

Evidence of *Helicobacter* spp. in freshwaters from Roraima Tepui, Guayana Shield, South America

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Abstract *Helicobacter* presence and viability in waters is not well characterized. The identification of natural reservoirs and infection sources may provide novel insights into its waterborne transmission. The goal of this study was to investigate the occurrence of *Helicobacter* spp. in natural freshwaters from Roraima Tepui, a little studied and unique ecosystem of the Guayana Shield. Freshwaters collected from two localities at Roraima Tepui were cultured in HP selective broth and agar for *Helicobacter pylori* and analysed by fluorescent in situ hybridization (FISH), specific PCR assays, 16S rRNA gene sequencing and phylogenetic analysis. The presence of other bacteria in freshwater enrichments was determined using clone

library sequencing of the 16S rRNA gene and phylogenetic inferences. *Helicobacter* spp. were detected by semi-nested PCR and FISH in freshwater enrichments from both sites. Coccoid viable but nonculturable (VBNC) cells were evidenced using 16S rRNA gene *Helicobacter* species and *H. pylori*-specific probes. Partial 16S rRNA gene sequences of two HP enrichments showed high similarity to *H. pylori* and *Helicobacter nemestrinae* (99–100 %). Other bacteria such as *Serratia*, *Aquitalea*, *Chromobacterium*, *Mycobacterium*, *Acinetobacter*, *Curvibacter* and *Dysgonomonas* were also detected using complete 16S rRNA gene sequences, with *Serratia*, *Aquitalea* and *Chromobacterium* the most common genera (40.9, 18.2 and 15.2 %, respectively). This is the first time that *Helicobacter* spp. have been reported in freshwaters of a tepui ecosystem. Our results contribute to the current knowledge of these bacteria in the aquatic environment and expand their known/potential sites outside the human host.

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Introduction

The genus *Helicobacter* (Goodwin et al. 1989) has been expanding since the discovery of *Helicobacter pylori*, the first bacterium known to colonize the

gastric environment (Marshall and Warren 1984) and the causative agent of gastritis, peptic ulcer disease and gastric carcinoma (van Amsterdam et al. 2006). Over half of the world's population are carriers of *H. pylori*, and it represents one of the most common bacterial infections. Studies from developing countries have shown a high prevalence of *H. pylori* infection (70–90 % of population), mainly attributed to low socioeconomic status, age, and the poor management of drinking water (García et al. 2014). The exact mode of *H. pylori* transmission in humans remains unclear. Several authors have suggested that fecal-oral transmission occurs through drinking water supplies, groundwater, recreational waters, freshwaters streams, and estuary and marine waters contaminated by sewage (Mazari-Hiriart et al. 2001; Moreno et al. 2003; Cellini et al. 2004; Carbone et al. 2005; Voytek et al. 2005; Fernández et al. 2007; Twing et al. 2011; Cunachi et al. 2015; Santiago et al. 2015). Although many studies have found *Helicobacter* DNA in the environment, reports of live cells are extremely rare and highly inconclusive (Percival and Thomas 2009). Water may also act as a reservoir for the bacteria where they can remain for periods of time in a viable but nonculturable state (VBNC), before being ingested through drinking water (Adams et al. 2003; Bellack et al. 2006; Assadi et al. 2015). As *H. pylori* is one of the EPA drinking water contaminant candidates (USEPA 2009), the detection of reservoirs and sources of infection is important for the understanding of this bacterial presence and persistence in natural environments.

Tepuis (or Tepuys) are table-top mountains that rise from 400 to 3000 m above the surrounding savannas or forests in the Guayana highlands in northern South America (Gansser 1954; Santos et al. 2003). Tepuis have been characterized as biological islands due to their shape and isolation from each other (Berry et al. 1995). Roraima is the highest (2700 m), best-known tepui and the only one open for tourism in the Canaima National Park, Venezuela, in the common border with Brazil and Guyana (Aubrecht et al. 2011). The tepui is located in one of the world largest tropical rainforest (Berry et al. 1995; Aubrecht et al. 2011). As Roraima Tepui is a preserved habitat of ecological relevance, the goal of this study was to determine whether *Helicobacter* was present in the freshwaters of this tepui ecosystem using a combination of enrichment cultures, fluorescent in situ hybridization (FISH),

specific PCR assays, 16S rRNA gene sequencing and phylogenetic analysis.

Materials and methods

Site description and sample collection

The study was conducted on the summit of Roraima Tepui, Canaima National Park, at the south eastern corner of Bolivar State in southern Venezuela. The Tepui is formed by a layer of sandstone that lies over an igneous basement (the Guayana Shield) and presents particular topographical, lithological, hydrological and structural features that have promoted the development of an extensive groundwater system, which probably might emerged through localized upwelling on the base of tepui outer wall (600–700 m below the level of the summit) (Galan et al. 2004). The regional climate is semi-humid/tropical with high rainfalls (5700 mm/y), being the rainiest months between May and September, and a relatively dry season lasting the first three months of the year (Nimer 1989).

Sampling was authorized with permissions issued by the Environmental Ministry of Venezuela. In October 2007, freshwater samples from two sites were collected at the south central part of the Roraima summit, relatively close to the great wall or south escarpment of the plateau (5°11'52.17"N 60°45'12.45"W): Sampling site 1 named in this study as Laguna de la Puerta (LP), the entrance lagoon of Ojos de Cristal Cave or Roraima Sur Cave System (the world longest cave in quartzite), and sampling site 2 or Los Jacuzzis (LJ), a freshwater well used as a bathing place or pool for visitors (Fig. 1). Both sites are located at the end of a surface sinkhole on Roraima Tepui that takes a permanent stream, which drains through surface vegetation. The water flows are product of surface drainage from precipitation and underground movement (Galan et al. 2004). The adverse environmental conditions and remote geographical location of the tepui difficult the access to the sampling sites and the collection of a large volume and number of water samples to be transported to the laboratory.

A volume of 50 mL of freshwater samples were collected with sterile plastic containers (Fisher brand®, USA) in duplicate from each locality

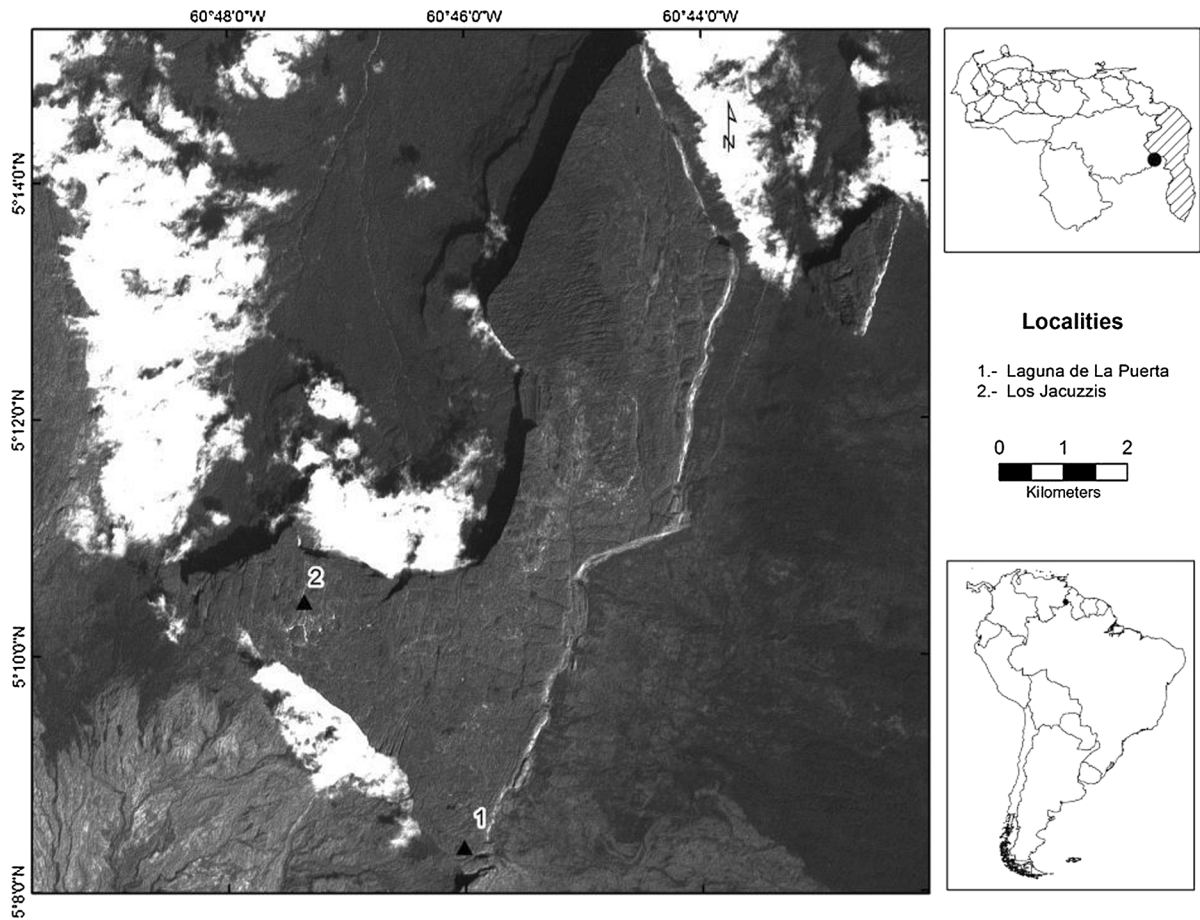


Fig. 1 Location map of Roraima Tepui and the sampling sites tested. Images were provided by the SPOT satellite, TS-5 (LPAIS, Fundación Instituto de Ingeniería para Investigación y

Desarrollo Tecnológico, Caracas, Venezuela) and processed with ArcGIS 10.0 Software (Stanford University)

($n = 4$), about 10 cm below the surface. Particulates in the water were allowed to settle before the water was tested for pH using an Accumet AP61 portable pH meter (Fisher Scientific, USA). Temperature was measured using a RH300 Pshycrometer (Extech instruments, USA).

Bacterial culturing

Bacterial enrichments were established from 1 mL of collected water samples and used to inoculate serum bottles with 9 mL of HP broth containing a mixture of nutritive components (special peptone, beef extract, yeast extract, calf serum with iron, and NaCl), antibiotics (vancomycin, trimethoprim, cefsulodin, amphotericin B, and polymixin B), and a colour indicator system for urease production (urea, phenol

red, and HCl) in the presence of a microaerobic gas mixture (5 % CO₂, 10 % H₂, 85 % N₂; Praxair, Inc., Caracas, Venezuela) (Degnan et al. 2003). These enrichments were incubated at 37 °C for 3–5 days in the laboratory. Four freshwater enrichments were obtained from Laguna de la Puerta (LP1 and LP2), and Los Jacuzzis (LJ1 and LJ2) and used for *Helicobacter* detection by FISH and PCR assays. An aliquot (50 µL) from each enrichment was streaked on HP agar plates (Degnan et al. 2003) and incubated at 37 °C under microaerobic conditions as described above to attempt *H. pylori* isolation. Eleven urease positive colonies were analysed by biochemical assays and API ATB/Plus system (BioMérieux, France) at Centro Venezolano de Colecciones de Microorganismos (CVCM, Venezuela), cloning and complete 16S rRNA gene sequencing.

Fluorescence in situ hybridization (FISH)

FISH assays were performed by triplicate according to Trebesius et al. (2000) and Contreras et al. (2012) on freshwater enrichments (LP1, LP2, LJ1 and LJ2) obtained in HP broth and clinical strains: *H. pylori* isolated from a Venezuelan patient with gastritis as positive control and *Shigella* sp. (ATCC11126) as negative control. Four fluorescent oligonucleotide probes targeting the bacterial 16S rRNA gene were used in this study: two *Helicobacter* genus-specific probes, HEL274 (5'-GGCCGATACCCGTCATWGCT-3') and HEL717 (5'-AGGTCGCCTTCGCAATGAGTA-3'), both Cy3-5' labeled (Chan et al. 2005), an *H. pylori*-specific probe Hpy-1 (5'-CACACCTGACTGACTATCCCG-3'; Cy3-5' labeled), and a probe for the universal bacteria domain EUB338 (5'-GCTGCCTCCCGT-3'; Alexa Fluor 488 N-5' labeled) as a positive control to simultaneously visualize the bacteria (Trebesius et al. 2000; Contreras et al. 2012). The hybridization was performed at 54 °C for 90 min and stringent washing at 56 °C for 10 min (Contreras et al. 2012). The slides were air-dried and mounted with anti-fading mounting medium (Immuno-Mount, Thermo Fisher Scientific Inc., USA). The bacteria were visualized with a Nikon Eclipse E600 epifluorescence microscope, which was equipped with filter blocks: G2A for Cy3 (excitation filter: 510–560 nm; beam splitter: 565 nm; barrier filter: 580 nm) and B2A for Alexa Fluor 488 N (excitation filter: 450–490 nm; beam splitter: 500 nm; barrier filter: 515 nm). A Nikon Coolpix 8700 camera digitally acquired the images. Two photographs of the same field were taken with G2A and B2A. The ImageJ 1.34 s (<http://rsb.info.nih.gov/Ij>) public domain Java image processing package (National Institutes of Health) was used for produces merged images. Positive hybridizations for *Helicobacter* species were visible as orange labeled-cells, which hybridized with the HEL274 and HEL717 (red) and EUB338 (green) probes, and for *H. pylori* as red labeled-cells when hybridized with the Hpy-1 (red) probe. Representative images were chosen from about 10 random positions for each sample.

Helicobacter PCR assays and 16S rRNA partial gene sequence analysis

Aliquots of 1 mL from the freshwater enrichments (LP1, LP2, LJ1 and LJ2) were centrifuged at

16,000×g for 1 min. The supernatant was discarded and the DNA was then purified from the pellet using the QIAamp DNA Mini Kit (QIAGEN, USA). *Helicobacteriaceae* and *Helicobacter* DNA was amplified using family (Bohr et al. 2002) and genus-specific (Germani et al. 1997) PCR assays for the 16S rRNA gene, respectively. *Helicobacteriaceae* DNA was amplified by nested PCR; the first with two external universal primers: 8F and 1525R (Contreras et al. 2007), and the second PCR using two internal primers *Helicobacteriaceae*-specific: BohrF (C97-20) and BohrR (H3A-20), as previously described by Bohr et al. (2002). The initial PCR reaction used a Ready-To-Go PureTaq PCR kit (Amersham Biosciences, USA) and contained 4 µL of extracted DNA and 3 µL of primers (5 µmol L⁻¹) and sterile distilled water to 25 µL. The reaction mixture of the second step (25 µL) contained 3 µL of *Helicobacteriaceae*-specific primers (5 µmol L⁻¹) and 2 µL of PCR final product from the first step.

Helicobacter DNA was amplified by semi-nested PCR; the first PCR reaction used the *Helicobacter* genus-specific (HeliF) forward primer and a reverse primer targeting the Epsilon branch of *Proteobacteria* (EpsilR). The second PCR reaction used the *Helicobacter* genus-specific internal primers HeliF and HeliR (Germani et al. 1997). The PCR reactions used a Ready-To-Go PureTaq PCR kit and contained 1 µL of extracted DNA and 3 µL of the HeliF/EpsilR primers (5 µmol L⁻¹) and sterile distilled water to 25 µL. The reaction mixture of the second step (25 µL) contained 3 µL of the HeliF/HeliR primers (5 µmol L⁻¹) and 2 µL of the first PCR product. For both nested and semi-nested PCR, the positive control of the first step was prepared by adding 1 µL of *H. pylori* DNA, the product of which served as the positive control in the subsequent PCR reaction.

The presence of *H. pylori* DNA was determined using specific PCR assays targeting the *glmM* (Kansau et al. 1996), *ureaA* (Peek et al. 1995) and *cagA* (Rugge et al. 1999) genes. The PCR reactions were performed using the Ready-To-Go PureTaq PCR kit on a GeneAMP PCR System 9700 (Applied Biosystems, USA). Each reaction contained PCR reagents plus 4–6 µL of the extracted DNA, 3 µL of primer mix (5 mol L⁻¹), and sterile distilled water for a final volume of 25 µL. A volume of 1 µL from the positive control was used as a template for each PCR assay. The negative control was a no-template PCR reaction.

Two *Helicobacter*-specific fragments of the 16S rRNA gene (LP1 and LJ1) were amplified and purified for sequencing using the CONCERT Rapid PCR Purification System kit (GibcoBRL, USA), according to the manufacturer recommendations. Purified amplicons were sequenced at the CeSAAN facility (IVIC, Venezuela) with an ABI PRISM 3130xl Sequencer (Applied Biosystems, USA). The 16S rRNA gene sequences (~292–304 bp) were deposited in the GenBank (Table 1).

Helicobacter sequences together with closest GenBank matches were aligned using the SINA software (Pruesse et al. 2012). The phylogenetic tree was constructed using neighbor-joining method and the Jukes-Cantor model provided in Molecular Evolutionary Genetics Analysis 2.1 software (MEGA, version 5.0) (Tamura et al. 2011), and the stability of grouping was estimated by bootstrap analysis (10,000 replicates).

Cloning, 16S rRNA gene sequencing and phylogenetic inferences

Genomic DNA from eleven urease positive colonies grown on HP agar plates under microaerobic conditions (five colonies obtained from LP and six from LJ) was extracted by ZR Fungal/Bacterial DNA MiniPrep (Zymo Research Corporation, USA). To amplify the 16S rRNA complete gene sequence, a PCR reaction of 40 µL containing 10µL 2X Taq Master Mix (New England Biolabs, USA), ~100 mM of each bacterial primer: 8F (5'-AGAGTTTGATCMTGG CTCAG-3') and 1391R (5'-GACGGGCGGTGWGTRCA-3'), and 50 ng of DNA template was performed. The PCR amplification was carried out with a hot-start at 94 °C for 8 min, followed by 30 s at 94 °C, 45 s at 58 °C and 1 min at 72 °C for 30 cycles. This was followed by an elongation cycle at 72 °C for 8 min. The PCR products were purified with a ZR DNA Clean & Concentrator-25 Kit (Zymo Research), and cloned into a pTOPO-TA vector and transformed into competent *E. coli* according to manufacturer's protocol (Invitrogen, Life Technologies, USA). Clones were picked and screened for unique phylotypes as previously described (Barton et al. 2004). Sequencing was carried out commercially by Agencourt Bioscience and assembled together using DNA Baser software. Assembled sequences were aligned and chimeras

removed using the Greengenes NAST algorithm (<http://greengenes.lbl.gov>). Complete 16S rRNA gene sequences were submitted to the NCBI GenBank database under the accession numbers detailed on Table 1.

A set of sixty-four 16S rRNA sequences (1279–1401 bp) were analysed using QIIME pipeline dependencies (Caporaso et al. 2010). Operational taxonomic units (OTUs) were selected and analysed based on sequence similarity with a 97 % as cut off value. Taxonomic identity was assigned using the RDP classifier (Wang et al. 2007) and taxonomic diversity plots were obtained using QIIME plot generator. Charts were generated at different taxonomic levels.

Results

Environmental parameters

At the collecting sites and during the sampling period the physicochemical parameters tested for freshwater samples were pH 6.9 and temperature 13.3 °C. These parameters were similar to previous reports (Mecchia et al. 2014).

Bacterial culture

At microaerobic conditions described previously (Degnan et al. 2003), growth was observed in four freshwater enrichments in HP broth, two from each site (LP1, LP2, LJ1 and LJ2), and eleven urease positive colonies selected as putative *Helicobacter* spp. from HP agar (five from LP and six from LJ). Gram staining showed mixed Gram-negative bacteria without the typical spiralled morphology of our target genera. Further attempts to isolate *Helicobacter* or *H. pylori* from HP broth enrichments were performed without success.

The eleven urease positive colonies were preliminary identified by API ATB/Plus (BioMérieux, Lyon, France) as *Chromobacterium violaceum* (99.9 % identity), *Photobacterium damsela* (86.0 % identity), *Pantoea* sp. (85.7 % identity), *Moraxella lacunata* (90.9 % identity) and *Serratia liquefaciens/plymouthica* (85.9–99.9 % identity), the latter being the most commonly isolated (7/11) from HP enrichments.

Table 1 Identified bacteria in freshwaters of Roraima Tepui based on 16S rRNA gene sequencing

Sequence ID	16S rRNA gene sequence similarity (%)	Length (bp)	GenBank accession number
<i>Helicobacter</i> sp. enrichment culture LP1Q	<i>Helicobacter pylori</i> KF717598 (100 %); <i>Helicobacter nemestrinae</i> AF363064 (100 %).	292	KC633951
<i>Helicobacter</i> sp. enrichment culture J1Q	<i>Helicobacter pylori</i> KC759147 (99 %); <i>Helicobacter nemestrinae</i> AF348617 (99 %).	304	KC633951
A02_R2K_LP1.1	<i>Chromobacterium</i> sp. JF904885 (99 %)	1384	KC633951
A03_R12G_LJ2.2.2	<i>Serratia</i> sp. JQ954965 (99 %)	1393	KC633951
A03_R3J_LP1.2	<i>Chromobacterium</i> sp. JF904885 (99 %)	1394	KC633951
A04_R5C_LP2.2.1	<i>Acinetobacter</i> sp. HQ659186 (99 %)	1394	KC633951
A05_R7B_LJ1.1	<i>Chromobacterium</i> sp. GU216192 (98 %)	1354	KC633951
A06_R8F_LJ1.2	<i>Serratia</i> sp. GU084119 (99 %)	1392	KC633951
A07_R9D_LJ2.1.1	<i>Serratia proteamaculans</i> JF94823 (99 %)	1364	KC633951
A08_R11B_LJ2.2.1	<i>Serratia</i> sp. JN859196 (99 %)	1391	KC633951
B02_R2L_LP1.1	<i>Chromobacterium</i> sp. KJ576860 (98 %)	1394	KC633951
B03_PLACA1_R12L_LJ2.2.2	<i>Serratia proteamaculans</i> JF94823 (99 %)	1344	KC633951
B03_R3K_LP1.2	<i>Paludibacterium</i> sp. JF327660 (98 %)	1394	KC633951
B04_R5D_LP2.2.1	<i>Aquitalea</i> sp. EU287928 (98 %)	1393	KC633951
B05_R7E_LJ1.1	<i>Chromobacterium</i> sp. KJ576864 (99 %)	1338	KC633951
B06_R11I_LJ2.2.1	<i>Serratia</i> sp. FN556576 (99 %)	1339	KC633951
B06_R8H_LJ1.2	<i>Serratia</i> sp. JN106438 (99 %)	1393	KC633951
B07_R9E_LJ2.1.1	<i>Serratia</i> sp. JN106438 (99 %)	1394	KC633951
B08_R11C_LJ2.2.1	<i>Aquitalea</i> sp. KF441626 (99 %)	1387	KC633951
C01_R2A_LP1.1	<i>Chromobacterium</i> sp. KF137653 (99 %)	1385	KC633951
C02_R3B_LP1.2	<i>Acinetobacter</i> sp. DQ452476 (99 %)	1383	KC633951
C03_R4B_LP2.1.1	<i>Aquitalea</i> sp. AB277847 (98 %)	1394	KC633951
C04_R6A_LP2.2.2	<i>Mycobacterium</i> sp. AB355701 (99 %)	1357	KC633951
C05_R7G_LJ1.1	<i>Chromobacterium</i> sp. KF137653 (98 %)	1370	KC633951
C06_R8I_LJ1.2	<i>Serratia</i> sp. JN106438 (99 %)	1375	KC633951
C07_R9F_LJ2.1.1	<i>Serratia</i> sp. JN106438 (99 %)	1395	KC633951
C08_R11D_LJ2.2.1	<i>Aquitalea</i> sp. KF441626 (99 %)	1371	KC633951
D01_R2B_LP1.1	<i>Paludibacterium</i> sp. JF327660 (98 %)	1388	KC633951
D02_R3D_LP1.2	Uncultured <i>Actinobacterium</i> clone EF016798 (98 %)	1369	KC633951
D03_R4D_LP2.1.1	Uncultured <i>Aquitalea</i> sp. clone EU159475 (98 %)	1371	KC633951
D04_R6B_LP2.2.2	<i>Mycobacterium</i> sp. AB355701 (99 %)	1374	KC633951
D05_R5G_LP2.2.1	<i>Acinetobacter</i> sp. DQ452476 (99 %)	1396	KC633951
D05_R8A_LJ1.2	Uncultured <i>Serratia</i> sp. clone JN032368 (99 %)	1401	KC633951
D06_R8J_LJ1.2	<i>Serratia proteamaculans</i> NR074820 (99 %)	1383	KC633951
D07_R9H_LJ2.1.1	Uncultured <i>Serratia</i> sp. clone EC34BG09 (99 %)	1373	KC633951
D08_R11H_LJ2.2.1	<i>Serratia</i> sp. FN556576 (99 %)	1347	KC633951
E01_R2D_LP1.1	<i>Curvibacter</i> sp. KF441650 (99 %)	1389	KC633951
E02_R3ELP1.2	<i>Chromobacterium</i> sp. KF137653 (99 %)	1396	KC633951
E03_R4F_LP2.1.1	<i>Aquitalea</i> sp. EU287928.1 (98 %)	1327	KC633951
E04_R6D_LP2.2.2	<i>Mycobacterium</i> sp. JQ518348 (99 %)	1342	KC633951
E05_R5H_LP2.2.1	<i>Aquitalea</i> sp. AB277847 (98 %)	1391	KC633951

Table 1 continued

Sequence ID	16S rRNA gene sequence similarity (%)	Length (bp)	GenBank accession number
E06_R8K_LJ1.2	<i>Serratia</i> sp. JN859196 (99 %)	1399	KC633951
E07_R10A_LJ2.1.2	<i>Serratia</i> sp. GU084119 (99 %)	1401	KC633951
F01_R2E_LP1.1	<i>Chromobacterium</i> sp. KJ576860 (98 %)	1394	KC633951
F02_R3F_LP1.2	<i>Acinetobacter</i> sp. HQ659186 (99 %)	1375	KC633951
F03_R4J_LP2.1.1	Uncultured <i>Aquitalea</i> sp. EU159475 (98 %)	1394	KC633951
F04_R6I_LP2.2.2	<i>Mycobacterium</i> sp. AB355701 (99 %)	1363	KC633951
F05_R5I_LP2.2.1	<i>Mycobacterium</i> sp. JQ518348 (99 %)	1347	KC633951
F05_R8C_LJ1.2	Uncultured <i>Serratia</i> sp. JN032368 (99 %)	1328	KC633951
F06_R8L_LJ1.2	<i>Serratia</i> sp. HM063908 (99 %)	1337	KC633951
F07_R10C_LJ2.1.2	Uncultured <i>Serratia</i> sp. JN860406 (99 %)	1401	KC633951
F08_R11K_LJ2.2.1	<i>Aquitalea</i> sp. KF441626 (99 %)	1374	KC633951
G02_R12B_LJ2.2.2	<i>Serratia</i> sp. GU084119 (99 %)	1397	KC633951
G02_R3H_LP1.2	<i>Aquitalea</i> sp. AB695196 (98 %)	1326	KC633951
G03_R2K_LP1.1	<i>Aquitalea</i> sp. AB277847 (98 %)	1388	KC633951
G04_R6L_LP2.2.2	<i>Mycobacterium</i> sp. JQ518348 (99 %)	1305	KC633951
G05_R8D_LJ1.2	<i>Serratia</i> sp. GU084119 (99 %)	1358	KC633951
G06_R9A_LJ2.1.1	<i>Serratia</i> sp. JN106438 (99 %)	1367	KC633951
G07_R10E_LJ2.1.2	<i>Serratia</i> sp. GU084119 (99 %)	1398	KC633951
G08_R11L_LJ2.2.1	<i>Serratia</i> sp. HM468066 (99 %)	1333	KC633951
H01_R2H_LP1.1	<i>Bacteroidetes</i> JX828414 (97 %)	1369	KC633951
H02_R12D_LJ2.2.2	Uncultured <i>Serratia</i> sp. JN032368 (99 %)	1321	KC633951
H02_R3I_LP1.2	Uncultured bacterium AB637342 (95 %)	1279	KC633951
H04_R7A_LJ1.1	<i>Chromobacterium</i> sp. KJ576864 (99 %)	1328	KC633951
H05_R8A_LJ1.2	<i>Serratia</i> sp. JN106438 (99 %)	1370	KC633951
H06_R9B_LJ2.1.1	<i>Serratia</i> sp. HM468066 (99 %)	1317	KC633951

Identification was assigned with similarities above 97 % to known sequences in the NCBI database

Detection of *Helicobacter* spp. by Fluorescence in situ hybridization (FISH)

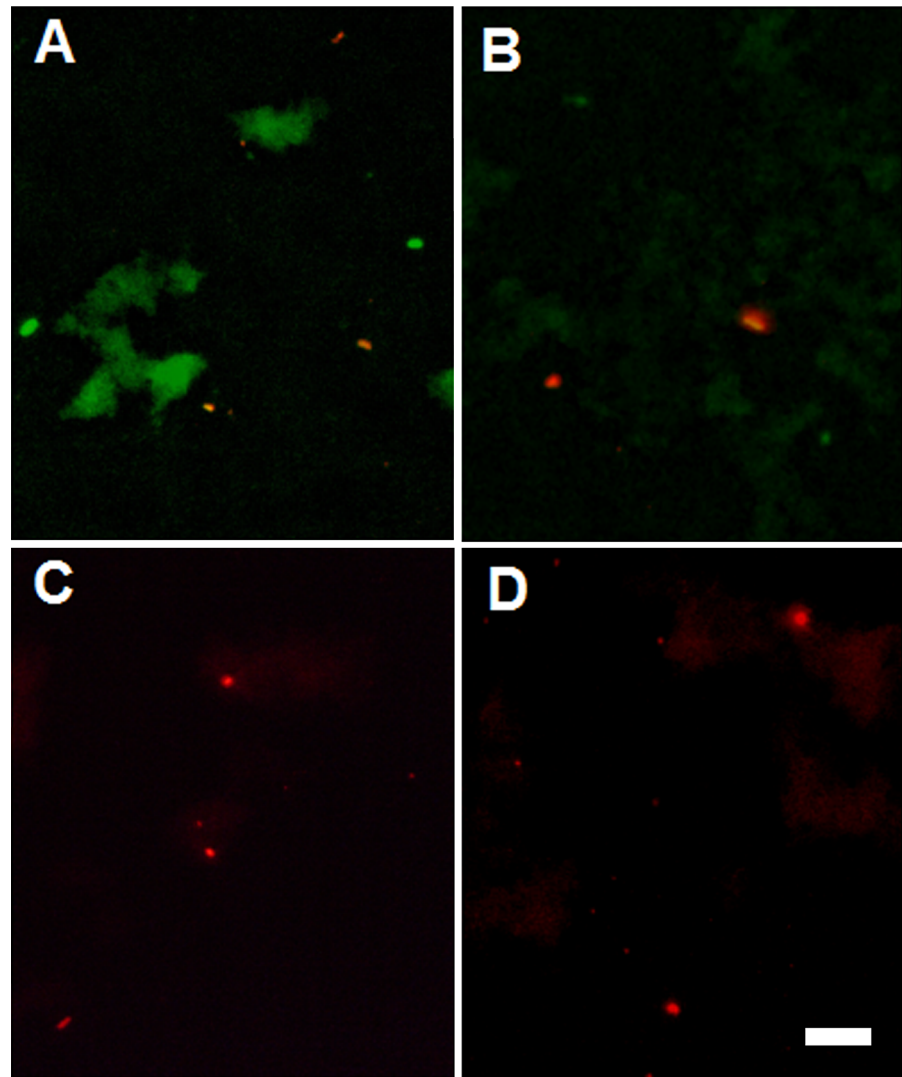
While no putative *Helicobacter* spp. were isolated, the presence of these bacteria was evaluated by FISH on the freshwater enrichments LP1, LP2, LJ1 and LJ2 grown with HP broth. The probes HEL274/HEL717 and Hpy-1 were able to detect *Helicobacter* and *H. pylori* in all enrichments, as is shown in Fig. 2. *Helicobacter* spp. and *H. pylori* were identified as orange (mixed colour of green and red) (A-B) or red (C-D) whole-cells with different morphological types, predominantly coccoid cells. The presence of other non-*Helicobacter* bacteria was also detected with the EUB338 (green) probe (A-B).

Detection of *Helicobacter* spp. by PCR and 16S rRNA partial gene sequences

Although *H. pylori*-specific genes (*glmM*, *cagA* and *ureA*) were not found in any of the enrichments studied, *Helicobacteriaceae* and *Helicobacter* DNA were detected by nested and semi-nested PCR in LP (LP1 and LP2) and LJ (LJ1 and LJ2) confirming the presence of members of the genus as observed by FISH.

Helicobacter-specific fragments of the 16S rRNA gene from LP1 and LJ1 enrichments were sequenced and the phylogenetic relationships of these sequences (*Helicobacter* sp. Roraima enrichment culture LP1Q and J1Q, respectively) with other *Helicobacter* species based on 16S rRNA sequence data is shown in Fig. 3.

Fig. 2 Detection of *Helicobacter* spp. and *H. pylori* by FISH in freshwater enrichments from Roraima Tepui. **a, b** *Helicobacter* spp. (orange) and eubacteria (green) cells detected with the mixture of fluorescent probes HEL274+HEL717 (red) and EUB338 (green) in LP and LJ, respectively. **c, d** *H. pylori* cells observed with Hpy-1 probe (red) in LP and LJ, respectively. In the images can be seen predominant coccoid cells indicative of the VBNC state. Bar **a–d** 8 μ m



Our freshwater enrichments formed a cluster with different primate *H. pylori* strains, mainly from humans, or *H. nemestrinae* from pig tailed macaque, both with 99–100 % of homology.

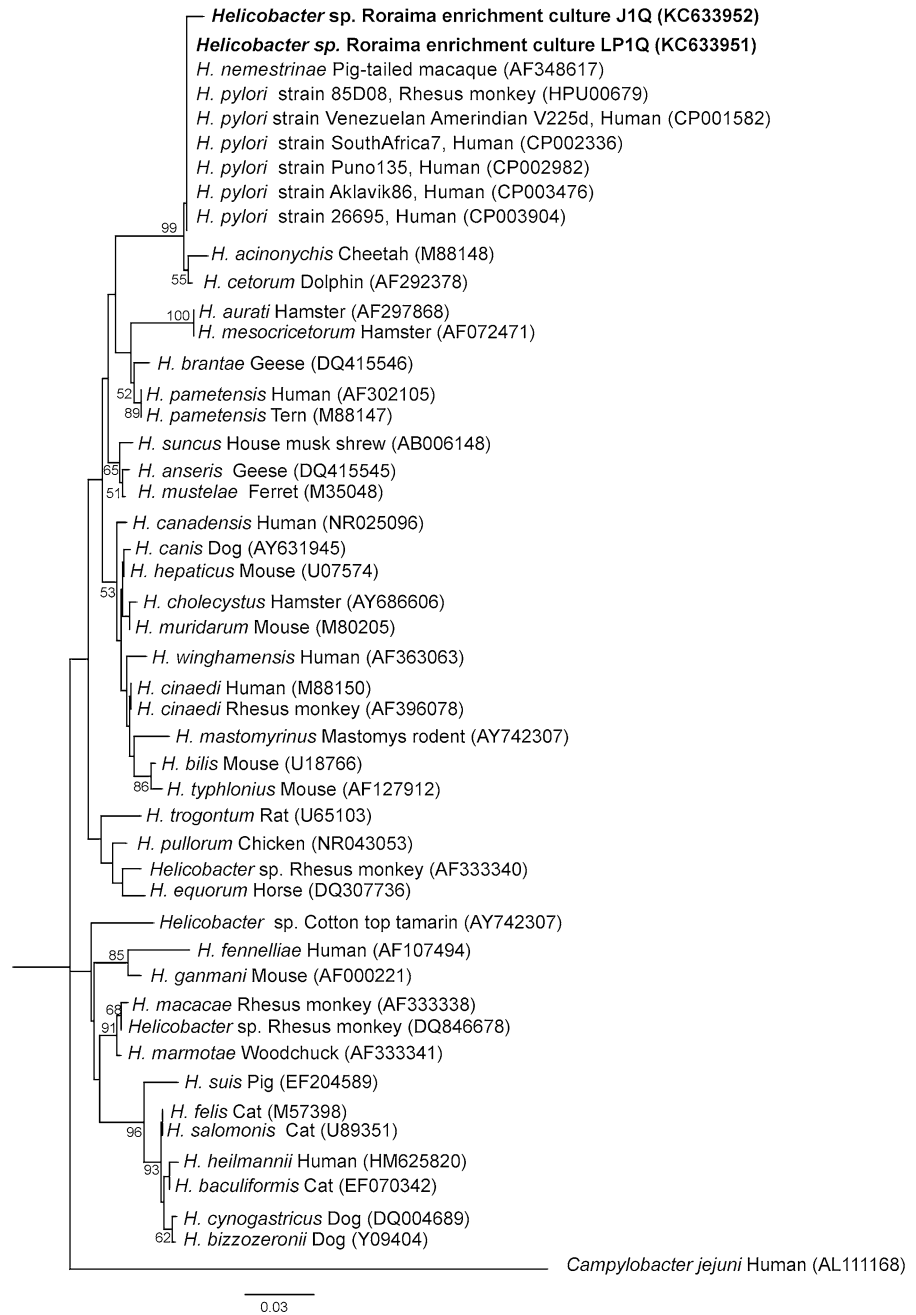
Complete 16S rRNA gene sequences of freshwater cultures

In order to identify the presence of other bacterial genera in the eleven cultures obtained from HP agar under microaerobic conditions, we carried out a molecular phylogenetic assessment of the cultures using bacterial clone libraries from 16S rRNA gene sequences and obtained 30 and 34

clones for LP and LJ, respectively. Within each clone library, the OTUs were identified aligning obtained sequences by BLAST using the NCBI database as reference.

These cultures were dominated by two phyla: the *Proteobacteria* and *Actinobacteria*. Bacteria from the genera *Serratia* (40.9 %), *Aquitalea* (18.2 %), *Chromobacterium* (15.2 %), *Mycobacterium* (9.1 %), *Acinetobacter* (6.1 %), *Curvibacter* (1.5 %) and *Dysgonomonas* (1.5 %) were identified on the basis of the complete 16S rRNA gene sequences. Phylogenetic inferences show representative sequences grouped mainly in the *Gammaproteobacteria*, *Actinobacteria* and *Neisseriales* (Table 1; Fig. 4).

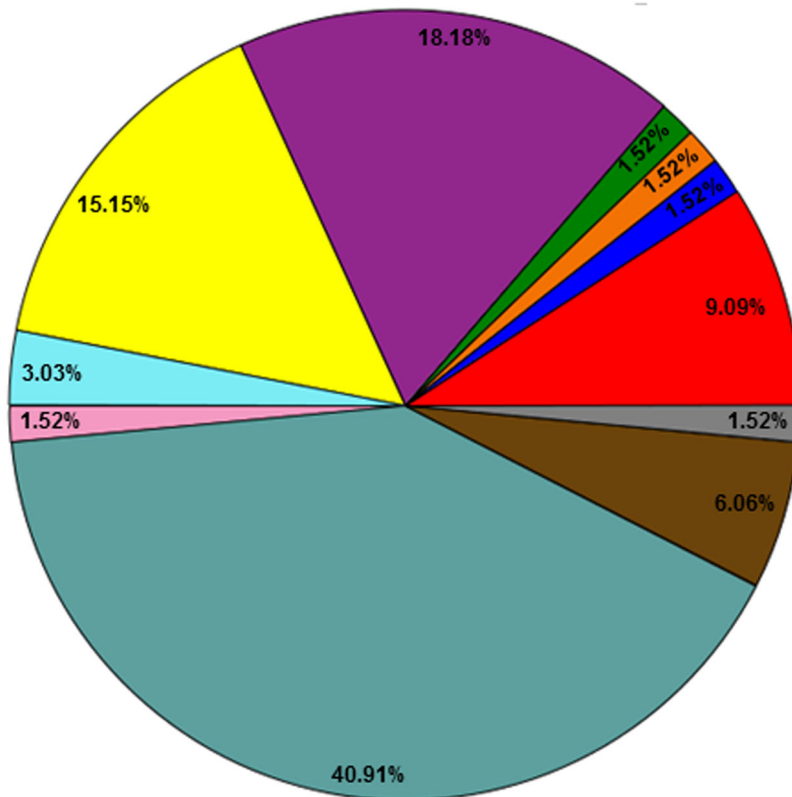
Fig. 3 Phylogenetic tree of partial 16S rRNA sequences of *Helicobacter* obtained from Roraima freshwater enrichments. Tree was constructed using Neighbor-Joining algorithm. Bootstrap values are based on 10,000 replicates and no values are given for groups with Bootstrap values less than 50 %. The scale bar represents 0.03 (3 %) nucleotide sequence difference



Discussion

In this study the presence of *Helicobacter* spp. was evidenced in freshwaters of a tepui ecosystem. The unsuccessful isolation of these bacteria from HP agar was associated with the large number of coccoid cells observed in the FISH images (Fig. 2), indicating the

entrance into a VBNC state. It has been suggested that transformation to a coccoid form enables the bacterium to resist the potentially adverse effects of entering aquatic environments (Bellack et al. 2006). Similarly, several studies have failed to isolate and culture live *Helicobacter* or *H. pylori* from environmental samples (Bellack et al. 2006; Delpont and van



- Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Mycobacteriaceae;Mycobacterium
- Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Other;Other
- Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;Dysgonomonas
- Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Curvibacter
- Bacteria;Proteobacteria;Betaproteobacteria;Neisseriales;Neisseriaceae;Aquitalea
- Bacteria;Proteobacteria;Betaproteobacteria;Neisseriales;Neisseriaceae;Chromobacterium
- Bacteria;Proteobacteria;Betaproteobacteria;Neisseriales;Neisseriaceae;Other
- Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacteriales;Enterobacteriaceae;Other
- Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacteriales;Enterobacteriaceae;Serratia
- Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Acinetobacter
- Bacteria;TM7;TM7_genera_incertae_sedis;Other;Other;Other

Fig. 4 Phylogenetic composition represented by complete 16S rRNA bacterial sequences of Roraima freshwater cultures grown on HP agar. *Pie charts* were generated by taxa plotting

in QIIME pipeline. The percentage of bacteria from each division was calculated, and results of 1.52 % are shown

der Merwe 2007). The hostile environment and difficult access to Roraima Tepui determined the low number and volume of water samples and could contribute to the failed isolation of *Helicobacter* spp.

Good agreement was observed between the detection of *Helicobacter* by FISH and semi-nested PCR in freshwater enrichments from the two studied sites.

Despite our ability to identify *H. pylori* by FISH and partial sequence analysis of the 16S rRNA, we did not detect the specific genes *glmM*, *ureA* and *cagA* by PCR in the freshwater enrichments. The inability to amplify these typical *H. pylori* genes could be due to (i) low concentration or fragmented DNA (Fernández et al. 2008) in the freshwater cultures from Roraima, and (ii)

the clinical design of primers used in this study which might not be sufficiently robust to provide the accurate detection of *H. pylori* genes from waters (Voytek et al. 2005). FISH has shown to yield more positive results than PCR for the detection of *H. pylori* specific genes in environmental samples (Moreno et al. 2003). On the other hand, the discrepancy between the 16S rRNA and specific genes could also be due to variation in primer specificity and sensitivity of the assays (Carbone et al. 2005; Twing et al. 2011). The 16S rRNA PCR assay, while possibly not as specific, is more sensitive than single genes that may vary more in their primer sequence because of their conserved nature within a genome (Twing et al. 2011). The 16S rRNA genes are present in high copy numbers as essential constituents of living cells; therefore, there is no problem with gene loss, as has been previously proposed for *H. pylori* specific genes (Carbone et al. 2005).

As inferred from the 16S rRNA partial sequencing and phylogenetic analysis, the *Helicobacter* sequences (LP1 and LJ1) from the two sites examined in the tepui showed a high homology with different *H. pylori* strains and *H. nemestrinae* (99–100 %). It is important to note that this part of the analysis was based on a partial 16S rRNA sequencing (292–304 bp). Several reports have probe that 16S rRNA gene analysis are useful for defining prokaryotic relationships from the species to phylum levels. However, cases of very closely related species have highlighted the need to use other molecules to better resolve species identity. For example, *H. nemestrinae*, once believed to be a novel gastric helicobacter isolated from a pig tailed macaque, was later recognized to be a strain of *H. pylori* by correction of the 16S rRNA gene sequence and analysis of seven housekeeping and two flagellin genes (Suerbaum et al. 2002). In fact, the 16S rRNA target sequence for *H. pylori* probe used in our FISH analysis was found to be identical to several *H. pylori* strains and *H. nemestrinae*, but in no other species (Trebesius et al. 2000). The presence of these two species of *Helicobacter* suggests the possibility of anthropogenic or wildlife sources into the freshwaters of the Roraima tepui. These natural aquatic environments might have been secondary contaminated with human waste from tourists or Pemon community. Epidemiological reports of Amerindians groups (Native American peoples) from Venezuela indicate a high prevalence of *H. pylori* (*cagA* antigen seropositivity of 95 % in Guajibó-Piaroa) (Contreras et al. 2008). However, a low prevalence of *H. pylori*

(11 %) was found recently in Paraitepuy Pemon community, the aboriginal group established in the basement of Roraima Tepui whom serve as porters and guides for tourists (data not shown). This new information suggests a major influence of tourists in the *Helicobacter* entrance to Roraima freshwaters. The other possibility is the wildlife origin of helicobacters in Roraima freshwater samples. The high endemism of wildlife described in the Roraima Tepui includes invertebrates (McDiarmid and Gorzula 1989; Macculloch and Lathrop 2007; Brewer-Carias and Audy 2012) and birds (Herrera 2003). Although, there are some reports of mammals from the Canaima National Park represented by lions (*Puma concolor*), coatías (*Nassua nassua*), melero bears (*Tamandua tetradactyla*), and small howler monkeys (*Alouatta*) (Brewer-Carias and Audy 2012), no other primates than humans are common visitors at Roraima summit. The limited amount of suitable habitat may not sustain primates or other mammal life on Roraima summit because of the adverse environmental conditions existing in the area (Mecchia et al. 2014). Only a rodent named Roraima mouse (*Podoxymys roraimae*) has been described as original and collected from the tepui summit, and nowadays is found in a vulnerable condition (Pérez-Zapata et al. 1992). Our evidence placed rodent's *Helicobacter* sequences in other clusters far away from Roraima enrichment cultures LP1Q and J1Q (Fig. 3); however, the possibility of Roraima wildlife as *Helicobacter* carriers is not ruled out.

In addition to *Helicobacter* detection in these freshwater enrichments, the complete 16S rRNA gene sequencing analysis allowed the identification of other bacteria. The most abundant phylum founded was *Proteobacteria* (86.37 % of the total sequenced reads), followed by *Actinobacteria* and *Bacteroidetes*. These phyla were similar to those reported in water samples (freshwater, seawater, drinking water and wastewater) using high throughput sequencing technology such as 454 pyrosequencing and Illumina (Caporaso et al. 2010; Gilbert et al. 2009; Hong et al. 2010; Kirchman et al. 2010; Ye and Zhang 2013). In our study, Gammaproteobacteria (48.49 %) was the most abundant among the Proteobacteria, followed by Betaproteobacteria (37.88 %); however, Alphaproteobacteria was absent in the freshwater enrichments from Roraima Tepui, while it has been generally dominant in seawater, drinking water and wastewater samples (Gilbert et al. 2009; Hong et al. 2010;

Kirchman et al. 2010; Ye and Zhang 2013). The abundance of some members of Gamma and Betaproteobacteria may be favoured by nutrients and selective components present in the HP enrichment broth as well as microaerophilic growth conditions. At the same time, these culture conditions could restrict the growth of genera encompassed within Alphaproteobacteria producing a bias on predominance of certain groups of Proteobacteria. Furthermore, the majority of the sequences of our study were classified to the genus level as *Serratia*, *Aquitalea*, *Chromobacterium* and *Mycobacterium* (97–99 % of 16S rRNA similarity; Table 1) with the highest percentages of appearance in the two sites (Fig. 4). *Serratia*, *Mycobacterium*, *Acinetobacter* and *Dysgonomonas* have been reported in abundance in wastewaters (Wiedmann-Al-Ahmad et al. 1994; Wéry et al. 2010; Ye and Zhang 2013), whereas *Curvibacter* has been only reported in well water (Ding and Yokota 2004). Although the majority of these genera occur in sewage, they are also common in unpolluted water and soil as free-living members (Wiedmann-Al-Ahmad et al. 1994; Ding and Yokota 2004; Wéry et al. 2010; Ye and Zhang 2013).

This represents the first report of *Helicobacter* in aquatic environments of a little studied and unique ecosystem as the Roraima Tepui. Although further research is necessary to elucidate the human or wildlife *Helicobacter* origin, our results contribute to the current knowledge of these bacteria in the aquatic environment and expand their known/potential sites outside the human host. Additional attempts should be incorporated to isolate *Helicobacter* from environmental samples and further research is necessary to assess the bacteriological quality of Roraima freshwaters in order to estimate the possible fecal contamination sources.

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