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Additional Information

Detection of *Helicobacter pylori* in drinking water treatment plants in Bogotá, Colombia, using Cultural and Molecular Techniques

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Abstract

Helicobacter pylori is one of the most common causes of chronic bacterial infection in humans, and a predisposing factor for peptic ulcer and gastric cancer. The infection has been consistently associated with lack of access to clean water and proper sanitation. *H. pylori* has been detected in surface water, wastewater and drinking water. However, its ability to survive in an infectious state in the environment is hindered because it rapidly loses its cultivability. The aim of this study was to determine the presence of cultivable and therefore viable *H. pylori* in influent and effluent water from drinking water treatment plants (DWTP). A total of 310 influent and effluent water ||samples were collected from three drinking water treatment plants located at Bogotá city, Colombia. Specific detection of *H. pylori* was achieved by culture, qPCR and FISH techniques. Fifty-six positive *H. pylori* cultures were obtained from the water samples.

Characteristic colonies were covered by the growth of a large number of other bacteria present in the water samples, making isolation difficult to perform. Thus, the mixed cultures were submitted to Fluorescent *in situ* Hybridization (FISH) and qPCR analysis, followed by sequencing of the amplicons for confirmation. By qPCR, 77 water samples, both from the influent and the effluent, were positive for the presence of *H. pylori*. The results of our study demonstrate that viable *H. pylori* cells were present in both, influent and effluent water samples obtained from drinking water treatment plants in Bogotá and provide further evidence that contaminated water may act as a transmission vehicle for *H. pylori*. Moreover, FISH and qPCR methods result rapid and specific techniques to identify *H. pylori* from complex environmental samples such as influent water.

1. Introduction

Helicobacter pylori is a pathogenic bacterium which colonizes human gastric mucosa, and is known to affect >50% or the world population (Aziz et al., 2015; Khean-Lee et al., 2011; USEPA, 2015a, 2015b). According to Hooi et al. (2017) there were approximately 4.4 billion individuals with H pylori infection worldwide in 2015. Prevalence is highest in Africa (79.1%), Latin America and the Caribbean (63.4%), and Asia (54.7%). In contrast, HP prevalence is lowest in Northern America (37.1%) and Oceania (24.4%). In Colombia, 77-80% of the population is infected (Campuzano-Maya et al., 2007). Infection with this bacterium has been associated with the development of chronic gastritis, peptic ulcer disease, atrophic gastritis, intestinal metaplasia, B cell MALT lymphoma and gastric adenocarcinoma (Backert et al., 2016). In 1994, *H. pylori* was classified as a Group 1 carcinogen by the WHO International Agency for Research on Cancer (IARC, 1994). The clinical outcome of *H. pylori* infection has been associated with the presence of specific *H. pylori* virulence factors, including cytotoxin-associated gene A (CagA) and the vacuolating cytotoxin (VacA) (Winter et al., 2014). The Vacuolating cytotoxin A secreted by *H. pylori* enhances the ability of the bacteria to colonize the stomach and contributes to the pathogenesis of gastric adenocarcinoma and peptic ulcer disease.

Currently, the route of transmission of *H. pylori* remains unclear, evidence supporting both the fecal-oral and oral-oral route (Leja et al., 2016). 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 (Carbone et al., 2005; Cellini et al., 2004; Cunachi et al., 2015; Mazari-Hiriart et al., 2001; Moreno et al., 2003a; Santiago et al., 2015; Twing et al., 2011; Voytek et al., 2005). The risk of acquisition of *H. pylori* appears to be multifactorial and potentially contaminated

environmental sources, such as local drinking water, swimming in rivers, or the ingestion of fecally contaminated vegetables have been reported as risk factors for *H. pylori* infection (Leja et al., 2016).

At the moment, *H. pylori* is included in the U.S. Environmental Protection Agency (EPA) Contaminant Candidate List (CCL), which includes new chemicals and microorganisms contaminants that may pose risks for drinking water, based on information about known and suspected public health risk and the occurrence of the contaminant in water (USEPA, 2004, 2015b).

Studies about the survival of *H. pylori* in the environment have shown that, under stress conditions, *H. pylori* acquires the coccoid form and enters the viable but nonculturable state (VBNC), in which the organism could be metabolically active and keeps most virulence genes (Bai et al., 2016; Hulten et al., 1998) but that cannot be cultured *in vitro* (Bode et al., 1993; Nilius et al., 1993). Thus, in the VBCN state *H. pylori* could survive in water for several months (Bode et al., 1993; Percival and Suleman, 2014). Some authors have suggested that some cocci can revert to their original spiral, culturable shape (Cellini et al., 1994; She et al., 2003). It has been also reported that *H. pylori* is able to remain viable in water storage systems, possibly held in the biofilms (Percival and Suleman, 2014). This VBNC forms cannot be detected by culture, which means that the potential of acquisition through environmental reservoirs, as drinking water, may be undervalued (Azevedo et al., 2007).

Thus, the main challenge when conducting environmental monitoring is to demonstrate the existence of viable *H. pylori* in water samples. Very few attempts to culture *H. pylori* from environmental waters have been successful (Al-Sulami et al., 2012; Bahrami et al., 2013; Degnan et al., 2003; Lu et al., 2002; Moreno and Ferrús, 2012; Santiago et al., 2015), which

has led to the use of molecular methods to detect and identify the organism (Assadi et al., 2015; Khadangi et al., 2017; McDaniels et al., 2005).

Several authors have reported the use of conventional Polymerase Chain Reaction (PCR) and Quantitative Real-Time Polymerase Chain Reaction (qPCR) for the amplification of the *vac*A, *cag*A, *ure*A, *glm*M genes to detect *H. pylori* in different types of water, including drinking water, surface water, treated and untreated wastewater, marine water, ground water and biofilms (Bahrami et al., 2013; Fujimura et al., 2004; Lu et al., 2002; Moreno and Ferrús, 2012; Queralt et al., 2005; Santiago et al., 2015). Due to the high sensitivity of the qPCR reaction, a minimum amount of any inhibitory substance present in the sample can trigger a false negative or a low rate of detection (Schrader et al., 2012; Wilson, 1997). Inhibitors can come from the own sample or arise during the processing of the sample or extraction of nucleic acids (Schrader et al., 2012). Some approaches such as an enrichment step of the sample or Immunomagnetic Separation (IMS) have been proposed to avoid the qPCR inhibition due to substances present in the samples (Lu et al., 2002).

Although qPCR and PCR techniques have a high sensitivity, they present the disadvantage of not being able to discriminate between viable and nonviable cells. To achieve the detection of viable cells by molecular methods, different methods have been developed, such as the use of the intercalating agent of DNA fluorophore propidium monoazide (PMA) (Santiago et al., 2015; Villarino et al., 2000). However, assessing bacterial viability using PMA-qPCR remains a challenge, as this technique only demonstrates membrane integrity and can lead to overestimation of the viable bacteria population under some inactivation conditions (Lee and Bae, 2017).

Fluorescent *In Situ* Hybridization (FISH) with rRNA oligonucleotide probes has been used for detection and identification of *H. pylori* in water samples (Fernandez Delgado et al., 2016; Moreno et al., 2003a; Piqueres et al., 2006; Tirodimos et al., 2014). FISH method has the advantage of not being inactivated by sample inhibitors. FISH in combination with direct viable count incubation (DVC-FISH) has been recently reported as a complementary technique for successfully detecting viable cells of *H. pylori* in wastewater and drinking water (Moreno y Ferrús, 2012; Moreno-Mesonero et al., 2016; Piqueres et al., 2006).

The objective of the present work was to determine the occurrence of *H. pylori* in influent and effluent water samples from three drinking water treatment plants (DWTP) from Bogotá, Colombia, by using a combination of enrichment culture, specific qPCR assays of *vac*A gene and Fluorescent *in situ* Hybridization (FISH).

2. Experimental procedures

2.1. Sampling sites

Water samples were collected from three Drinking Water Treatment Plants (DWTP 1, 2 and 3), located at north, south and northeastern of Bogotá city, Colombia. These plants receive water from various sources and apply conventional disinfection treatment consisting in pre-treatment, coagulation-flocculation, sedimentation, down flow filtration and disinfection with chlorine. DWTP 1, located in northern Bogotá, takes the water from the Bogotá river shipside in which a sedimentation process is generated to reduce the microbial load. It treats an average flow of 10.5 m^3/s and distributes the water to 8 municipalities and part of the north of Bogotá. DWTP 2, located in southern Bogotá, receives water from the upper Tunjuelo River basin, La Regadera reservoir and Chisacá. This plant treats an average flow of 1.6

m^3/s and distributes water to the southern sector of Bogotá. DWTP 3, located in northeastern Bogotá, receives water from Chingaza reservoir and the Teusacá River, which are connected in the San Rafael reservoir. This plant treats an average flow of 14 m^3/s and distributes water to the municipality of the Calera and 70% of the city of Bogotá.

2.2. Water sampling and Helicobacter pylori concentration

Sampling was carried out between July 2015 and August 2016 and included 155 influent and 155 effluent water samples: fifty-three samples were taken from DWTP 1; fifty-two samples were taken from DWTP 2 and fifty samples from DWTP 3.

Three hundred mL of the influent samples were collected into 500 mL sterile bottles. Each water influent sample was centrifuged at 3000 x g for 20 minutes and the pellet was resuspended in 2 mL of phosphate-buffered saline (PBS 1X: 130 mmol/L sodium chloride, 10 mmol/L sodium phosphate, pH 7.2).

For the effluent samples, we used the "Moore swab" method (OPS/WHO, 2010). Briefly, a swab was kept in contact with effluent flow for 72 hours, removed and placed into a sterile bottle, held at 4°C and processed within a few hours. The swab was transferred to 200 mL of Brucella Broth (Becton Dickinson BBL^{TM}, USA) supplemented (BBS) with 0,4% Isovitalex (Becton Dickinson BBL^{TM}, USA) and 0,2% Dent (Oxoid, USA), shaken at 500 rpm for 30 min and finally incubated at 37°C under microaerophilic conditions (5% O_2, 11% CO_2, 85% N_2) for 24 hours. After this pre-enrichment step, 150 mL of each sample was centrifuged at 3000 x g for 20 minutes and the pellet was resuspended in 2 mL of PBS 1X.

One mL of both, influent and effluent PBS suspension samples, were concentrated by Immunomagnetic Separation (IMS). Treatment of the beads for IMS was conducted according to Enroth y Engstrand protocol (Enroth and Engstrand, 1995). Briefly, 3 µl the polyclonal Rabbit anti-HP IgG (5.3 mg of protein per mL) (Dako, Denmark) was incubated with 500 µl of magnetic beads (6 to 7 x 10^8 beads per mL) precoated with sheep anti-rabbit IgG (Dynabeads™ M-280; Norvex by Life technologies, Norway) for 24 h at 4°C with gentle agitation.

For the concentration of samples, 20 µl of coated beads were added to 1mL of PBS 1X suspension. The samples were gently agitated for 1 h at 4°C. With the aid of a magnet (MPC-s, Invitrogen Inc.) beads were separated from the rest of the sample and rinsed three times in 1 mL of PBS containing 0.1% bovine serum albumin (BSA) with gentle agitation (18rpm) at 4°C for 30 min. After the third washing, the beads-bacteria aggregates were resuspended in 1 mL PBS 1X and subsequently analyzed by culture, qPCR and FISH.

One mL aliquots of PBS suspensions of each sample without IMS were also tested by culture.

2.3. Helicobacter pylori culture conditions and bacterial strains

Reference strains *H. pylori* 11637 NCTC and 11638 NCTC (National Collection of Type Cultures, UK), and the *H. pylori* strain 301A, isolated from gastric biopsy of a patient from Bogotá city, were cultured in Supplemented Brucella Agar (SBA): BBL™ Brucella agar (Becton Dickinson, USA), 5% defibrillated horse blood, 0,2% DENT Supplement (Oxoid, UK) and 0,4% Isovitalex (Becton Dickinson, USA). Plates were incubated under microaerophilic conditions (5% oxygen, 11% carbon dioxide, and 85% nitrogen) with 95% humidity (Bayona

Rojas, 2013) in an anaerobic incubator (Thermo Scientific, USA) at 37 °C for 3 days. Reference strains were used as positive controls for culture, qPCR, and FISH.

2.4. Culture methods for Helicobacter pylori isolation

2.4.1. Preliminary assays

For the isolation of *H. pylori* from water samples, different culture media were evaluated: Supplemented Brucella Agar (SBA), described above, HP agar (Degnan et al., 2003) and HP agar supplemented with different antibiotics (nalidixic acid, streptomycin sulfate and kanamycin). Aliquots of 0.1 mL of water samples spiked with the three reference strains, *H. pylori* NCTC 11637, NCTC 11638 and *H. pylori* 301A, were spread on the different agar media plates and incubated under microaerophilic conditions at 37 °C for 5-14 days, to determine the optimal bacteria growth time. In addition, 2 different pore sizes membrane filters (0,45 µm and 0,22 µm) were tested for filtration effectiveness. Reference strain *E. coli* ATCC 25992, from the American Type Culture Collection (ATCC) was used as a culture negative control.

2.4.2. Helicobacter pylori isolation from water samples

Based on the results of the culture preliminary assays above described (data not shown), three different strategies were used for the detection of *H. pylori* from influent samples: 1) 0,1 mL of the suspension of samples in PBS 1X after centrifugation were directly spread onto SBA plates; 2) 0,1 mL of the concentrated IMS suspension were spread on to SBA plates and 3) 0,4 mL of concentrated from IMS were enriched in Supplemented Brucella Broth (BBS: BBL™ Brucella broth (Becton Dickinson, USA), 0,2% DENT Supplement

(Oxoid, UK) and 0,4% Isovitalex (Becton Dickinson, USA)) for 24h at 37°C under microaerophilic conditions and centrifuged at 3000 x g/ for 20min. The pellet was then resuspended in PBS 1X, and 0,1 mL were spread onto SBA plates (Fig. 1A).

To analyze water effluent samples, the procedures was as follows: 1) 20 mL of swab pre-enriched Brucella broth was filtered through a membrane filter of 0,22 μm pore size and the membrane was transferred on to SBA plates; 2) 0,1 mL of the PBS suspension containing the concentrated pre-enriched broth were directly spread onto SBA plates and 3) 0,1 mL of the IMS concentrated suspension were spread on to SBA plates (Fig. 1B).

In all the cases, agar plates were incubated under microaerophilic conditions for 10 days at 37°C and examined for the presence of characteristic colonies. Presumptive *H. pylori* colonies were subculture on SBA plates and biochemical tests (catalase, urease and oxidase) and Gram-stained were performed. When plates contained high amounts of non-*H. pylori* bacterial growth, they were also analysed by collecting the whole surface content and examined this using FISH and PCR analysis (Moreno and Ferrús, 2012). Positive results obtained by PCR were confirmed by sequencing the amplified fragment of the *vac*A gene as described below.

2.5. Detection of Helicobacter pylori using qPCR analysis and DNA sequencing

DNA was purified from a 1mL aliquot of each IMS concentrated sample using the DNeasy Blood & Tissue kit (Qiagen, USA), according to the manufacturer's instructions. Specific *H. pylori* qPCR based on SYBR green I fluorescence was conducted using VacA primers to amplify a 372 bp fragment (Table 1) in a LightCycler® 2.0 Instrument (Roche Applied Science, Spain).

The final reaction volume of 20 µl contained: 2 µl of Light-Cycler® FastStart DNA SYBR Green I (Roche Applied Science, Spain), 1.6 µl of MgCl$_2$ (50 mM), 0.5 µl of each primer (20 mM) and 2 µl of DNA template. The amplification consisted of an initial DNA denaturation step at 95 °C for 10 min, followed by 40 cycles of: 95 °C for 10 s, 62 °C for 5 s and 72 °C for 16 s; and finally, one cycle at 72 °C for 15 s and one at 40 °C for 30 s (Santiago et al., 2015). Amplifications were conducted in duplicate.

DNA from *H. pylori* strain NCTC 11637 was used as a positive control and qPCR mix without DNA served as negative control in all the qPCR analysis. Two samples of DNA isolated from both, influent and effluent sterilized water, were inoculated with NCTC 11637 *H. pylori* DNA in order to use as controls of the presence of qPCR inhibitors in each reaction running.

The melting temperature (Tm) for the VacA primers was 85°C (Santiago et al., 2015). Thus, we considered potential positive samples as those with Tm values ranging between 85.5 and 84.5. For quantification, a standard curve was performed as previously described by Santiago et al. (2015), taking into account that there is only one copy of the *Vac*A gene in the genome of *H. pylori* (Foegeding et al., 2016). Briefly, ten-fold serial dilutions of the DNA from *H. pylori* NCTC 11637 were prepared from 10^6 down to 10 genomic units (GU)/µL. An aliquot of 2 µL was used for each qPCR reaction, by using VacA primers. Triplicate analyses were run for each DNA dilution. After the assays, the limit of quantification was established in 10 log genomic units or cells of *H. pylori*/reaction (data not shown).

Additionally, for confirmation of all the qPCR positive results, products were visualized in 2% agarose gel electrophoresis prepared with 0.01% GelRed (Biotium, USA). Moreover, the homology of the amplified sequences with the corresponding *H. pylori vac*A gene fragment

was determined by sequencing, using the Sanger method (Macrogen, Korea). Sequences were compared to the sequences published in GenBank according to Altschul et al. (1997) using BLAST software alignment tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.6. Detection of Helicobacter pylori by FISH

For FISH analysis, *H pylori* presumptive colonies from mixed cultures in SBA were resuspended in 1 mL of PBS 1X and immediately fixed with three volumes of 4% paraformaldehyde for 2 hours at 4 °C. One mL aliquots of each influent and effluent water samples that were *H. pylori* positive by culture were also fixed.

The fixed samples were centrifuged, washed with PBS buffer, and finally resuspended in 1:1 PBS/ethanol (v/v) as previously described (Moreno et al., 2003a). Thereafter, slides were dehydrated by successive immersions in 50%, 80% and 100% ethanol for 3 min each. Then, each well was covered with 10 mL of hybridization buffer (0.9 M NaCl, 20 mM HCl-Tris, 0.01% SDS and 30% formamide, pH 7.5) containing 50 ng of each probe. The reaction was carried out in the dark at 46 °C for 1.5 h. A combination of three EUB338 probes, complementary to a region of the Eubacteria domain 16S rRNA was used as a positive control (Amann et al., 1990; Moreno et al., 2003b). For *H. pylori* detection, a specific 16S rRNA LNA (Locked Nucleic Acid) probe designed by Moreno et al. (2003a) and synthesized by EXIQON (Exiqon A/S Vedvaek, Denmark) was used (Table 1).

After hybridization, slides were washed in the dark at 48 °C for 15 min in 50 mL of wash solution (0.10 M NaCl, 0.02 M HCl-Tris, 0.01% SDS and 0.005 M EDTA). Finally, they were washed with distilled water and air-dried in the dark. Slides were mounted with FluoroGuard Antifade Reagent (Bio-Rad, Spain) between the coverslip and the slide and were visualized

using an Olympus BX 50 fluorescence microscope with U-MWB, U-MWIB and U-MWIG filters. Images were taken using an Olympus DP-12 camera. A pure culture of fixed *H. pylori* NCTC 11637 cells was used as a positive control for the FISH process.

3. Results

3.1. Detection of Helicobacter pylori in influent and effluent water using culture

Characteristic colonies were observed on SBA plates following direct samples, from enrichment samples and from the membrane filters. Nevertheless, in all cases, presumptive colonies were covered by a mass of other bacterial species (non-specific growth). Therefore, after performing Gram stain and biochemical test, the presence of *H. pylori* within the mixed culture was confirmed by amplification of *vac*A gen by qPCR and sequencing, as previously described. Sequencing confirmed the growth of *H. pylori* on the SBA plates (98–100% similar to *H. pylori vac*A gene sequence in GenBank) in 56 samples.

Direct spreading of the centrifuged influent samples resulted in the detection of the highest percentage of *H. pylori* positive samples (14 samples, 9% of positive samples). Regarding effluent water samples, the culture strategy of membrane filtration after pre-enrichment of the sample (strategy 1) proved to be the best method (15 samples, 9.7%). (Table 2).

In total, *H. pylori* was cultured in 26 of 155 (16.8%) of the influent water and 30 out of 155 (19.4%) of the effluent water samples. 18.9% (10/53) of the effluent and 11.3% (6/53) of the influent samples from DWTP 1 were positive for *H. pylori* culture. In DWTP 2 *H. pylori* was cultured from 15.4% (8/52) of both types of water. In DWTP 3, 24% (12/50) of both, influent and effluent water samples were positive for *H. pylori* culture. (Table 3).

1.2. Helicobacter pylori detection by qPCR analysis

Among the 155 influent water samples analyzed, 35 samples (22.6%) were positive for *H. pylori* by qPCR (Table 3). For each sample, the amount of DNA or genomic units for *vac*A gene was calculated using *H. pylori* standard curve above described. The quantification of *H. pylori* cells was possible in 13 (8.4%) of the positive samples, with concentration between 1.28×10^1 and 4.69×10^2 genomic units (GU). The remaining 22 positive samples were not quantified as the Ct values were above the reliability threshold (>35 cycles).

Forty-two out of the 155 (27.1%) effluent water samples were positive for *H. pylori* (Table 3 and 4). *H. pylori* could only be quantified in 20 (12.9%) of the positive samples, with concentration values ranging from 5.77 to 2.12×10^3 GU per sample.

Results of the sequencing of all amplicons indicated that all of them were 98–100% similar to *H. pylori vac*A gene (GenBank accession numbers AF049653.1 – CP003904.1 AJ438914.1 – U95971.1) (Complete results, see Supplementary materials).

1.3. FISH analysis of water samples

Cultured *H. pylori* positive samples were analysed by FISH. Specific hybridization showed the presence of rod-shaped and coccoid forms of *H. pylori* cells in both, influent and effluent samples (Fig.2) (Complete results, see Supplementary materials).

2. Discussion

Over the past two decades many authors have suggested that water could be an important route of *H. pylori* transmission (Leja et al., 2016). However, demonstrating this issue is hard, as *H. pylori* is difficult to culture from aquatic environments. By PCR or qPCR *H. pylori* has been frequently detected in environmental samples such as river water (Flores-Encarnación et al., 2015; Fujimura et al., 2004; Moreno et al., 2003a), seawater (Cellini et al., 2004; Mazari-Hiriart et al., 2001), groundwater (Flanigan and Rodgers, 2003), sewage water and drinking water (Benson et al., 2004; Cunachi et al., 2015; Hulten et al., 1996; Moreno et al., 2003a; Santiago et al., 2015).

Cultural methods are extremely limited to recover *H. pylori* from water samples, mainly due to the absence of an optimal selective culture medium (Al-Sulami et al., 2012; Azevedo et al., 2004; Bahrami et al., 2013; Degnan et al., 2003; Lu et al., 2002). The fact that other bacteria present in the environmental samples are able to grow on the *H. pylori* selective media makes its isolation a mayor challenge. Given that in environmental samples *H. pylori* is commonly in the VBNC state, this makes its isolation even more difficult (Azevedo et al., 2004; Fernández et al., 2007).

In this study, several strategies of sample concentration and culture allowed us to recover *H. pylori* from influent and effluent water samples of three DWTP (Table 2). As we expected a very low concentration of *H. pylori* in the effluent samples, we used the Moore swabs method, and a pre-enrichment step to increase the effectiveness of detection. This method has proven to be useful for isolating *Vibrio cholerae* and enteric bacteria from low contaminated waters, such as *Salmonella* from surface (McEgan et al., 2012) and fountain water (Fernández-Escartin et al., 2002), *E. coli* O157 from soil and water (Ogden et al., 2001) and *V. cholerae* from sewage (Barrett et al., 1980). Fernández et al. (2003) showed that samples recovered by "Moore swabs" yielded 24.3% of *Campylobacter* positive river

samples compared to the 7.2 % of positive samples recovered by filtration. In this work, a greater number of positive samples were obtained from the effluent than from the influent, probably due to the use of this concentration method, the pre-enrichment of samples or both of them.

According Enroth and Engstrand (1995), concentration by IMS was applied to PBS concentrates of both influent and effluent samples. This step allows, not only for recovering *H. pylori* based on its immunological properties, but also to eliminate contaminating substances that may interfere with detection tools. Nevertheless, this strategy was not as effective as expected, and it showed the lowest percentage of recovery of the 3 evaluated strategies. This fact could be due to the low concentration of *H. pylori* cells in the samples, as assessed by qPCR, and that probably they were in the VBNC form. Enroth and Engstrand (1995) reported a detection limit of 10^4 bacteria/mL in inoculated water samples when cells were recovered from old cultures. Nilsson et al. (1996) reported also a detection limit of 10^4 cells/mL in inoculated faeces. Lu et al. (2002) obtained 23 *H. pylori* isolates from untreated municipal wastewater by using IMS, probably because a greater load of *H. pylori* in wastewater than in drinking water.

When an enrichment step was applied to the influent samples analysis, the percentage of positive results was much lower than that obtained by direct spread. It could be due to the massive growth of competitive microbiota in the samples because of the lack of specificity of the enrichment broth (Fernández et al., 2007).

In summary, the most effective method for the influent samples was spreading directly 0,1 mL of the PBS suspension from the centrifuged samples. The most effective method for

recovering *H. pylori* from the effluent water samples was the membrane filtration of the pre-enriched sample.

Culturable *H. pylori* cells were detected in 26 out of 155 (16.8%) influent samples and in 30 out of 155 (19.4%) effluent water samples (Table 3). Due to the different methods used to detect *H. pylori*, results about prevalence of *H. pylori* in both types of samples cannot be compared. Moreover, the fact that we just focused on three distribution plants from a restricted geographical area is limiting the comparability of the presented work to broader international context. Other studies have shown that *H. pylori* is rarely isolated by culture from environmental water samples. For example, Al Sulami et al. (2012) reported the isolation of *H. pylori* only in 2% of 198 drinking waters. Fernandez et al. (2007) detected 3 positive mixed cultures from seawater samples, confirming the identification of the bacteria by specific PCR, although *H. pylori* isolation was unsuccessful.

In this work, water samples positive for *H. pylori* yielded also positive results by qPCR and FISH. To detect small concentrations of a pathogen, usually it is necessary to concentrate large volumes of water, which can lead to the simultaneous concentration of qPCR inhibitors (Moreno et al., 2003b). However, the controls of inhibition included in our analysis indicated that samples seem not to contain inhibitor substances that interfered with the qPCR reaction. By qPCR, 35 out of 155 influent water samples (22.6%) and 42 out of 155 effluent water samples (27.1%) were positive (Table 3). Concentration of *H. pylori* cells could only be quantified in 13 (8.4%) influent samples, with concentrations between 1.28×10^1 and 4.69×10^2 genomic units (GU)/reaction, what means levels from 10^2 to 10^3 *H. pylori* cells/mL in these water samples. For effluent samples, although we were able to quantify the amount of cells in 20 (12.9%) samples, these results do not represent the actual level of contamination of the waters, as the sampling method included a pre-enrichment step.

Although qPCR technique has been used previously to detect *H. pylori* in drinking water in the United States (McDaniels et al., 2005) and in drinking water or reclaimed wastewater in Spain, Costa Rica and Japan (Horluchi et al., 2001; Montero-Campos et al., 2015; Santiago et al., 2015; Yáñez et al., 2009), to our knowledge, no study has successfully reported the detection of *H. pylori* by qPCR in both influent and effluent water of different DWTP. Our results show that qPCR is more sensitive than culture to determine the presence of *H. pylori* in water samples This finding may be related to the fact that qPCR does not differentiate between viable, viable but non-culturable (VBNC) and non-viable cells.

It is suggested that *H. pylori* persists in the environment in a viable but nonculturable coccoid form (Nayak and Rose, 2007; Saito et al., 2003). However, the infectious capability of *H. pylori* under environmental conditions is controversial. Wang et al. (2004) showed that the *Vac*A gene sequence and expression in the coccoid forms coincides with that of *H. pylori* helical forms. Saito et al. (2003) reported that the coccoid forms of *H. pylori* are not passive cells since they were able to infect actively the gastric epithelial cells of man, showing that these non culturable cells are infective forms.

Some authors have detected the presence of *H. pylori* DNA only, but not cultivable cells, in drinking water (Khan et al., 2012; Watson et al., 2004). Santiago et al. (2015) used specific techniques as PMA-qPCR and DVC-FISH, for detecting *H. pylori* viable cells in drinking water samples. Their results showed the presence of viable *H. pylori* cells, which could not be detected by culture, in 25% of samples. They also were able to culture *H. pylori* from one sample, confirming that cultivable *H. pylori* can be present in drinking water distribution systems.

In this study, the FISH technique was used for definitive identification of presumptive colonies in mixed cultures. It was also applied to culture-positive samples, in order to observe the morphology of *H. pylori* cells present in the samples. Apart from the current study, few groups were capable of detecting *H. pylori* in non-inoculated water samples using FISH technology (Braganca et al., 2007; Moreno et al., 2003a; Piqueres et al., 2006). The detection of *H. pylori* by FISH can enable rapid analyses of water and sewage, thus improving its safety and quality, and contributing to elucidate the role of fecal contaminated water in the transmission of *H. pylori* infection (Moreno et al., 2003a).

Our work not only demonstrates the presence of *H. pylori* in drinking water in a viable, culturable stage, but also shows that water treatment in DWTP is not totally effective to eliminate *H. pylori*. Information on the chlorination agent and its concentration in the treatment process as well as before discharge into the distribution system, or on the processing time in each facility was not provided to us. Despite this limitations of the study, our results show that *H. pylori* is present in a high percentage of effluent water samples even after chlorination. Previous studies have reported that *H. pylori* loses its cultivability in contact with chlorine for short periods of time (1–5 min), although it remains potentially pathogenic in the VBNC state (Moreno et al., 2007). Our findings are in accordance with a number of other studies reporting that *H. pylori* can survive disinfection practices commonly used in drinking water plants (Johnson et al., 1997; Santiago et al., 2015).

3. Conclusions

Currently, there are no reports of studies evaluating the presence and survival of *H. pylori* in DWTP after disinfection treatment of water. Our study is the first to show the presence of cultivable *H. pylori* cells in DWTP from Bogotá, Colombia.

Both, FISH and qPCR methods are rapid and specific techniques to identify this pathogen in raw and drinking water samples. The combination of a culture procedure with a molecular method, such as qPCR and/or FISH, is a very specific tool for the detection, identification, and direct visualization of cultivable *H. pylori* cells from complex mixed communities such as water samples from DWTP.

Our findings demonstrate that cultivable *H. pylori* cells are present in influent and effluent water samples from DWTP and contribute to elucidate the role of drinking water in transmission of *H. pylori*.

Acknowledgments

Conflict of interests

The authors have no competing interests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version.

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Table 1

Sequences of probes and primers used for FISH analysis.

Probes/ Primers	Sequence	Target	Reference
LNA-HPY	5´-CTG GAG AGA C + TA AGC CC + T CC-3´	16S rRNA of *H. pylori*	Moreno et al., 20003b
EUB338-I	5´-GCTGCCTCCCGTAGGAGT-3´	16S rRNA of Eubacteria domain	Amann et al., 1990
EUB338-II	5´-GCTGCCTCCCGTAGGAGT-3´	16S rRNA of Eubacteria domain	Amann et al., 1990
EUB338-III	5´-GCT GCC ACC CGT AGG TGT- 3´	16S rRNA of Eubacteria domain	Amann et al., 1990
VacA	VacF: 5´-GGC ACA CTG GAT TTG TGG CA- 3´ vacR: 5´-CGC TCG CTT GAT TGG ACA GA- 3´	372 bp fragment of *Vac*A gene	Nilsson et al., 2002

Table 2

Summary of positive *H. pylori* results in influent and effluent samples of the DWTP by culture strategies.

2a: Results in influent samples

Strategy	Positive samples % (n)
1. Direct culture after centrifugation	9% (14/155)
2. IMS	5.2% (8/155)
3. IMS + enrichment	3.9% (6/155)

[n] (n positive samples /n total samples)

2b: Results in effluent samples*.

Strategy	Positive samples % (n)
1. Membrane filtration	9.7% (15/155)
2. Direct culture after centrifugation	7.5% (10/155)
3. IMS	7.1% (11/155)

[n] (n positive samples /n total samples)

*For all methods, samples were submitted to a previous concentration and pre-enrichment steps.

Table 3

Summary of positive *H. pylori* results in influent and effluent samples by culture and qPCR.

Type of water	Positive *H. pylori* cultivation % (n)	Positive *H. pylori* qPCR % (n)	Positive *H. pylori* qPCR quantification % (n)
Influent water	16.8 (26/155)	22.6 (35/155)	8.4 (13/155)
Effluent water	19.4 (30/155)	27.1 (42/155)	12.9 (20/155)
TOTAL	18.1 (56/310)	24.8 (77/310)	10.6 (33/310)

[n] (n positive samples /n total samples)

Fig. 1.

Workflow of detection of *H. pylori* using culture methods. **A**. Workflow from influent samples.

B. Workflow from effluent samples.
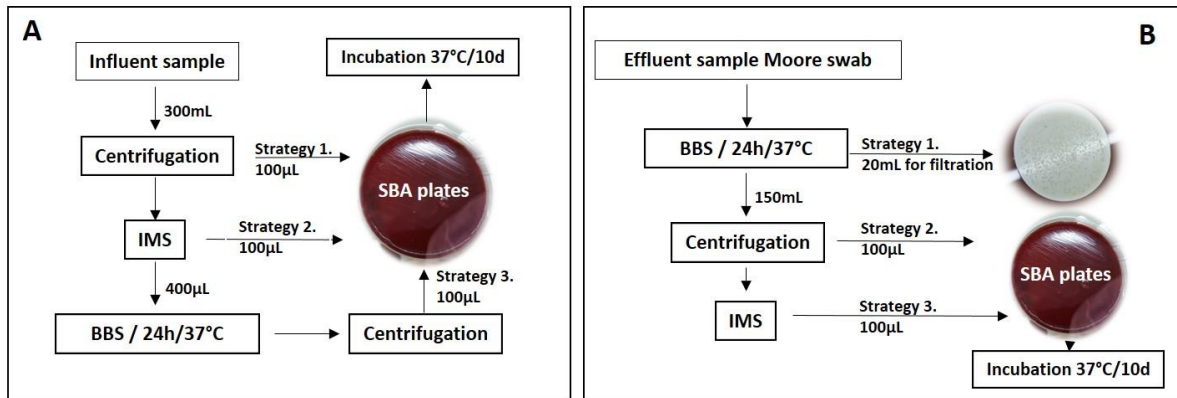
Fig. 2.

Detection of *H. pylori* by FISH in an influent water sample (1000X). **4A**. *H. pylori* hybridized with specific *H. pylori* LNA/rRNA probe. **4B**. The same sample hybridized with EUB 338 mix of probes