by diatoms (over 85%) – *Aulacoseira granulata*, *Cyclotella meneghiniana*, *Cymbella* sp., *Diatoma vulgare*, *Gomphonema parvulum*, *Nitzschia palea*, *Pleurosira laevis* and *Ulnaria ulna* – for the most part of the year. In second place, the green algae were represented mainly by several species of the genera *Closterium*, *Cosmarium* and *Scenedesmus* followed by the cyanobacterium mainly represented by *Leptolyngbya foveolarum*. Euglenids (*Euglena ehrenbergii* and *Euglena proxima*) were more important in samples obtained from June to October. Rhodophyceans were only found in the Lules River in February and April 2005. In the mesozooplankton fraction, there were registered few species and few specimens of each species per sample, with the exception of those recovered in November 2003 and January 2004 in the Salí River. In most of the samples, only genera associated with the riverbed such as the rotifers *Lecane* and *Brachionus* and cladocera quidorids, especially *Leydigia striata*, were registered. Copepods were the most important group, represented by the calanoid *Notodiaptomus incompositus* and the cyclopoids *Acanthocyclops robustus*, *Metacyclops* sp., *Eucyclops neumani* and *Paracyclops chiltoni*, being the two latter the more frequently identified. Other invertebrates were detected in these samples, being the most relevant the larvae of Diptera, especially *Chironomidae*.

Distribution of virulence factors

The genes that code for the virulence factors CT and TCP (*ctxA* and *tcpA* El Tor) traditionally associated with epidemic *V. cholerae* O1 and O139, were absent in all the 311 isolates from the Tucumán rivers and the freshwaters of the Río de la Plata. In contrast, almost all the isolates carried the following virulence factors: *rtxA* (99·4%) that codes for the pore-forming toxin Rtx, the hemolysin gene *hlyA* (99%) and *toxR* (99%), which codes for ToxR. The gene *stn/sto* that codes for the heat-stable toxin was found in only six of the isolates (1·9%) recovered in the three sampling sites of Tucumán in different campaigns during 3 years. Thus, all isolates recovered from the Río

de la Plata and 95·1% of isolates from Tucumán had the same virulence pattern: *ctxA*-, *tcpA* El Tor-, *toxR*+, *rtxA*+, *hlyA*+, *stn*-. The second more frequent virulence pattern with only 1·9% was *ctxA*-, *tcpA* El Tor-, *toxR*+, *rtxA*+, *hlyA*+, *stn*+. Six isolates lacked one or more of the frequent virulence factors, *hlyA*, *toxR* and *rtxA*, including one isolate recovered from the Salí River (Banda) in July 2004 that was negative for all the studied virulence factors.

Antimicrobial patterns

The percentage of resistance to the antimicrobial agents tested for the *V. cholerae* non-O1, non-O139 isolates recovered in Tucumán was: AMP – 21·3%; TET – 1·9%; SXT – 0·6% and NIT – 3·9%. All these isolates were susceptible to CHL. From the strains isolated in the freshwater sector of the Río de la Plata River, percentage of resistance to AMP and NIT was observed as 45·6% and 7·3%, respectively. In summary, 94% and 83% of the isolates of the Río de la Plata and Tucumán Rivers, respectively, are susceptible to all antibiotics or resistant only to AMP.

Discussion

Vibrio cholerae has been recognized as part of the bacterial flora inhabiting marine coasts, rivers and estuarine waters in different world areas (Morris 1994; Chakraborty *et al.* 2000).

In this work, we have identified *V. cholerae* in two different areas of Argentina: the Salí and Lules Rivers in the Province of Tucumán and the freshwater area of Río de la Plata.

In Tucumán rivers, significant differences were observed between the presence of *V. cholerae* and the time of the year. One possible factor could be water temperature, as *V. cholerae* was predominantly isolated in the warmer months from November to March as has been reported in other regions (Lipp *et al.* 2002). In addition, in the months where the sugar cane and citric industries are more active (from June to October) conductivity and pH values were lower, and together with lower temperatures and low-dissolved oxygen concentration, these conditions might not have been favourable for *V. cholerae*.

In these rivers, there was also a difference in the number of positive samples for *V. cholerae* according to the sampling site. This difference could not be clearly attributed to any of the factors analysed, being conductivity the only parameter that appeared to be lower in the Lules River, where *V. cholerae* was less frequently found. This is in agreement with results found by Huq *et al.* (2005) who suggested that higher conductivities in lake waters are associated with increases in cholera cases.

In the Río de la Plata, *V. cholerae* strains were only isolated from the freshwater area. At these sampling sites, high temperatures were registered as the sampling cruises were carried out in summer months. However, the frequency of isolation was lower than in Tucumán rivers, and this finding could be due to the low conductivity values observed in the Río de la Plata. No isolates were recovered in the brackish waters of the Río de la Plata. Although this pathogen had been previously isolated in this area during warm months (Costagliola *et al.* 2000; Binsztein *et al.* 2004), no sampling cruises were conducted in the summer months in the present study.

Data from Tucumán samples showed a similar percentage of positive results for the three sample types (water, microplankton and mesozooplankton). In contrast, results obtained from Río de la Plata River indicated that *V. cholerae* non-O1, non-O139 was detected more frequently in water samples than in plankton fractions, even when the volumes sampled and inoculated in APW were much bigger for plankton than for water. The methodology used in the study cannot differentiate free-living bacteria from bacteria attached to microscopic substrates, and both can be present in water samples. Hence, the higher frequency of isolation in water samples does not necessarily mean that the bacteria are found free in this environment. As collection of water is easier and cheaper than the plankton sampling, we suggest, considering these results, the use of this methodology for the surveillance of *V. cholerae* in aquatic environments.

Previous studies suggested that *V. cholerae* is present in the environment in association with plankton, mainly copepods and some aquatic hydrophytes (Islam *et al.* 1996). In this study, this micro-organism was identified in plankton samples both in Tucumán and in the freshwaters of the Río de la Plata. These samples contained mainly freshwater species, including microalgal groups such as diatoms, chlorophytes, euglenophytes and cyanophytes, and calanoidea and cyclopoidea copepod species, some of which have been associated with the presence of *V. cholerae* in other regions of the world (Huq *et al.* 1990; Islam *et al.* 1996). However, as was recently observed in the Adriatic Sea (e.g. Baffone *et al.* 2006), we could not find any relationship between detection of *V. cholerae* and the presence of any species of plankton in the present study. In Tucumán rivers, where low numbers of mesozooplankton species were found, arthropods like Chironomid larvae were detected. Chironomidae have been recently recognized as a reservoir of *V. cholerae* and could act as an alternative source of carbon for *V. cholerae* (Halpern *et al.* 2003).

All the isolates presented very similar patterns of virulence factors, in which *ctxA*, *tcpA* were absent and *hlyA*,

rtxA and *toxR* present independently of the site of isolation in agreement with studies from other regions of the world (Sharma *et al.* 1998; Rivera *et al.* 2001). One exception was the detection of the heat-stable toxin gene, found in 1·9% of the isolates. The appearance of this toxin could not be correlated with any of the factors evaluated in this study: it appeared in different sampling sites and at different times with a very low frequency. The strains recovered possessed similar virulence traits as those present in pathogenic isolates recovered in the Province of Tucumán from children who suffered diarrhoea (data not shown), suggesting that environmental isolates possess the ability to cause disease.

In different world regions, resistance to AMP, TET, SXT, CHL and polimixin B was reported for environmental isolates of *V. cholerae* non-O1, non-O139 (Dalsgaard *et al.* 1995). In Argentina, both human and environmental isolates of *V. cholerae* recovered from 1992 to 2000 showed very low resistance to antimicrobial agents, except for a group of multi-resistant *V. cholerae* O1 strains that caused an outbreak in the north of the country in 1997 (Petroni *et al.* 2002). In our study, a high percentage of resistance was only found for AMP, being 21% for isolates recovered in Tucumán and 45% for isolates recovered in Buenos Aires. As different populations of bacteria coexist in aquatic ecosystems, *V. cholerae* may acquire antibiotic resistance plasmids from neighbouring micro-organisms. Therefore, continuous surveillance of antimicrobial resistance in *V. cholerae* should be performed to detect the emergence of multi-resistant strains.

No single environmental parameter or plankton species/group could be clearly related with the isolation of *V. cholerae*. This implies that either multiple factors could be involved in the generation of a favourable environment for the growth of this micro-organism or there could be other factors involved that were not considered in this study.

We have identified this pathogen in two different areas of the country, recovering isolates that present both virulence factors and resistance to antimicrobial agents. For these reasons, a continuous and active surveillance of *V. cholerae* is necessary, both in humans and in the environment.

Acknowledgements

We gratefully acknowledge Seeligmann, C.T., Locascio de Mitrovich, C. and Mirande, V. for their assistance in identification of plankton species. In addition, we would like to thank Angela Jure for performing antimicrobials susceptibility testing; Insaurralde, C. and Filiol, A. for supplying the cruises from Prefectura Naval Argentina; and Panagopulo, M. for her technical assistance. This research was supported by the grant PICTR2000-00010 from the Agencia Nacional de Promoción Científica y Tecnológica, Secretaría de Ciencia y Tecnología, Ministerio de Cultura y Educación, Argentina.

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