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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 (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.,

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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 VBNC 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 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 16S rRNA oligonucleotide probes has been used for detection and identification of *H. pylori* in water samples (Fernández-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 and 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 min 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 h, 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™, USA) supplemented (BBS) with 0,4% Isovitalex (Becton Dickinson BBL™, 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 h. After this pre-enrichment step, 150 mL of each sample was centrifuged at 3000 x g for 20 min and the pellet was resuspended in 2 mL of PBS 1 ×.

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 (6–7 × 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 1 mL 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