ARTIGO/ARTICLE

Detection of viable and viable nonculturable *Vibrio cholerae* O1 through cultures and immunofluorescence in the Tucumán rivers, Argentina

Detecção de *Vibrio cholerae* O1 viável e viável não cultivável, através de técnicas de cultivo e imunofluorescência nos rios de Tucumán, Argentina

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ABSTRACT

Vibrio cholerae *has been sporadically isolated from rivers in Tucumán, Argentina, since the outbreak in 1991. The aim of this study was to determine the environmental reservoir of the bacterium in these rivers, assessing the presence of* Vibrio cholerae *non-O1 and O1 (the latter both in its viable culturable and non culturable state) and its relationship to environmental physicochemical variables. 18 water samplings were collected in the Salí River (in Canal Norte and Banda) and the Lules River between 2003 and 2005. Physical-chemical measurements (pH, water temperature, electrical conductivity and dissolved oxygen) were examined.* Vibrio cholerae *was investigated with conventional culture methods and with Direct Immunofluorescence (DFA-VNC) in order to detect viable non culturable organisms. All isolated microorganisms corresponded to* Vibrio cholerae *non-O1 and non-O139 (Lules 26%, Canal Norte 33% and Banda 41%). The majority was found during spring and summer and correlated with temperature and pH. Non culturable* Vibrio cholerae *O1 was detected year round in 38 of the 54 water samples analyzed. Application of the Pearson correlation coefficient revealed that there was no relationship between positive immunofluorescence results and environmental physicochemical parameters. Genes coding for somatic antigen O1 were confirmed in all DFA-VNC-positive samples, whereas the virulence-associated* ctx*A and* tcp*A genes were confirmed in 24 samples.*

Key-words: Vibrio cholerae *O1. Culture and immunofluorescence. Tucumán rivers.*

RESUMO

Vibrio cholerae *tem sido isolado esporadicamente nos rios da Província de Tucumán, Argentina, desde outubro de 1991. O objetivo deste estudo foi localizar os reservatórios nestes rios, identificar a presença de* Vibrio cholerae *O1 (em estado cultivável e não cultivável) e relacionar a presença desta bactéria com as variações físico-químicos da água. Foram coletadas dezoito amostras de água do rio Salí (nas localidades de Canal Norte e Banda) e do rio Lules, entre 2003 e 2005. Estas foram submetidas a análises físico-químicos como determinação de pH, temperatura, condutibilidade elétrica e oxigênio dissolvido. A presença de* Vibrio cholerae *foi verificada por métodos de cultivo convencional e por imunofluorescência direta (DFA-VNC). Todos os microrganismos isolados foram não O1 e não O139 (Lules 26%, Canal Norte 33% e Banda 41%). A maioria foi encontrada na primavera e verão, indicando uma relação com a temperatura e pH. Das 54 amostras analisadas por DFA-VNC, 38* Vibrio cholerae *não cultivável, foram detectadas em todas as épocas do ano. As amostras positivas foram confirmadas por PCR para o antígeno somático O1 e para os genes de virulência* ctx*A e* tcp*A. Coeficiente de correlação de Pearson revelou que não há relação entre os resultados obtidos por imunofluorescência e a variação dos parâmetros físico-químicos.*

Palavras-chaves: Vibrio cholerae *O1. Culture and immunofluorescence. Tucumán rivers.*

Cholera continues to be an important and devastating disease transmitted by water and food, especially in those regions of the world where it is endemic[8 12]. Before its reemergence in Peru and subsequent spreading throughout Latin America in 1991, the disease had been absent from the Americas for nearly 100 years[14]. There has been much speculation as to the cause of this reemergence and whether there has always been an environmental reservoir for *Vibrio cholerae* in Latin America. Since 1991, seasonal patterns of cholera outbreaks have been well documented in Central and South America, with the largest

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numbers of cases occurring during the warm months (January to March)[28 39]. In Argentina, there have been seven epidemics since 1992. These cholera outbreaks occurred mainly during the summer months. *Vibrio cholerae* O1 strains were isolated from water samples collected from rivers during epidemic periods, but also found in marine waters and the La Plata River estuaries[2].

Standard bacteriological procedures for isolation of *Vibrio choleare* O1 from environmental samples (including water) between epidemics were generally unsuccessful[19]. *Vibrio cholerae* requires salt for growth and can revert to a viable but non culturable state (VNC) in response to adverse environmental conditions. These VNC bacteria do not grow on conventional culture media, but remain intact and retain metabolic activity and respiration[6 27 29 30 42]. However, the method of Kogure *et al* can be used to demonstrate that these cells retain viability and their pathogenic potential[16 25 32]. Techniques employing microscopy, with either direct or indirect fluorescent antibody staining, have been developed and provide important data on the occurrence of viable but nonculturable *Vibrio cholerae* O1[19].

Since the outbreak in Tucumán, a province in the northwest of Argentina, sporadic cases of diarrhea by *Vibrio cholerae* have been detected in areas close to the Salí and Lules rivers. This study aimed to detect *Vibrio cholerae* O1 in these environments using conventional culture techniques to isolate the microorganism and direct immunofluorescence to detect the viable nonculturable state and associate its presence to 4 environmental physicochemical variables.

MATERIAL AND METHODS

Site description and water sampling. Samples were collected at the Salí River (two sites: Canal Norte (CN) and Banda (B)) and at the Lules River (one site). They were taken during 18 campaigns in a three-year period (2003-2005) with 6 campaigns per year. Water temperature, pH and electrical conductivity were determined *in situ* with a mercury thermometer, a portable digital pH meter (TPA-I) and an Altronix conductivity meter (Ct-1), respectively. Dissolved oxygen (DO) was measured at the laboratory according to the methods by Winkler[1].

Water samples were collected in sterile 5-liter bottles, and then immediately transported to the laboratory and subjected to bacteriological examination not more than 5h.

Bacteriological assaying. Two liters of water were filtered through 0.22µm membranes, using 12 to 15 membranes per sample. Membranes were subsequently incubated in 100ml of alkaline peptone water, pH 8.6, for 6-8h at 35°C. Two loopfuls of broth were streaked on Thiosulfate Citrate Bile agar (TCBS agar, Difco) and incubated for 18h at 37°C. Six to 12 typical colonies (yellow and 1 to 3mm diameter) were transferred to nutritive soft agar (T_1N_1, 0.75% agar) and incubated for 24h at 37ºC. All colonies were stored at room temperature for further testing. Isolates were identified biochemically and serotyped (O1 and O139 antisera from the National Institute of Infectious Diseases-INEI. ANLIS. "Dr Carlos G Malbran", Buenos Aires, Argentina)[6].

Direct immunofluorescence of *Vibrio cholerae* O1 (DFA-DVC). Two liters of water were membrane-filtered (0.22µm). Afterwards, the membranes were washed with 8ml of phosphate buffer, and this buffer was fractioned for direct immunofluorescence of *Vibrio cholerae* O1 (DFA-DVC) analysis. Samples were previously incubated in the dark for 6 to 8h at room temperature in the presence of yeast extract and nalidixic acid. Under these conditions, viable but nonculturable bacteria elongate from a coccoid shape to rod-like cells, yet they do not multiply due to the inhibitory effect of nalidixic acid (a DNA gyrase inhibitor). After incubation, samples were fixed with 4% formaldehyde and processed with cholera DFA kits (New Horizons Diagnostics Corporation) for detection of *Vibrio cholerae* O1[7 17]. Stained preparations were observed under an epifluorescence microscope (1000X) at 490 (maximum excitation) and 520nm (maximum emission) with a blue filter. All procedures were carried out in the dark. Readings were carried out within 24h after preparation of the samples.

Confirmation of *Vibrio cholerae* O1 with polymerase chain reaction. Because DFA-DVC is a presumptive technique presence of the microorganism was confirmed using PCR in water to detect genes coding for the somatic antigens O1 and virulence-associated *ctx*A genes that code for the A subunit of the *cholera* toxin (CT) and *tcp*A El Tor, that codes for the toxin co-regulated pilus (TCP) pilin subunit[5 9 33 34 40 41]. This method was carried out at the Bacteriology Department of the Institute of Infectious Diseases (INEI) ANLIS "Dr. Carlos G. Malbrán", Buenos Aires, Argentina.

Statistical analysis. The relationship between detection of *Vibrio cholerae* and the physicochemical variables assayed was assessed with the Pearson Correlation Coefficient ($\alpha = 0.05^*$ or 0.01^{**}), using the SPSS statistics program (version 10.0 for Windows).

RESULTS

Physical and chemical parameters of the water. The water temperature oscillated between 15 and 26°C, pH between 5.5 and 9.0, DO between 0 and 9mgl^{-1} and the conductivity between 400 and 1.600µS cm^{-1}. pH values at the Salí River sampling sites (CN and B) oscillated during the three years between acid and alkaline, whereas fluctuations at the Lules River sampling site were between 6.59 and 9.06. Regarding dissolved oxygen, anoxia stages were only observed in the Salí River, which also showed highest conductivity (Figures 1a, b, c).

Viable culturable *Vibrio cholerae*. A total of 613 suspicious colonies (yellow in TCBS) were isolated from the different sites and 385 were biotypified as *Vibrio cholerae* non-O1, non-O139. The percentage of isolates from each site was: RS (CN) 33%, RS(B) 41% and RL 26%.

Figure 2 shows *Vibrio cholerae* non-O1, non-O139 isolates according to their sampling site during the different months and seasons. The microorganism was mainly isolated during the warm months, corresponding to spring and summer, with a percentage of 30 or more.

When analyzing the correlation between isolation of *Vibrio cholerae* non-O1, non-O139 and physicochemical variables it was found that the highest number of isolations in the Lules River in January, March, November and December with a water temperature over 25°C and pH more than 7.7. DO was between 8.8 and 9mgl^{-1} and conductivity between 426 and 658µS cm^{-1}. No isolates were found in June and September with a water

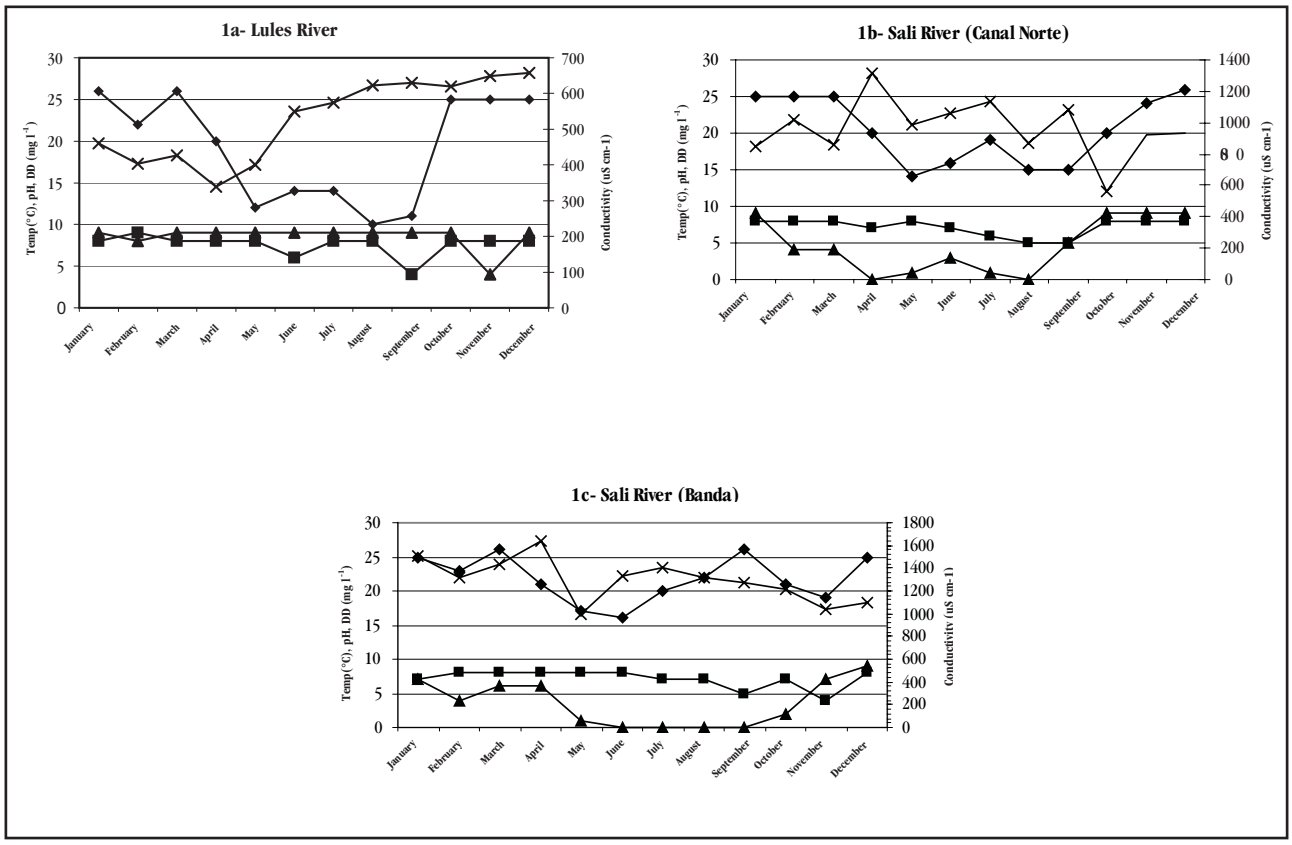

Figure 1 - Physicochemical parameters of a) Lules River, b) Salí River (Canal Norte) and c) Salí River (Banda). (◆) Temperature, (■) pH, (▲) Dissolved Oxygen (DO) and (x) Conductivity.

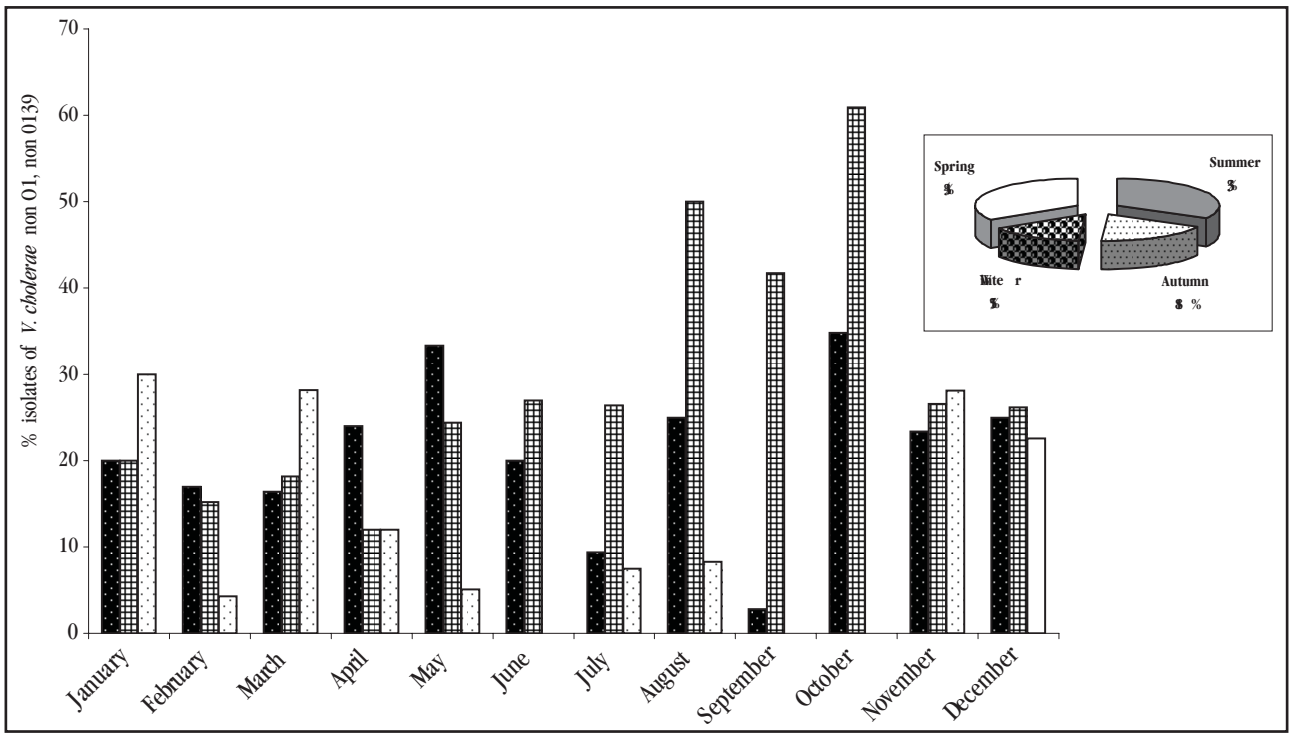

Figure 2 - Percentage of isolation of Vibrio cholerae *non-O1 at each sample site and during the different months and seasons. Salí River (Canal Norte), Salí River (Banda) and Lules River.*

temperature under 14°C and pH below 6.5. At the Canal Norte sampling site (Salí River) the microorganism was isolated all year round with temperatures and pH values that oscillated between 14 and 26°C and 5 and 8.5 respectively. Furthermore, periods of anoxia were observed and conductivity was generally less than 900µS cm^{-1}. The highest number of *Vibrio cholerae* non-O1, non-O139 isolates was recovered at the Banda sampling site (Salí River) during the period researched. Water temperature varied from 16 to 26°C and the pH was generally higher than 7. There were also anoxia periods (June to September) and conductivity was over 1.000µS cm^{-1}.

Figure 3 demonstrates that from the 54 water samples from the different sites analyzed per month 38 were positive for *Vibrio cholerae* O1 using the direct immunofluorescence (DFA-DVC) technique. *Vibrio cholerae* O1 (VNC) was detected all year round in rivers in Tucumán with the highest numbers during January, February and June. Water temperature in January and February was over 22°C and pH over 7.5, figures that are different from those obtained in June with temperatures under 16°C and pH values below 6.5. Conductivity and DO varied considerably in January, February and June with values between 400 and 1,550µS cm^{-1} and 2.8 and 9mgl^{-1} respectively. The Pearson correlation coefficient revealed that there was no relationship between positive immunofluorescence results and environmental physicochemical parameters.

Viable but nonculturable *Vibrio cholerae*. Even though no *Vibrio cholerae* O1 strains were obtained by conventional culture methods, DFA-DVC revealed the presence of *Vibrio cholerae* O1 (VNC), which appeared as rod-shaped bacteria after incubation with yeast extract and nalidixic acid (Figure 4).

PCR confirmed presence of genes coding for the somatic antigen O1 in the 38 positive samples for viable nonculturable *Vibrio cholerae* using immunofluoresence, but the virulence-associated *ctx*A and/or *tcp*A genes were only confirmed in 24 of them.

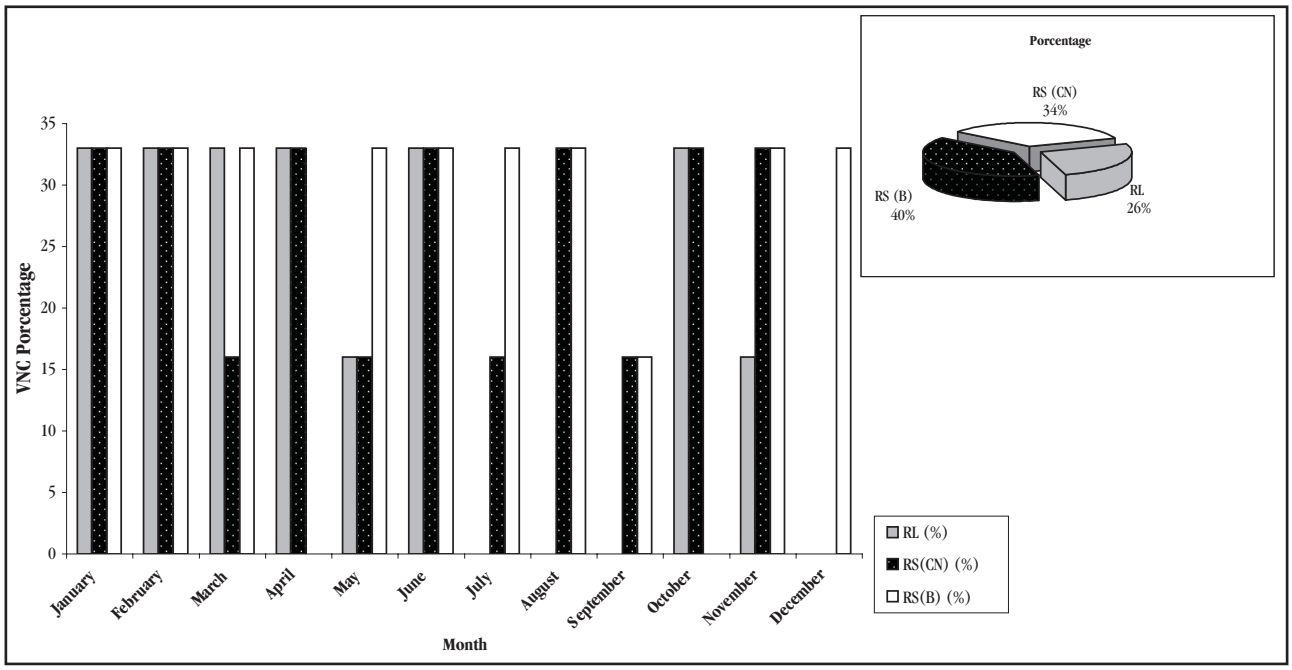

Figure 3 - Detection of* Vibrio cholerae *O1 VNC using direct immunofluorescence assaying at the different sample sites according to the month. Lules River, Salí River (Canal Norte) and Salí River (Banda).

Figure 4 - Detection of viable nonculturable* Vibrio cholerae *O1 through direct inmunofluorescence.

DISCUSSION

In 1977, Colwell *et al* first hypothesized that coastal waters were an important reservoir of *Vibrio cholerae*[10]. Other authors also detected *Vibrio cholerae* in seawater and other environmental sources around the world, both in cholera-endemic and in cholera-free areas[11 18 20 23 24 36 47].

Borroto, Lee *et al*, Tamplin and Carrillo isolated *Vibrio* from water with temperatures between 25 and 12ºC, which is in agreement with our results[3 27 43 44].

Singleton *et al* have concluded that presence of *Vibrio* O1 in aquatic environments is not limited to estuaries, because its salinity requirements can be met through an adequate nutrient concentration in fresh water environments. It has been reported that this microorganism is able to survive in fresh water for prolonged periods of time[4 13 22 35 37 38 42 45]. Feachem *et al* and Miller *et al* have demonstrated that various biological and physicochemical factors influence growth, survival, and distribution of *Vibrio cholerae* in aquatic environments[13 31]. Isolation of the microorganism with classical culture methods may fail. This depends on the physicochemical properties of the water or the physiological state of *Vibrio cholerae* O1 itself, either with actively growing cells or cells in a latent or dormant state[31 37]. The DFA-DVC technique has shown to be useful for detection of viable but nonculturable *Vibrio cholerae* O1 in water samples[30 44 46]. Huq *et al* isolated *Vibrio cholerae* O1 from fresh water environments (rivers) using immunofluorescence, but they too were unable to isolate culturable forms with conventional culture methods in Bangladesh. Gonçalves *et al* found viable nonculturable forms of the *Vibrio* organism in two river estuaries in Brazil and Binsztein et al detected it for the first time in the La Plata River and close to a marine platform in Argentina[2 15 19].

The rivers in Tucumán are affected by effluents of a variety of industries (sugar cane, citric fruit processing and paper among others) that, together with agricultural activities, modify the aquatic environments, thus generating conditions that allow survival of *Vibrio cholerae* non-O1, non-O139 and persistence of the viable nonculturable state of *Vibrio cholerae* O1. The fact that *Vibrio cholerae* O1 was not detected with classical culture methods agrees with results obtained by other researchers[21 30].

Kurazono *et al* and Sharma *et al* sustain that the epidemiological impact of environmental *Vibrio cholerae* strains is not clearly understood, because most of them do not produce the cholera toxin and have also lost significant pathogenic factors[26 41]. Similarly, 37% of the total number of samples that confirmed somatic antigen O1 tested negatively for the virulence-associated *ctx*A and *tcp*A genes.

Our study has for the first time provided evidence of isolation of *Vibrio cholerae* non-O1, non-O139 and presence of the viable nonculturable state of *Vibrio cholerae* O1 in rivers in Tucumán all year round. Consequently, it can be inferred that the Lules and Salí rivers constitute a reservoir for the microorganism in our province.

The warm temperatures in addition to a high concentration of organic nutrients from agro-industrial waste, as is the case in the rivers in Tucumán, create in these developing areas with poor sanitary conditions an adequate environment so that *Vibrio cholerae* can persist. Considering that this water is used for human consumption in rural areas, and that drinking water constitutes an important transmission vehicle of the pathogen, exhaustive monitoring studies would be necessary to determine how these bacteria, present in the rivers, affect public health now and in the future.

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