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Quantification of human infection risk caused by rotavirus in surface waters from Córdoba, Argentina

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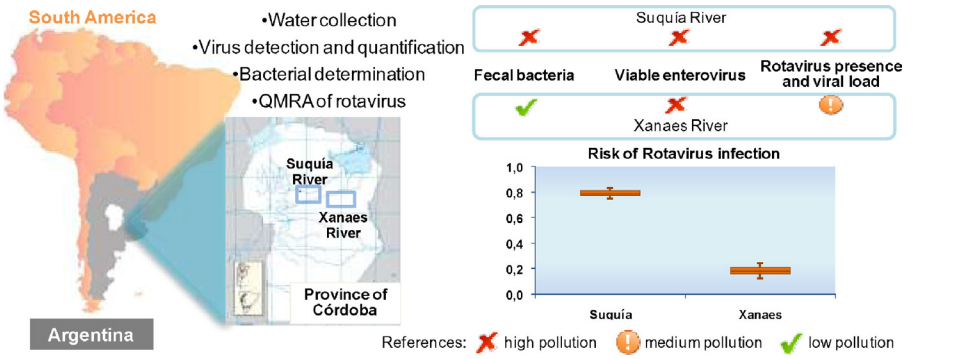
HIGHLIGHTS

- Enteroviruses were frequently detected in both rivers of Córdoba.
- Enteric viruses were identified in recreational waters with low bacterial loads.
- QMRA revealed that recreational rivers represented a public health hazard.
- Bacterial indicators do not reflect the risk from rotavirus infection.
- Viral monitoring should be included to determine microbiological water quality.

GRAPHICAL ABSTRACT



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ABSTRACT

Fecal contamination of water is a worrying problem because it is associated with the transmission of enteric pathogenic microorganisms that can cause many infectious diseases. In this study, an environmental survey was conducted to assess the level of viral contamination by viable enterovirus and rotavirus genome in two recreational rivers (Suquía and Xanaes) of Córdoba, Argentina. Quantitative microbial risk assessment (QMRA) was calculated to estimate the risk of rotavirus infection. Water sampling was carried out during a one-year period, the presence of total and fecal coliforms was determined and water samples were then concentrated for viral determination. Cell culture and indirect immunofluorescence were applied for enterovirus detection and RT-qPCR for rotavirus quantification. Coliform bacteria levels found in Suquía River often far exceeded the guideline limits for recreational waters. The Xanaes exhibited a lower level of bacterial contamination, frequently within the guideline limits. Enterovirus and rotavirus were frequently detected in the monitoring rivers (percentage of positive samples in Suquía: 78.6% enterovirus, 100% rotavirus; in Xanaes: 87.5% enterovirus, 18.7% rotavirus). Rotavirus was detected at a media concentration of 5.7×10^5 genome copies/L (gc/L) in the Suquía and 8.5×10^0 gc/L in the Xanaes. QMRA revealed high risk of rotavirus infection in the Suquía, at sampling points with acceptable and non-acceptable bacteria numbers. The Xanaes showed significantly lower health risk of rotavirus infection but it proved to be a public health hazard. The viral occurrence was not readily explained by the levels of bacteria indicators, thus viral monitoring should be included to determine microbiological water quality. These findings provide the first data of QMRA for recreational waters in Argentina and reveal the need for public awareness of the health implications of the use of the river waters.

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1. Introduction

Water is the most precious natural resource on our planet, essential for basic human existence. However, environmental waters face a wide variety of stressors that affect not only the ecosystem but also human health (Lipp et al., 2001). Studies involving the analysis of microbial contamination of water have been conducted in different parts of the world (Luyt et al., 2012; Xiao et al., 2013; Haack et al., 2013; Widmer et al., 2013). In most of these studies, it is noticeable the pollution of surface waters by bacteria and protozoa, but also some studies address the detection of viruses (Chigor and Okoh, 2012; Vieira et al., 2012; Allmann et al., 2013; Lee et al., 2014). Enteric viruses are transmitted by the fecal-oral route, being shed in high numbers through feces (10^5 to 10^{11} viral particles per gram of stool) of infected individuals. They are highly stable in the environment because they lack the lipid envelope, being able to persist for long time in waters. Despite the relatively low concentration of viruses in fecal impacted waters, its presence carry health risks since they have very low infectious doses (10–100 virions) and therefore even a few viral particles in water can infect a person (La Rosa et al., 2012). Gastrointestinal symptoms are the most commonly encountered manifestations, but also respiratory diseases, conjunctivitis, hepatitis, central nervous system infections and chronic diseases can occur (La Rosa et al., 2012).

Among the enteric viruses, enteroviruses (EV) and rotaviruses (RV) are studied as environmental contaminants and listed as relevant waterborne pathogens by the World Health Organization (WHO, 2011). The EV comprises a large genus within the *Picornaviridae* family. They affect millions of people worldwide each year, and are often found in respiratory secretions and in stool of infected persons. Enteroviral infections in humans are reported to peak in summer and early fall, which also coincides with increased water recreational activities and water contact (Kocwa-Haluch, 2001). Because most of the human EV can replicate in cell cultures, they are good indicators to confirm the presence of viable and infectious viruses in environmental samples. On the other side, RV is the most important cause of gastrointestinal infection in children under 5 years and can have severe consequences, including hospitalization and death, with the latter being far more frequent in low-income regions (Tate et al., 2012). Protection to RV disease can be achieved by both symptomatic and asymptomatic natural infection, as well as by vaccination. Two live-attenuated vaccines (one pentavalent, RotaTeq® by Merck; and one monovalent, Rotarix® by GlaxoSmithKline) have been successfully introduced in a growing number of countries since 2006 (Ruiz-Palacios et al., 2006; Vesikari et al., 2006). The monovalent vaccine has been included into the Argentinean National Immunization Program in January 2015.

Microbiological parameters for recreational water quality have included over time the use of coliforms bacteria (Papaioannou et al., 2014). The Sub-Secretary of Water Resources of Argentina and the Secretary of Water Resources and Coordination of the Province of Córdoba have adopted the approach of considering the guide levels recommended by the United States Environmental Protection Agency (U.S. EPA) of the United States for fecal coliforms (≤200 MPN/100 mL) and the European Union Law for total coliforms (≤5000 MPN/100 mL). However, bacteriological criteria have shown to be not ideal to evaluate the level of fecal pollution in water. The absence of these pathogens could not exclude the presence of enteric viruses which are generally more resistant than bacteria to sewage treatment procedures (Blatchley et al., 2007; Pusch et al., 2005). Moreover, several cases of severe viral gastroenteritis resulting from exposure to recreational water with bacterial loads within the guideline limits have been reported worldwide (Leclerc et al., 2002).

Quantitative microbial risk assessment (QMRA) is a probabilistic modeling technique that is now widely used in assessing health risks associated with exposure to waterborne pathogens. In the present study, an environmental survey was conducted in order to assess the level of viral contamination by viable EV and RV genome in surface waters of Córdoba, Argentina, impacted by different urban populations, and to calculate the probability of risk of rotavirus infection by contact with these urban surface waters which are highly used for recreational activities. The viral contamination results were compared to the level of coliform bacteria in the river waters. To our knowledge this is the first QMRA reported for recreational waters in Argentina. The finding of this study would provide the first data for Argentina to make strategic investments to improve sanitary conditions in the local rivers.

2. Materials and methods

2.1. Background

Córdoba city is the capital of the Province of Córdoba, located in the Central Region of Argentina and has approximately 1,317,298 inhabitants with a population density of 2308 habitants/km^2 (INDEC, 2010). Suquía River rises in the San Roque Dam and traverses Córdoba city from west to east. Suquía water flow is 10 m^3/s, subject to a seasonal fluctuation: high flow during the wet season (WS, October–March, average temperature 21.5 °C with 723.8 mm of rainfall, water flow 24 m^3/s) and very low during the dry season (DS, April–September, average temperature 13.6 °C with 146.1 mm of rainfall, water flow 1.5 m^3/s). The river is the main intake source of Córdoba city water potabilisation plants, and it also serves for irrigation, industrial use, electricity production, recreation and some sport fishing (Pesce and Wunderlin, 2000; Castelló et al., 2000). Villa del Rosario is the head town of Río Segundo Department (Province of Córdoba). It has 15,313 inhabitants and a population density of 86 habitants/km^2. Villa del Rosario is located 80 km east-southeast from Córdoba city, on the right-hand banks of the Xanaes River, which is born in the Paravachasca Valley at the confluence of the Los Molinos and Anizacate rivers, on the eastern slopes of the Cumbres de Achala (Sierras Grandes). Xanaes River flows west–east with an average inflow of 12.2 m^3/s. The river serves for many purposes, such as the intake source of water potabilisation plant, irrigation, industrial use and recreation (Castelló et al., 2000).

2.2. Monitoring sites and frequency

Two different monitoring areas of the Province of Córdoba were selected for water sampling (Fig. 1A). The first area, named Córdoba city included seven monitoring stations over the Suquía River (Fig. 1B). Three stations were located upstream from Córdoba city (1-Funnel, where the river borns at the San Roque Dam, 2-Villa Warcalde and 3-San Antonio ford), three throughout Córdoba city (4-Zipoli bridge, 5-Centenario bridge and 6-Sargento Cabral ford) and one located downstream from both Córdoba city and the main sewage treatment plant of the city (7-San José bridge). The sampling was carried out in 2010 twice during the DS and also twice during the WS, collecting a total of 28 surface water samples. The second monitoring area, named Villa del Rosario included four monitoring stations over the Xanaes River (Fig. 1C). One station was located upstream Villa del Rosario city (1-Farm), two throughout the city (2-Coast and 3-Bridge) and one downstream the city (4-Stage). A monthly monitoring was done during a one-year period (December 2011–November 2012), collecting a total of 48 water samples (six samples per monitoring station during the DS and six during the WS). For each water sample (from the Suquía and Xanaes Rivers), 2 L were taken on weekday mornings in sterile bottles and were transported within 12 h at 4 °C to 8 °C to the laboratory, for further processing and analysis (0.5 L for bacteria detection and 1.5 L for virus concentration).

2.3. Bacteriological analysis

The number of total coliforms and thermotolerant coliforms was determined by the Most Probable Number (MPN) technique, according to Standard Methods (APHA, 2005).

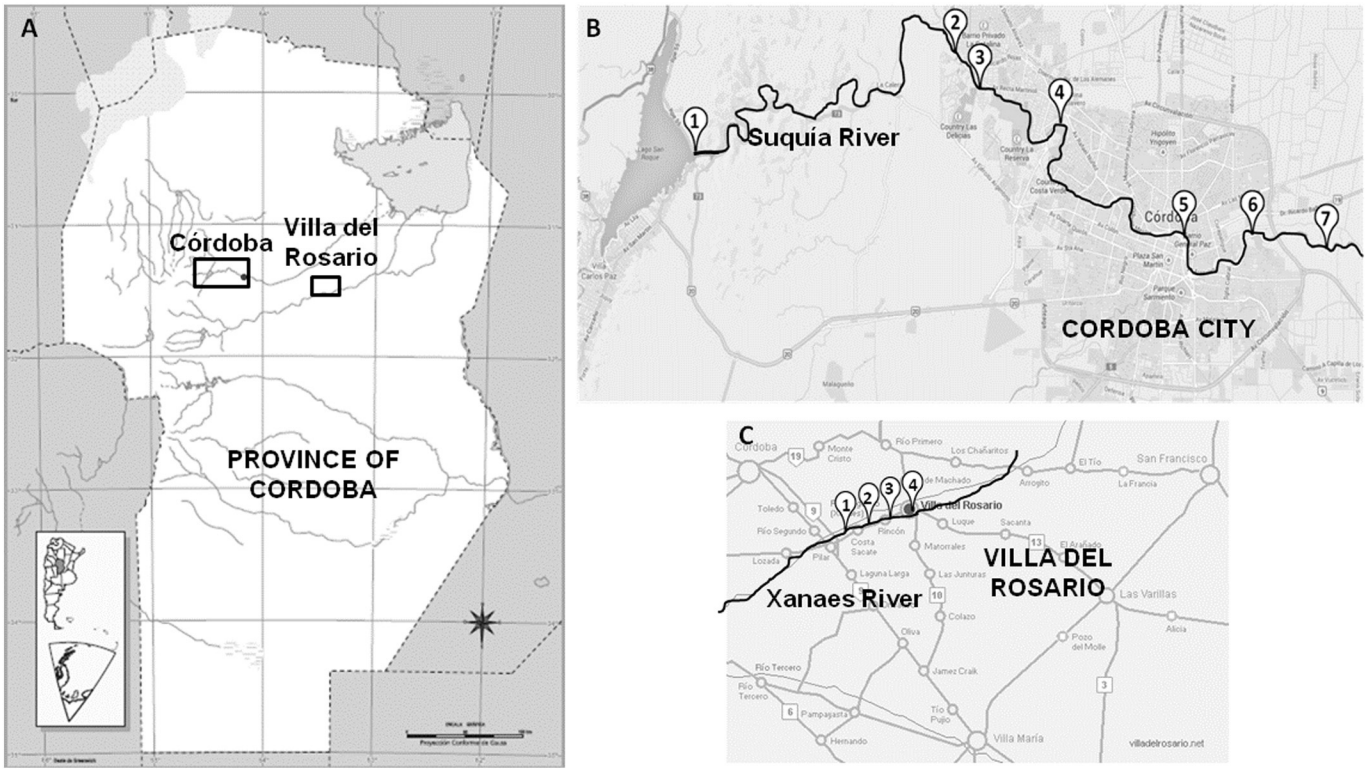

Fig. 1. Sampling location sites in Córdoba bathing waters. A) Sampling geographic areas indicated in the Province of Córdoba (Argentina) map: Córdoba city and surrounding area and Villa del Rosario city. B) Suquía River: 1-Funnel; 2-Villa Warcalde; 3-San Antonio ford; 4-Zipoli bridge; 5-Centenario bridge; 6-Sargento Cabral ford; 7-San José bridge. C) Xanaes River: 1-Farm; 2-Coast 3-Bridge; 4-Stage. Thick lines in black indicate the rivers and numbers above them depict the monitoring stations.

2.4. Virus concentration

The concentration of surface water samples was performed using methods described previously by Lewis and Metcalf (1988) and Greening et al. (2002), with modifications described by Huang et al. (2005). A total of 1.5-liter samples were concentrated 100-fold to 15 mL by high-speed centrifugation, elution, and polyethylene glycol precipitation.

2.5. Viable enterovirus detection

Enterovirus infectivity was evaluated by cell culture in HEp-2 cell line. Prior to spiking the concentrated samples into the culture flasks, water concentrates were quickly thawed at 37 °C, treated twice with chloroform (in a relation 1:1) and antibiotics and antimycotics were added (penicillin 100.000 IU/mL; streptomycin 2.5% and amphotericin B 250 μg/mL). Then, each water sample (1 mL/flask) was analyzed twice in Hep-2 cell flasks using standard operating procedures (WHO, 2004). After inoculation they were kept at 36 °C under 5% CO_2 atmosphere for 5–7 days and cytopathogenic effects (CPE) were examined by inverted microscope every day. Possible CPE were identified and EV was confirmed by indirect immunofluorescence assay. Monoclonal antibody blend used for EV detection consisted of coxsackievirus type A9 monoclonal antibody; coxsackievirus type B monoclonal antibody blend: B1, B2, B3, B4, B5 and B6; echovirus monoclonal antibody blend: serotypes 4, 6, 9, 11, 30 and 34; poliovirus monoclonal antibody blend: serotypes 1, 2 and 3; and enterovirus monoclonal antibody blend: serotypes 60, 71 and Cox A16. The monoclonal antibody reagents were commercially prepared and were purchased from Chemicon International (Temecula, CA).

2.6. Rotavirus genome detection, characterization and quantification

2.6.1. Nucleic acid extraction and cDNA synthesis

Viral RNA was extracted from 140 μL of the concentrated sample using the commercial QIAamp Viral RNA kit (Qiagen Inc., Hilden, Germany). The manufacturer's protocol was followed, and the purified viral RNA was eluted in 30 μL of elution buffer. Extracted RNA was reverse-transcribed into cDNA using random hexamer primers and AMV reverse transcriptase (Invitrogen, CA, USA).

2.6.2. Molecular detection and characterization of rotavirus

cDNA products were used as templates for PCR VP7 gene amplification with the Beg9/End9 pair of primers (Gouvea et al., 1990) and VP4 gene amplification with the Con2/Con3 primers (Gentsch et al., 1992). Multiplex heminested PCRs with genotype-specific primers for VP7 (G genotypes) and VP4 (P genotypes) were used for detection and genotype characterization of RV (Gouvea et al., 1990; Gentsch et al., 1992; Iturriza-Gomara et al., 2000). Positive and negative controls were included in all PCR runs. The PCR products were resolved on 10% polyacrylamide gel electrophoresis (Laemmli, 1970) followed by silver staining (Herring et al., 1982), to achieve high resolution of the products obtained.

2.6.3. Rotavirus quantification

Water samples were quantified in duplicate by qPCR using the ABI 7500 Real-Time PCR System (Applied Biosystems, CA, USA). qPCR was performed as described by Fumian et al. (2010) using primers designed by Zeng et al. (2008). A standard curve (10^6 to 10^1 copies per reaction) was generated using tenfold serial dilutions of pTOPO vectors (Invitrogen, USA) containing the NSP3 target region. The qPCR reaction was performed in a final volume of 25 μL by using Environmental PCR

Master Mix (Applied Biosystems, CA, USA). Amplification data were collected and analyzed using Sequence Detection Software version 1.0 (Applied Biosystems, CA, USA). A test result was considered positive if a sigmoidal amplification curve crossed the threshold before 45 cycles and all positive and negative control reactions gave expected results. In order to establish the amplification efficiency and the limit of detection of the real-time PCR assays, ten-fold dilutions (10^6 to 10 copies) of the plasmid NSP3 standard were tested by duplicate as described above. Assay efficiency ($10^{(-1/\text{slope})}$) was calculated from the slope of the standard curve which was generated by plotting the log copy number versus the cycle threshold (Ct) value. The recovery efficiencies of the nucleic acid extraction and concentration procedures were considered for the determination of rotavirus concentration in the initial waters (Poma et al., 2013).

2.7. Statistical analysis

Total coliforms, fecal coliforms and rotavirus load were transformed into $\log_{10}$. Microbiological dataset obtained was divided in two seasonal groups: WS and DS. χ^2 test was used to compare categorical variables and a non parametric Kruskal Wallis test to assess if significant differences existed between WS and DS. The descriptive statistics for the bacteriological and virological quality variables were used. Means and confidence intervals of 95% were calculated. *P*-values lower than 0.05 indicated significant differences for the variables measured. Statistical analyses were performed with InfoStat (Di Rienzo et al., 2014).

2.8. Quantitative microbial risk assessment

Haas et al. (1999) identified four formal procedures of determining QMRA, namely:

2.8.1. Hazard identification

Rotavirus was chosen for the QMRA based on the following criteria: occurrence and persistence in the environment (Abad et al., 1994); low infectious doses (Graham et al., 1987); possibilities for detection and quantification (Zeng et al., 2008); adequate literature on the organism (Sanborn and Takaro, 2013; Parashar et al., 2013); representativity of a major group of pathogens (Health Canada, 2011); and the occurrence of diseases such as diarrhea and gastroenteritis in the population (Gomez et al., 1998; Giordano et al., 2001; Estes, 2001).

2.8.2. Exposure assessment

The potential exposure routes in the study area were identified to determine the critical points to quantify the microbial risks to human health. In this study, we assumed that the microbial risks from dermal contact and inhalation were minor relative to exposure through ingestion. Moreover, the exposure analysis was based on three principles: i) the average concentration of rotavirus in the water samples from each sampling sites on the rivers, ii) the average volume of water consumed per individual during recreational activities, and iii) the viability of the viruses. To calculate the total exposure or dose for a particular sampling point, the pathogen concentration in a sample was multiplied by the volume of water ingested and by the fraction of detected particles capable of cause an infection. The concentrations of rotavirus were determined as described under the "Rotavirus genome detection, characterization and quantification" procedure. The following underlying assumptions were used in the exposure assessment for rotavirus: i) exposure through direct contact by exposed population playing and swimming in the river; ii) involuntary ingestion of 10 mL of water per exposure, and the annual volume of involuntary water ingested was based on a frequency of 6 events per year of a total of 10 mL each (Steyn et al., 2004; Westrell et al., 2004; Labite et al., 2010; Schets et al., 2011); iii) the ratio of infectious virus particles to total detected virus particles was 1:10 (Chigor et al., 2014). A major drawback of the RT-PCR assay used in the detection of rotavirus is its inability to determine the viability and infectivity of viruses detected, as the presence of viral nucleic acids does not necessarily indicate the presence of infectious viruses (Hamza et al., 2009; Bofill-Mas et al., 2010). To circumvent this limitation the previously estimated ratio of infectious viruses to total rotavirus particles reported by Chigor et al. (2014) was used in this work. Nonetheless, the ratio between infectious viruses and genome copies likely varies with the matrix from which a sample was obtained, specific organism and primer/probe combinations (Ward et al., 1984; Rodriguez et al., 2009; Rutjes et al., 2009; Chigor et al., 2014). Thus, the use of this ratio brought with it an unknown level of uncertainty to our analysis.

2.8.3. Dose–response model

The β-Poisson dose–response model was used to estimate the probability of rotavirus (Haas et al., 1999: $\alpha = 0.2531$ and $N_{50} = 6.17$) infection. The following model equations were used:

(a) β-Poisson dose–response model:

$$P_I(d) = 1 - \left[1 + \left(\frac{d}{N_{50}}\right)\left(2^{1/\alpha} - 1\right)\right]^{-\alpha}. \quad (1)$$

(b) Annual risk of infection:

$$P_{I(A)}(d) = 1 - [1 - P_I(d)]^n. \quad (2)$$

where $P_I(d)$ is the probability or risk of infection for an individual exposed to a single pathogen dose d through ingestion; α is a parameter that characterize dose–response relationships referred to as pathogen infectivity constants; d is the pathogen dose; N_{50} is the median infective dose or the number of pathogens required to cause an infection in 50% of the exposed population. $P_{I(A)}(d)$ is the estimated annual probability or risk of an infection from n exposures per year due to a single pathogen dose d.

2.8.4. Variability and uncertainty in the data

The uncertainty was introduced through the analysis of data distribution by sampling points. Monte Carlo simulations were made for 10,000 iterations using @Risk software 6.3 (Palisade Corporation, Newfield, New York). In each iteration, samples were taken from the data distribution function. The output of the analysis was the mean and standard error of the risk of infection as well as the frequency distribution of the probabilities of infection. The recalculated values were plotted in a box and whisker plot in order to show the extreme values and the range of middle values.

3. Results

3.1. Microbiological quality of Suquía River waters

Viable EV was detected in 22 (78.6%) and RV in 28 (100%) out of the 28 surface water samples collected. RV media concentration was 5.7×10^5 gc/L (Table 1). Neither EV detection rates nor RV concentration showed significant differences along the river course ($P \geq 0.2482$ and $P \geq 0.2000$, respectively) (Fig. 2A). High bacterial loads, far exceeding the limits considered as microbiologically acceptable for recreational water quality, were observed in the Suquía River (Table 1). The bacterial contamination was more pronounced as the river ran right through the city (Fig. 2A). Indeed only the monitoring station 1-Funnel showed acceptable levels of both indicator bacteria, with numbers statistically lower than the monitoring sites 3 to 7 ($P = 0.0286$), and the monitoring station 7-San José bridge evidenced the highest level of bacterial contamination, statistically higher than the stations 1 to 4 ($P = 0.0286$). Although acceptable bacteria loads were detected at the monitoring site 1-Funnel, both viable EV and RV genome were identified.

Table 1
Microbiological detection in the Suquía River from each sampling site considering both wet (WS) and dry (DS) seasons.

Variables	Sampling sites							Suquía River (Global results)
	1-Funnel	2-V. Warcalde	3-SA. ford	4-Z. bridge	5-C. bridge	6-SC. ford	7-SJ. bridge	
Mean viable EV detection rate (%) (95% CI)[a]	50 (±49.0)	100 (±0.0)	75 (±42.4)	100 (±0.0)	75 (±42.4)	75 (±42.4)	75 (±42.4)	**78.6** (±15.2)
WS	50 (±69.3)	100 (±0.0)	100 (±0.0)	100 (±0.0)	50 (±69.3)	100 (±0.0)	50 (±69.3)	78.6 (±21.5)
DS	50 (±69.3)	100 (±0.0)	50 (±69.3)	100 (±0.0)	100 (±0.0)	50 (±69.3)	100 (±0.0)	78.6 (±21.5)
P-value (WS vs. DS)	1.0000	1.0000	0.2482	1.0000	0.2482	0.2482	0.2482	1.0000
Mean RV detection rate (%) (95% CI)	100 (±0.0)	100 (±0.0)	100 (±0.0)	100 (±0.0)	100 (±0.0)	100 (±0.0)	100 (±0.0)	**100** (±0.0)
WS	100 (±0.0)	100 (±0.0)	100 (±0.0)	100 (±0.0)	100 (±0.0)	100 (±0.0)	100 (±0.0)	100 (±0.0)
DS	100 (±0.0)	100 (±0.0)	100 (±0.0)	100 (±0.0)	100 (±0.0)	100 (±0.0)	100 (±0.0)	100 (±0.0)
P-value (WS vs. DS)	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Mean RV concentration (gc/L) (range)	1.1×10^6 (2×10^5–6.1×10^6)	1×10^6 (6.4×10^4–4.5×10^6)	7.4×10^4 (1.9×10^3–2×10^6)	2×10^5 (5×10^3–6.1×10^6)	9.3×10^5 (6.4×10^4–3×10^6)	1.4×10^6 (6.4×10^4–8×10^6)	9.8×10^5 (3.9×10^3–8.6×10^6)	$\mathbf{5.7 \times 10^5}$ (1.9×10^3–8.6×10^6)
WS	4.8×10^5 (2×10^5–1.1×10^6)	5.4×10^5 (6.4×10^4–4.5×10^6)	8.9×10^4 (6.2×10^3–1.3×10^6)	2.4×10^5 (1.3×10^4–4.5×10^6)	4.4×10^5 (6.4×10^4–3×10^6)	6.3×10^5 (6.4×10^4–6.1×10^6)	1.8×10^5 (3.9×10^3–8.6×10^6)	3.2×10^5 (3.9×10^3–8.5×10^6)
DS	2.6×10^6 (1.1×10^6–6.1×10^6)	2×10^6 (2×10^6)	6.2×10^4 (1.9×10^3–2×10^6)	1.7×10^5 (5×10^3–6.1×10^6)	2×10^6 (1.3×10^6–3×10^6)	3×10^6 (1.1×10^6–8×10^6)	5.2×10^6 (4.5×10^6–6.1×10^6)	1×10^6 (1.9×10^3–7.9×10^6)
P-value (WS vs. DS)	0.6667	0.1818	0.0424	0.1818	0.0424	0.6667	0.1818	0.0424
Mean fecal coliform number (MPN/100 mL) (range)	$6.6 \times 10^{1*}$ (4×10^0–2.4×10^2)	4.2×10^3 (1.5×10^1–4.6×10^4)	3.9×10^3 (9.3×10^2–1.5×10^4)	1.9×10^4 (4.6×10^3–4.6×10^4)	1.4×10^5 (1.1×10^4–1.1×10^7)	1.5×10^5 (1.5×10^4–9.3×10^5)	1.9×10^6 (1.5×10^5–1.1×10^7)	$\mathbf{1.9 \times 10^4}$ (4×10^0–1.1×10^7)
WS	$1.5 \times 10^{2*}$ (9.3×10^1–2.4×10^2)	2.1×10^4 (9.3×10^3–4.6×10^4)	3.7×10^3 (9.3×10^2–1.5×10^4)	2.6×10^4 (1.5×10^4–4.6×10^4)	9.1×10^5 (7.5×10^4–1.1×10^7)	8.3×10^4 (1.5×10^4–4.6×10^5)	1.3×10^6 (1.5×10^5–1.1×10^7)	3.2×10^4 (9.3×10^1–1.1×10^7)
DS	$3 \times 10^{1*}$ (4×10^0–2.4×10^2)	8.3×10^2 (1.5×10^1–4.6×10^4)	4.1×10^3 (1.1×10^3–1.5×10^4)	1.4×10^4 (4.6×10^3–4.6×10^4)	2.2×10^4 (1.1×10^4–4.6×10^4)	3×10^5 (9.3×10^4–9.3×10^5)	3×10^6 (9.3×10^5–9.3×10^6)	1.1×10^4 (4×10^0–9.3×10^6)
P-value (WS vs. DS)	>0.9999	>0.9999	>0.9999	>0.9999	0.3333	0.6667	>0.9999	0.6116
Mean total coliform number (MPN/100 mL) (range)	$8.3 \times 10^{1*}$ (4×10^0–2.4×10^2)	6.2×10^3 (1.5×10^1–4.6×10^4)	7.2×10^3 (1.1×10^3–1.5×10^4)	2.6×10^4 (4.6×10^3–4.6×10^4)	1.7×10^5 (2.1×10^4–1.1×10^7)	2.3×10^5 (4.6×10^4–9.3×10^5)	8.9×10^6 (4.6×10^5–1.1×10^8)	$\mathbf{3.2 \times 10^4}$ (4×10^0–1.1×10^8)
WS	$2.4 \times 10^{2*}$ (2.4×10^2)	4.6×10^4 (4.6×10^4)	1.3×10^4 (1.1×10^4–1.5×10^4)	4.6×10^4 (4.6×10^4)	9.1×10^5 (7.5×10^4–1.1×10^7)	1.4×10^5 (4.6×10^4–4.6×10^5)	2.2×10^6 (4.6×10^5–1.1×10^7)	5×10^4 (2.4×10^2–1.1×10^7)
DS	$3 \times 10^{1*}$ (4×10^0–2.4×10^2)	$8.3 \times 10^{2*}$ (1.5×10^1–4.6×10^4)	$4.1 \times 10^{3*}$ (1.1×10^3–1.5×10^4)	1.4×10^4 (4.6×10^3–4.6×10^4)	3.1×10^4 (2.1×10^4–4.6×10^4)	3.7×10^5 (1.5×10^5–9.3×10^5)	3.5×10^7 (1.1×10^7–1.1×10^8)	1.8×10^4 (4×10^0–1.1×10^8)
P-value (WS vs. DS)	>0.9999	>0.9999	>0.9999	>0.9999	0.3333	0.6667	0.6667	0.4287

Bold in the table depicts global results of the River along the whole study period.
[a] 95% CI = 95% confidence interval.
* Acceptable coliform numbers (≤200 MPN/100 mL fecal coliforms; ≤5000 MPN/100 mL total coliforms).

No statistically significant differences were observed in viable EV and rotavirus detection rates and concentration, nor in fecal and total coliform media numbers in relationship with WS and DS ($P > 0.05$) (Table 1).

The association between monitoring stations and sampling seasons was analyzed for the microbiological variables (Table 1). No association was observed for viable EV detection: a few monitoring stations (i.e. 3-San Antonio ford and 6-Sargento Cabral bridge) revealed higher EV detection rates in the WS than in the DS, meanwhile other stations (i.e. 5-Centenario bridge and 7-San José bridge) showed the opposite. In relation with RV concentration, although not statistically significant, higher concentrations were observed in almost all monitoring sites in the DS as compared with the WS ($P \geq 0.6667$).

3.2. Microbiological quality of Xanaes River waters

Viable EV was detected in 42 (87.5%) and RV in 9 (18.7%) out of the 48 surface water samples collected. RV media concentration was 8.5×10^0 gc/L (range 0–3×10^6) and neither EV detection rate nor RV concentration showed significant differences along the river course ($P \geq 0.1213$ and $P \geq 0.2298$, respectively) (Fig. 2B). The highest RV concentrations were observed in the monitoring station 1-Farm, while the

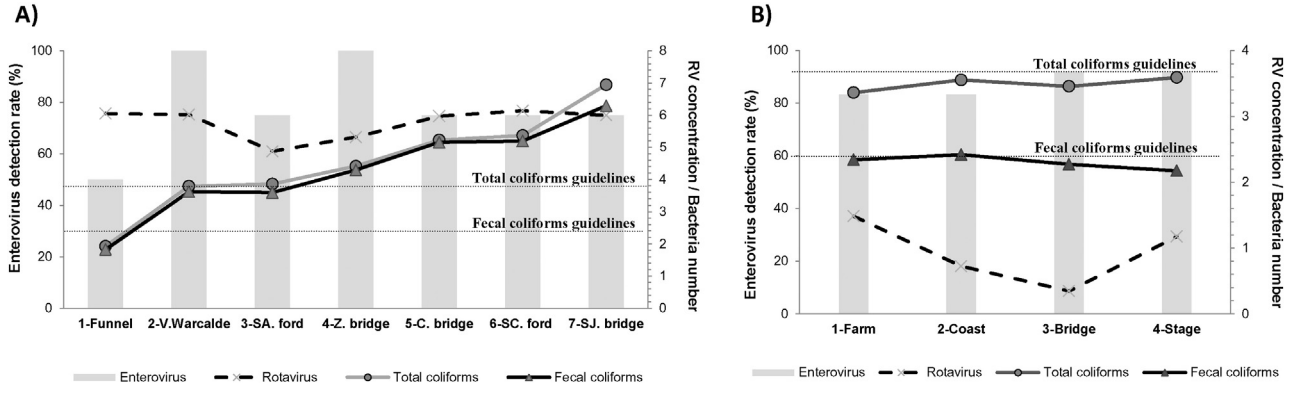

Fig. 2. Enteric viruses (viable EV and RV genome) and indicator bacteria (fecal and total coliforms) at each sampling site of the (A) Suquía River and (B) Xanaes River. Bacteria loads are depicted as $\log_{10}$ MPN/100 mL and rotavirus concentration as $\log_{10}$ gc/L. Bacteria guidelines are: fecal coliforms ≤200 MPN/100 mL (≤$\log_{10}$ 2.3) and total coliforms ≤5000 MPN/100 mL (≤$\log_{10}$ 3.7).

station 3-Bridge revealed the lowest RV concentrations, but the differences were not significant ($P = 0.2298$) (Fig. 2B). Fecal coliform bacteria were detected in 42 samples (87.5%) at a media number of 2×10^2 MPN/100 mL, while total coliform bacteria were detected at the four monitoring stations throughout the study period, at a media number of 3.1×10^3 MPN/100 mL. The bacterial contamination did not show significant differences along the river course ($P \geq 0.6222$) (Fig. 2B).

No significant differences in EV detection rate nor in indicator fecal bacteria were observed in relationship with WS and DS ($P \geq 0.3827$ and $P = 0.5285$, respectively). However, statistically significant differences were observed in RV concentration and total coliform load between WS and DS. RV was more frequently detected in the WS than in the DS and also at significantly higher concentrations ($P = 0.0424$). Total coliforms exhibited significantly higher numbers in the WS than in the DS ($P = 0.0063$) (Table 2).

The association between monitoring stations and sampling seasons was analyzed for the microbiological variables (Table 2). No association was observed for viable EV detection, some monitoring stations revealed higher EV detection rates in the DS (i.e. 1-Farm and 4-Stage) and others in the WS (3-Bridge). Regarding RV detection rate and media concentration, differences among the seasons were noted in monitoring stations 1-Farm and 4-Stage. However, the differences in RV concentration were not statistically significant ($P \geq 0.1818$). Bacterial coliforms showed a trend of higher numbers at each monitoring site in the WS, however this association was not statistically significant ($P \geq 0.0736$). Viable EV and RV genome were detected at monitoring sites that achieved acceptable bacterial load standards, but also RV genome was undetectable in water samples with bacteria presence.

3.3. Molecular characterization of RV strains

Molecular characterization of the 28 RV positive water samples detected in the Suquía River showed that G1 and P[8] were the most common genotypes detected in the environmental waters (G1 21/28, 75%; P[8] 26/28, 92.8%), followed by G3 (14/28, 50%), G9 (12/28, 42.8%) and P[4] (2/28, 7.2%). Other G genotypes were also detected at low frequency rates, like G4 (7/28, 25%), G2 (2/28, 7.2%) and G8 (1/28, 3.6%).

By the other hand, out of the 9 RV positive water samples from the Xanaes River G1, G2 and G3 were the most common G types detected (3/9 each, 33.3%) and also G9 genotype was detected in one sample (1/9, 11.1%). P types could only be determined for 3 of the RV-positive samples. P[4] genotype was detected in 2 samples (66.7%) and P[10] in one (33.3%).

3.4. Modeling the risk of infection

The probability of infection from waterborne rotavirus was estimated for each of the exposure source points over the Suquía and Xanaes Rivers (Fig. 4). The Suquía River revealed an extremely high health risk of RV infection that was observed along the whole length of the river, including monitoring sites with acceptable bacterial numbers (Fig. 3A–B). On the other side, the Xanaes River showed a significant lower mean risk of infection than the Suquía River for an individual exposure (Fig. 3A) as well as for annual exposure (0.7 vs 1). The lowest health risk of RV infection was observed at the monitoring site 3-Bridge of the Xanaes River (median risk 0.06), but the difference with the other monitoring stations was not statistically significant (Fig. 3C).

4. Discussion

Surface waters may be directly or indirectly contaminated with human enteric viruses by (un)treated sewage or washoff of animal manure (Lodder and de Roda Husman, 2005). Several communicable diseases of concern are commonly associated with recreational swimming, wading and use of contaminated bathing locations, especially associated with the gastrointestinal tract.

In the present study microbiological monitoring of two large rivers of the province of Córdoba, Argentina, was carried out. The results

Table 2
Microbiological detection in the Xanaes River from each sampling site considering both wet (WS) and dry (DS) seasons.

Variables	Sampling site				Xanaes River (Global results)
	1-Farm	2-Coast	3-Bridge	4-Stage	
Mean viable EV detection rate (%) (95% CI)[a]	83.3 (±21.1)	83.3 (±21.1)	91.7 (±15.6)	91.7 (±15.6)	**87.5** (±9.4)
WS	66.7 (±37.7)	83.3 (±29.8)	100 (±0.0)	83.3 (±29.8)	83.3 (±14.9)
DS	100 (±0.0)	83.3 (±29.8)	83.3 (±29.8)	100 (±0.0)	91.7 (±11.0)
P-value (WS vs. DS)	0.1213	1.0000	0.2963	0.2963	0.3827
Mean RV detection rate (%) (95% CI)	25 (±24.5)	16.7 (±21.1)	8.3 (±15.6)	25 (±24.5)	**18.7** (±11.0)
WS	50 (±40.0)	16.7 (±29.8)	0 (±0.0)	50 (±40.0)	29.2 (±18.2)
DS	0 (±0.0)	16.7 (±29.8)	16.7 (±29.8)	0 (±0.0)	8.3 (±11.0)
P-value (WS vs. DS)	0.0455	1.0000	0.2963	0.0455	0.0644
Mean RV concentration (gc/L) (range)	3.1×10^1 (0–3×10^6)	5.2×10^0 (0–6.4×10^4)	2.2×10^0 (0–1.5×10^4)	1.5×10^1 (0–6.4×10^4)	**8.5×10^0** (0–3×10^6)
WS	9.3×10^2 (0–3×10^6)	6.3×10^0 (0–6.4×10^4)	0 (0)	2.2×10^2 (0–6.4×10^4)	3.4×10^1 (0–3×10^6)
DS	0 (0)	4.4×10^0 (0–7.4×10^3)	5×10^0 (0–1.5×10^4)	0 (0)	2.2×10^0 (0–1.5×10^4)
P-value (WS vs. DS)	0.1818	>0.9999	>0.9999	0.1818	0.0424
Mean fecal coliform number (MPN/100 mL) (range)	2.2×10^2 (0–2.3×10^3)	2.6×10^2 (0–9.3×10^3)	$1.9 \times 10^{2*}$ (0–2.4×10^4)	$1.5 \times 10^{2*}$ (0–2.8×10^3)	**$2 \times 10^{2*}$** (0–2.4×10^4)
WS	2.7×10^2 (4×10^1–2.3×10^3)	2.6×10^2 (4×10^1–4.3×10^3)	$1.1 \times 10^{2*}$ (0–9×10^2)	$1.2 \times 10^{2*}$ (0–9×10^2)	$1.8 \times 10^{2*}$ (0–4.3×10^3)
DS	$1.8 \times 10^{2*}$ (0–2.1×10^3)	2.6×10^2 (0–9.3×10^3)	3×10^2 (0–2.4×10^4)	$1.9 \times 10^{2*}$ (0–2.8×10^3)	2.2×10^2 (0–2.4×10^4)
P-value (WS vs. DS)	0.8896	0.7587	0.5779	0.6883	0.5285
Mean total coliform number (MPN/100 mL) (range)	$2.3 \times 10^{3*}$ (4×10^1–4.8×10^4)	$3.5 \times 10^{3*}$ (4×10^1–1.5×10^5)	$2.9 \times 10^{3*}$ (4×10^1–4.8×10^4)	$3.9 \times 10^{3*}$ (4×10^1–1.1×10^5)	**$3.1 \times 10^{3*}$** (4×10^1–1.5×10^5)
WS	5.1×10^3 (9×10^2–4.8×10^4)	8.9×10^3 (7×10^2–1.5×10^5)	$4.6 \times 10^{3*}$ (7×10^2–4.8×10^4)	1.3×10^4 (7×10^2–1.1×10^5)	7.2×10^3 (7.1×10^2–1.5×10^5)
DS	$1 \times 10^{3*}$ (4×10^1–9.3×10^3)	$1.4 \times 10^{3*}$ (4×10^1–2.4×10^4)	$1.8 \times 10^{3*}$ (4×10^1–2.4×10^4)	$1.1 \times 10^{3*}$ (4×10^1–4.8×10^4)	$1.3 \times 10^{3*}$ (4×10^1–4.8×10^4)
P-value (WS vs. DS)	0.1688	0.2424	0.4459	0.0736	0.0063

Bold in the table depicts global results of the River along the whole study period.
[a] 95% CI = 95% confidence interval.
* Acceptable coliform numbers (≤200 MPN/100 mL fecal coliforms; ≤5000 MPN/100 mL total coliforms).

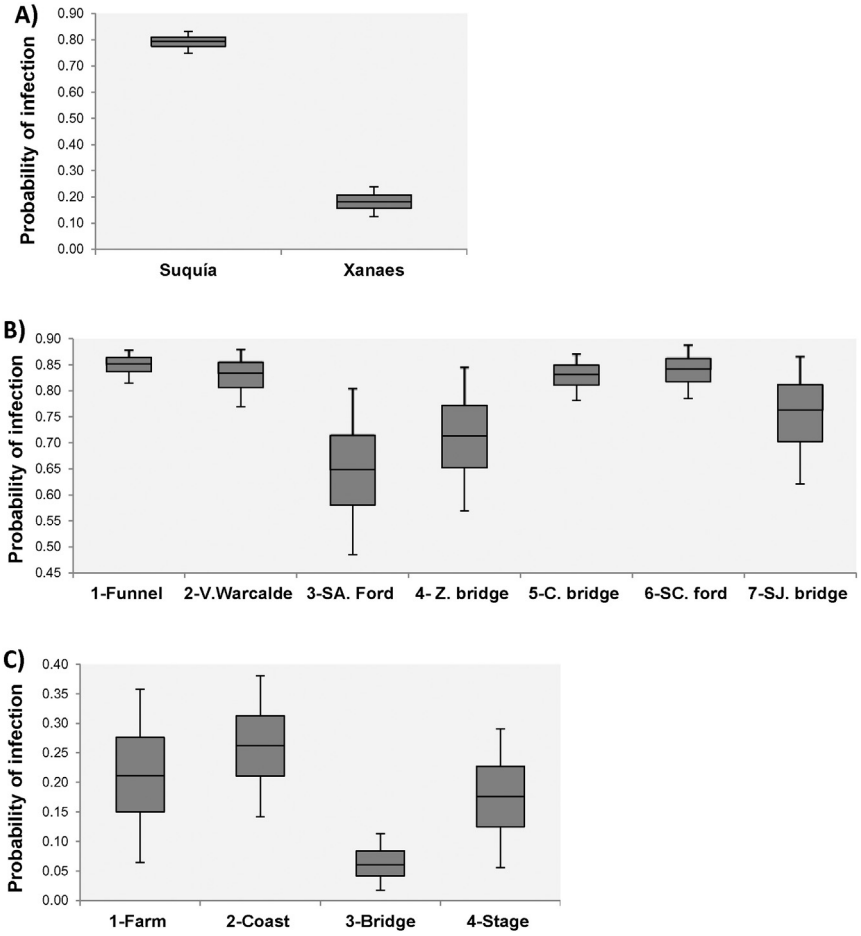

Fig. 3. Daily estimated risk of waterborne rotavirus infection associated with individuals exposed to the Suquía (A and B) and Xanaes River (A and C) waters. Boxplots represent 25th, 50th and 75th percentiles (bottom, middle and top edge of box) and outliers represent 5th and 95th percentiles. The probability of infection is established in the 0–1 range.

revealed the anthropogenic influence on these streams, since high fecal contamination was observed in the Suquía River, with coliform bacteria numbers that far exceed the acceptable limits for microbiological quality of recreational waters, meanwhile the Xanaes River exhibited a lower level of bacterial contamination, with a media number of fecal and total coliforms usually within the guideline limits. The difference in bacterial pollution of the rivers of Córdoba would be in relation to the population size of the cities that sit on the banks of both rivers.

The Suquía River showed acceptable numbers of thermotolerant and total coliform bacteria only at the site 1-Funnel but then, as the river passes through the city, an important deterioration in bacteriological water quality was noted. The reduction of water quality was particularly noted by an increase of bacteria in the monitoring station 5-Centenario bridge, where the river receives the impact of the Cañada Stream (which enters directly into the river after crossing the south area of the city). This rise in bacterial load persisted in points 6-Sargento Cabral ford and 7-San José bridge, being this situation aggravated in the monitoring site 7, where the river receives the discharge of the treated sewage from the Municipal Plant of Bajo Grande. Although the deterioration of bacterial water quality along the river course, enteric viruses did not revealed significant differences among the monitoring stations. Also, there was no significant seasonal variation for the measured microbiological variables in the different monitoring sites, which would reflect a permanent contribution of fecal contamination to the Suquía River. This result agrees with the circulation of RV all year round in the population of Córdoba, Argentina (Barril et al., 2015). In addition to this, it is noteworthy that some monitoring sites achieved acceptable bacterial load standards, but showed viable EV detection rates and RV concentrations similar to those obtained in monitoring sites which showed bacterial loads that exceed the guidelines for recreational water quality. This highlights the lack of correlation between bacterial and viral load and reinforces the need to incorporate the viruses as markers of fecal contamination.

The Xanaes River revealed a lower impact of microbiological contamination, with coliform loads that almost never exceeded the limits considered acceptable for recreational water quality, and low RV detection rates. However, viable EV was frequently detected in these waters. The difference between RV and EV detection rates could be because EV includes a large group of viruses and infects and causes illness at all age groups, which may lead to a higher circulation of the virus in the population, meanwhile RV is excreted primarily by primoinfected infants. Although this, significant seasonal variation was observed for the variable RV, with significantly higher concentrations in the WS than in the DS ($P = 0.0424$). This result is important because in the WS, especially in the summer, the river is crowded and the water is used for recreational purposes, increasing the risk of RV transmission. Interestingly, the monitoring site 1-Farm showed the highest RV concentrations, although the difference in concentration with the other monitoring sites was not statistically significant. It is noteworthy that close to this site is settled a cattle and equines breeding farm. Thus, it will be of interest to phylogenetically analyze the viral isolates in order to determine whether the detected viral strains correspond to animal or human species. Although rotaviruses infect particular species preferentially for which they have been defined as the homologous strains, heterologous rotavirus infections occur in both natural and experimental circumstances (Matthijnssens et al., 2006). Moreover, global epidemiologic surveys have identified G3 and G14 as the most common genotypes associated with diarrhea in horses but also rare genotypes which are commonly

associated with humans, like G1 and G8, were occasionally reported in horses (Isa et al., 1996; Garaicoechea et al., 2011; Gulati et al., 2007; Kobayashi et al., 2007). Similarly, the cattle is regarded as a likely reservoir for RV strains with G1–G3 and G8 genotypes, which also frequently infect humans (Kobayashi et al., 2007; Papp et al., 2013). Thus, the cattle and equine farm settled at the monitoring site 1-Farm may be the plausible source and likely reservoir for human rotavirus infections.

In the present study, the most prevalent RV VP7-genotype detected in the recreational rivers was G1, which correlated with the most common genomic variant associated with human gastrointestinal infections in our community (Barril et al., 2006). G1 plays a major role in infantile diarrhea worldwide and is frequently isolated from sewers in Córdoba and also in other geographical regions (Barril et al., 2010; Kamel et al., 2010; Kiulia et al., 2010; Kargar et al., 2013; Ruggeri et al., 2015). On the other side, the most common VP4-genotypes detected were P[8], in the Suquía River, and P[4], in the Xanaes; P genotypes which have frequently been associated with gastroenteritis cases in Córdoba (Barril, 2011). The molecular characterization of the RV strains revealed that the genotypes detected in these recreational waters match with the genotypes reported in clinical cases and sewage from Córdoba, which allows inferring that the Suquía and Xanaes Rivers are incorporated into the natural circulation of the virus and are potential water sources of viral infection. However, it was surprising that P[8] was not detected in the Xanaes River, despite several attempts to typify this genotype, and also the amplification of the VP4 gene in these environmental waters yielded poor results. Probably, there were low virus titers in the Xanaes waters which led to low rates of P typing and failure to detect the genotype P[8]. Moreover, it must be pointed out that the VP4 amplification has been previously described to be less sensitive than the VP6 or VP7 amplification (van Zyl et al., 2006; Vieira et al., 2012).

Many sources of uncertainty may arise in inputs to a risk assessment. In the assessment of exposure to recreational water, reliance is place on water consumption. In the present paper it was considered a minimum volume of 10 mL. In this way, the viral doses could be underestimated and so on, the probability of viral infection. Anyway, the Suquía River showed a high risk of RV infection along the whole length of the river, including monitoring sites with acceptable bacterial numbers. The Xanaes River showed statistically significant lower risk of RV infection for an individual exposure (Xanaes $P_I(d)$: 0.1822; Suquía $P_I(d)$: 0.7947), however both recreational rivers revealed a high annual probability of RV infection at all monitoring sites (median Xanaes $P_{I(A)}(d)$: 0.7010; median Suquía $P_{I(A)}(d)$: 1), which reveals that both rivers represent a public health hazard.

In Argentina there is a lack of complete information and data about the spectrum of human health outcome after rotavirus infection (ie. distribution of the population after rotavirus infection that would result asymptomatic, symptomatic that requires medical assistance or die). This impedes to reach the analysis of the risk characterization in order to estimate the magnitude of the public health problem of rotavirus infection. In addition, in the year 2015 the monovalent rotavirus vaccine (G1 [P8]) was introduced in the Argentine National Immunization Program. This vaccine has an estimated efficiency of 94.5% against severe disease and death, but it does not prevent symptomatic infections in secondary contacts with the virus nor viral transmission to susceptible hosts. Therefore, it is important to identify recreational waters as sources of RV infection.

Viable EV and RV genome were detected in the local rivers even in areas showing low levels of bacterial contamination. These data corroborate with previous studies that have shown no association between bacterial indicators and viral contamination and suggests the prolonged persistence of enteric viruses in the environment (Bosch, 1998; Noble and Fuhrman, 2001; Skraber et al., 2004; Pusch et al., 2005; Miagostovich et al., 2008; Espinosa et al., 2009). Because recreational waters are not subjected to any treatment and are considered suitable for swimming at certain bacterial levels, the presence of viruses reveals a potential burden to public health that cannot be disregarded. Thus, the analysis of enteric viruses seems to be a more reliable indicator for environmental monitoring of fecal pollution than bacterial indicators and should also be included to determine microbiological water quality.

The high frequency of detection of microbiological agents in the recreational waters of the Suquía River is consistent with the prevalence of fecal bacteria and enteric viruses in various aqueous matrices of the region and the world (Hamza et al., 2009; Rodriguez-Díaz et al., 2009; Fongaro et al., 2012; Assis et al., 2014). In the same way, the frequency of detection of microbiological pollutants in the Xanaes River is in line with reports of surface waters less impacted by human activities (Vecchia et al., 2012; Vieira et al., 2012; Kiulia et al., 2010; He et al., 2012). The viral contamination detected in the urban rivers of Córdoba provides an advice of the probability of rotavirus infection for the population exposed with to these polluted recreational waters. A combination of sanitation and hygiene intervention is required to minimize the risk of infection constituted by waterborne rotavirus in the identified sources of contamination.

5. Conclusions

The detection of viable EV and RV genome is a frequent event in the recreational rivers of Córdoba, Argentina, suggesting that people exposed to these surface waters are at risk for enteric viruses' waterborne infection. Enteric viruses were identified in the urban rivers in the absence of bacterial loads that exceed acceptable guideline values. Moreover, QMRA revealed extremely high risk of RV infection in the Suquía River, at sampling points with acceptable and non-acceptable bacteria numbers. Although the Xanaes River showed significantly lower health risk of RV infection than the Suquía, it represented a public health hazard. Thus, viral monitoring should be included to determine microbiological water quality. The findings of this study provide the first data of viral risk assessment in Argentina. The detection of enteric viruses in water sources will facilitate the provision of appropriate advice to public and responsible authorities regarding the use and treatment of water, in order to prevent waterborne viral infections outbreaks.

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