Survival of *Helicobacter pylori* in the wastewater treatment process and the receiving river in Michigan, USA

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ABSTRACT

Contaminated water may play a key role in the transmission of *Helicobacter pylori*, resulting in gastrointestinal diseases in humans. The wastewater treatment process is an important barrier to control the transmission of *H. pylori*. However, the presence and viability of *H. pylori* in the treatment process is not well known. In this paper, the real colony morphology of *H. pylori* was confirmed by two types of culture media. The survival of *H. pylori* through the tertiary wastewater treatment process, especially UV disinfection, and in the receiving Huron River in Ann Arbor, Michigan, was investigated by plates cultivation, regular polymerase chain reaction (PCR) assays and quantitative real-time PCR from DNA. The results demonstrated that *H. pylori* was not only present, but also viable in all processed wastewater samples in the Ann Arbor wastewater treatment plant (WWTP). *H. pylori* can be found in a higher concentration in the receiving Huron River. There are many kinds of antibiotic- and UV-resistant bacteria, including *H. pylori*, in the final effluent of Ann Arbor WWTP.

Key words | antibiotic resistance, anti-oxidation, health risk, *Helicobacter pylori*, wastewater treatment

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INTRODUCTION

Helicobacter pylori, a bacterium initially observed in 1893, was not recognized as an infectious agent until 1982 when Warren and Marshall isolated this pathogen (Marshall & Warren 1984). *H. pylori* is a bacterium that infects the stomach of 80% of people living in low socioeconomic areas of Latin America. By contrast, less than 20% of asymptomatic Caucasians carry *H. pylori* in the USA. The WHO estimates that in the world there are over 900,000 new diagnoses and 650,000 deaths from gastric cancer each year, mainly in Eastern Europe, Asia and Latin America. *H. pylori* is a leading cause of gastrointestinal diseases in humans, including gastric and duodenal ulcers, gastric cancer and primary gastric lymphoma (Aziz *et al.* 2015).

Over the preceding years and to date, the definitive mode of human infection by *H. pylori* has remained largely unknown and has thus gained the interest of researchers around the world. Fecal-oral and person to person contact have been suggested as possible routes of exposure to this organism. However, many investigations showed that contaminated water may play a key role in the transmission of *H. pylori* (Baker & Hegarty 2001; Engstrand 2001; Bellack *et al.* 2006).

Survival of *H. pylori* in different types of water has been reported to extend from days to weeks at temperatures between 4 and 15 °C over a wide pH range (Velazquez & Feirtag 1999; Adams *et al.* 2003; Gomes & De Martinis 2004). The water transmission possibility studied by Azevedo strongly argues that drinking water can pose a substantial threat of *H. pylori* infection based on the fulfillment of several essential criteria. These criteria include the ability of *H. pylori* to adhere to different materials, and to co-aggregate with other bacteria and form complex structures on pipes or other surfaces in contact with water (Azevedo *et al.* 2006, 2007, 2008). Biofilms in drinking water systems have been reported

as possible reservoirs of *H. pylori* (Watson *et al.* 2004; Braganca *et al.* 2007; Linke *et al.* 2010). Although different types of disinfectants have been used to maintain microbial safety, *H. pylori* was shown to retain its viability in chlorinated water (Moreno *et al.* 2007; Giao *et al.* 2008) and little is known about its viability in UV disinfected water.

Attempts to culture *H. pylori* cells from environmental water samples have been largely unsuccessful. The cellular morphology of this organism has been demonstrated to change from vegetative rod-shaped cells to a non-culturable coccoid form after storage in water (Keevil 2003; Piqueres *et al.* 2006). However, clear colony morphology has been rarely reported. Entrance of *H. pylori* into the viable but non-culturable state allows *H. pylori* to persist in water (Buck & Oliver 2010). Unsuccessful attempts to culture *H. pylori* from environmental waters have led to the use of molecular methods to detect and identify this organism (McDaniels *et al.* 2005; Guimaraes *et al.* 2007). Several articles have been published using conventional polymerase chain reaction (PCR) to detect *H. pylori* in different types of water including ground water, surface water, treated and untreated wastewater, marine waters and also biofilms (Liu *et al.* 2002; Fujimura *et al.* 2004; Queralt *et al.* 2005; Voytek *et al.* 2005). However, these methods could not differentiate between viable and dead cells.

In this paper, the real colony morphology of *H. pylori* was confirmed by plate cultivation with a selective medium. A molecular detection method for *H. pylori* by specific primers was established. The survival of *H. pylori* through a tertiary wastewater treatment process, especially UV disinfection, and in the receiving river, was investigated by plate culture, regular PCR using specific primers and quantitative real-time PCR.

METHODS

Study area and sample collection

The study area was the tertiary wastewater treatment plant (WWTP) in Ann Arbor, Michigan, USA, and the Huron River was the receiving water for the wastewater discharge, as shown in Figure 1. The plant was designed in 1995 for a population of 210,700, and the average daily flow is 134,000 m^3/day. The plant mostly treats domestic wastewater, with only limited amounts of industrial wastewater; hospital wastewater comprises a very small percentage of total raw influent and is not pretreated. Agriculture is not extensively practiced in this area. The receiving water has a pH value around 6.8 and a biochemical oxygen demand (BOD_5) value around 5 mg O_2/L.

The wastewater treatment process consisted of: (i) preliminary treatment (screening and grit removal); (ii) primary treatment (gravity sedimentation tanks); (iii) secondary treatment (activated sludge process utilizing anoxic/oxic

Figure 1 │ Location of Ann Arbor WWTP and Huron River sampling site.

biological nutrient removal in aeration basins (ferric chloride added as required to polish effluent for soluble phosphorus) followed by secondary sedimentation); (iv) tertiary treatment (rapid sand filtration of secondary effluent to remove particulates); and (v) disinfection (UV light). About 45% of BOD_5 in the raw influent was removed after primary treatment, 95% after secondary treatment, and 99.5% after tertiary treatment.

Samples were collected three times from four sites in the plant, namely raw water (RW), primary treated wastewater (PW), secondary treated wastewater (SW) and final effluent (FW) (after UV disinfection and ready for discharge). Samples were also collected from the Huron River site 100 m downstream (HRW) of the wastewater discharge point. There was no other discharge to the river before this sampling site.

Each time, five samples were collected in sequence on the same day. Each sample consisted of 1,000 mL water collected in a sterile bottle, which was then kept in an ice bath during transportation to the laboratory and processed within 12 h of collection.

Helicobacter pylori culture

Helicobacter pylori (ATCC 43504) was grown on Tryptic Soy Agar (TSA, ATCC 260 Agar) plates with 5% defibrinated sheep blood and a selective culture medium under microaerophilic conditions. Colonies were collected from the media after 7 days' growth at 37 °C.

TSA consists of 1 L water, 15 g Tryptone, 5 g Soytone, 5 g sodium chloride and 15 g agar. Culture conditions: temperature: 37 °C, 7 days; atmosphere: microaerophilic (3–5% O_2, 10% CO_2).

The selective culture medium for *H. pylori* required sequential addition of components. The mixture, containing special peptone, beef extract, yeast extract, NaCl, phenol red, agar and water, was autoclaved for 20 min at 121 °C and then tempered to 50 °C. Then calf serum with iron, antibiotics (vancomycin, trimethoprim, cefsulodin, amphotericin B, and polymixin B), and urea were aseptically added with constant stirring. Finally, 0.8 ml of 1 N HCl was added dropwise to the medium as the color changed from red to yellow-orange (final pH at 45 °C, 5.7; final pH at 22 °C, 6.0). The medium was then poured into Petri plates. The *Helicobacter* cultures were placed in a BBL anaerobe jar with a BBL CampyPak™ Plus sachet, which creates a microaerophilic atmosphere of 5–10% oxygen and 10% carbon dioxide, and incubated for one week at 37 °C (Degnan *et al.* 2003).

Sample pretreatment and DNA extraction

Because of the high density of suspended solids in raw wastewater and primary treated wastewater, water samples were centrifuged first and then transferred for DNA extraction by different kits. For secondary clarifier wastewater and final effluent, they were first passed through a cellulose filter (pore size = 0.45 μm) to retain all bacteria on the membrane, and then transferred for DNA extraction. At the same time, isopropanol treatment for bacterial concentration was compared with centrifugation and filtration methods. As there is much suspended solids in the raw wastewater and the primary clarifier wastewater, Ultraclean Soil DNA isolation kits (MOBIO laboratories, Inc., USA) were used to compare the effect of DNA extraction methods on DNA quality with the metagenomics DNA isolation kit for water (Epicentre Biotechnologies, USA). For the cells on the culture plates, the DNA was extracted using the Wizard Genomic DNA purification kit (Promega Corporation, USA).

Verification of *H. pylori* specific primers and *H. pylori* detection by PCR and q-PCR

As Table 1 shows, three pairs of specific primers were tested and verified for *H. pylori* DNA. HS primers were selected to detect the survival of *H. pylori* in the Ann Arbor wastewater treatment process by regular PCR. Thermal cycling of the PCR reaction was performed with an initial melting at 94 °C for 15 min, and 30 cycles of denaturation at 94 °C for 1 min, annealing at 65, 60, and 62 °C for 1 min and extension at 72 °C for 1 min with a final extension step at 72 °C for 10 min.

Specific *H. pylori* real-time PCR based on SYBR green I fluorescence was carried out using HPF and HPR primers to amplify a 180 bp fragment in a Mastercycler pro Instrument (Eppendorf AG, Germany) to measure *H. pylori* copies in the wastewater treatment process and the receiving river. In the meantime, 338F and 518R were used to measure total bacterial copies by q-PCR. A positive control with *H. pylori* DNA was added to the q-PCR analysis.

All PCR products were analyzed by gel electrophoresis in a 1% (w/v) agarose gel prepared in 1.0x Tris acetate

Table 1 │ Specific primer sequences for *H. pylori* identification in water

Reference	Sequence	Product size
Crystal (2010)	HS1 5′AACGATGAAGCTTCTAGCTTGCTAG HS2 5′GTGCTTATTCGTTAGATACCGTCAT	400 bp
Thoreson *et al.* (1995)	Act1 5′-CTT GCT AGA GTG CTG ATT A-3′ Act2 5′-TCC CAC ACT CTA GAA TAG T-3′	527 bp
Gramley *et al.* (1999)	HpF 5-GCG ACC TGC TGG AAC ATT AC-3 HpR 5-CGT TAG CTG CAT TAC TGG AGA-3	180 bp

EDTA (TAE) buffer. Gels were run for 30 min at 90 V (BIO-RAD Sub-cell GT Agarose Gel Electrophoresis system; BIO-RAD Laboratories, California) and DNA bands examined under ultraviolet illumination.

RESULTS AND DISCUSSION

### *H. pylori* colony morphology and specific primers confirmation

As attempts to culture *H. pylori* cells from environmental water samples have been largely unsuccessful, we had to find an effective culture medium to grow *H. pylori* and verify the colony morphology. We ordered *H. pylori* (ATCC 43504) and tried TSA with 5% defibrinated sheep blood and selective culture media to cultivate and collect *H. pylori* cells. As Figure 2 shows, the real *H. pylori* colony looks transparent and has a small circular bulge on the TSA plate. The color indicator system in the selective medium can produce urease, which results in hydrolysis of urea to ammonium and bicarbonate and neutralizes a discrete area around the *H. pylori* colonies and turns them red because of the pH change of the medium from about 6.0 to >7.5 (Degnan *et al.* 2003).

Figure 2 │ Morphology of *H. pylori* colony on TSA (left) and selective culture medium (right).

PCR reaction with three pairs of *Helicobacter pylori* 16S rRNA gene primers were tested with pure strain DNA samples (ATCC 43504) on different culture media to test the specificity (Table 1). As Figure 3 shows, these three pairs of specific primers for *H. pylori* detection were verified by regular PCR for the pure strains on TSA and a selective medium.

Effects of different DNA extraction methods on *H. pylori* DNA quality

As Table 2 shows, the metagenomics DNA isolation kit for water can give a better DNA quality for PCR assay when compared with the UltraClean soil DNA isolation kit for different pretreated water samples. However, there were no significant differences for DNA quality between conventional cell pre-treatment such as centrifuge or filtration and isopropanol treatment. As Figure 4 shows, when the extracted DNA was purified, it can give better PCR results for *H. pylori* detection. However, DNA extracted by soil kit cannot show a clear band on the electrophoresis gel.
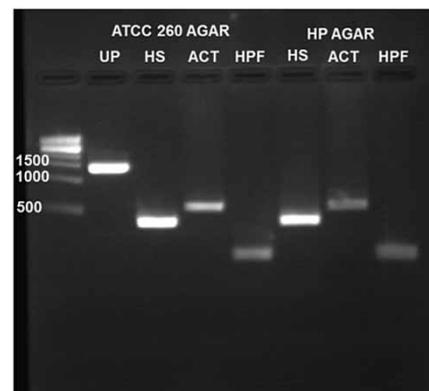
Figure 3 │ Specific primer verification of *H. pylori* by regular PCR (UP: universal primer).

Table 2 | DNA characters by different extraction methods

	CRW[a]	CPW[b]	FSW[c]	FFW[d]	IRW[e]	IPW[f]	ISW[g]	IFW[h]	CRS[i]	CPS[j]	IRS[k]	IPS[l]
C_{DNA}(ng/μL)	926.6	307.5	51.1	29.1	823.9	298.6	74.9	33.2	19.8	16.6	34.3	19.4
260/280	1.68	1.63	1.67	1.56	1.82	1.74	1.67	1.61	1.54	1.42	1.56	1.65
260/230	0.79	0.84	1.07	1.11	1.36	1.32	1.13	0.95	0.82	1.60	0.81	2.24

[a]CRW: Centrifuge raw wastewater and extract DNA by kit for water.
[b]CPW: Centrifuge primary clarifier wastewater and extract DNA by kit for water.
[c]FSW: Filtered secondary clarifier wastewater and extract DNA by kit for water.
[d]FFW: Filtered final effluent and extract DNA by kit for water.
[e]IRW: Isopropanol processed raw wastewater and extract DNA by kit for water.
[f]IPW: Isopropanol processed primary clarifier wastewater and extract DNA by kit for water.
[g]ISW: Isopropanol processed secondary clarifier wastewater and extract DNA by kit for water.
[h]IFW: Isopropanol processed final effluent and extract DNA by kit for water.
[i]CRS: Centrifuge raw wastewater and extract DNA by kit for soil.
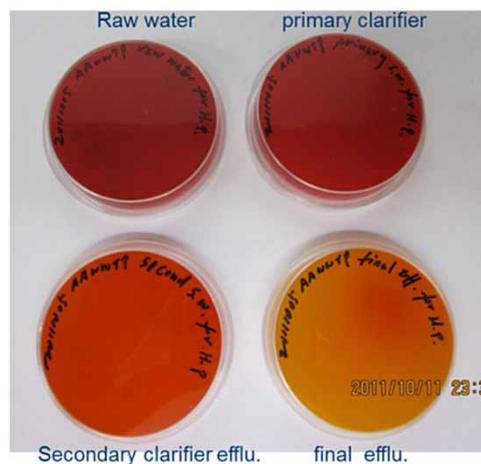[j]CPS: Centrifuge primary clarifier wastewater and extract DNA by kit for soil.
[k]IRS: Isopropanol processed raw wastewater and extract DNA by kit for soil.
[l]IPS: Isopropanol processed primary clarifier wastewater and extract DNA by kit for soil.
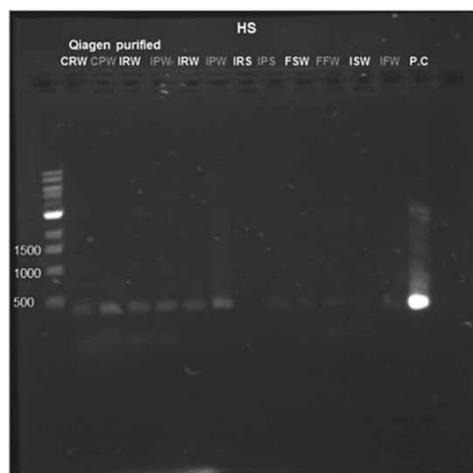
Figure 4 | Detection of *H. pylori* from wastewater in AA WWTP by HS specific primer.

Figure 5 | *H. pylori* cultivation for water samples from AA WWTP by selective agar plates.

Detection of *H. pylori* in the tertiary wastewater treatment process and Huron River

As Figure 4 shows, in all processed water samples in the tertiary wastewater treatment process in Ann Arbor, the DNA of *H. pylori* cells can be found. Although molecular techniques such as PCR can demonstrate the presence of *H. pylori* in water, DNA isolation alone fails to provide any indication about the viability of this pathogen. As Figure 5 shows, *H. pylori* cells in four main water processed samples along the Ann Arbor wastewater treatment process were detected by *H. pylori* selective culture media. The different color depth in the plates shows the different amount of

living *H. pylori* cells. The amount of *H. pylori* cells decreased through the treatment process. The existence of *H. pylori* cells in Ann Arbor WWTP and the Huron River had also been confirmed by quantitative real-time PCR for the natural water samples and cultured water samples in Figure 6. They had nearly the same variation tendency for natural water samples as shown in Figure 5. However, the cell numbers of *H. pylori* on culture plates did not show the same tendency according to the q-PCR results in Figure 6.

As there were five types of antibiotics (vancomycin, trimethoprim, cefsulodin, amphotericin B, and polymixin B) added in the selective medium, Figures 5–7 showed that there were many bacteria, including *H. pylori*, in the final effluent that were antibiotic resistant and UV resistant.
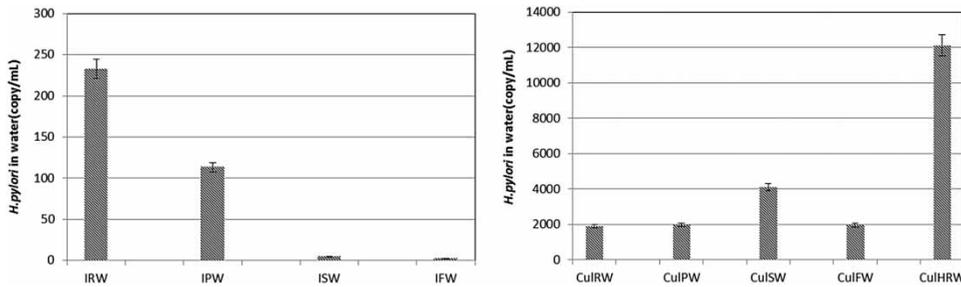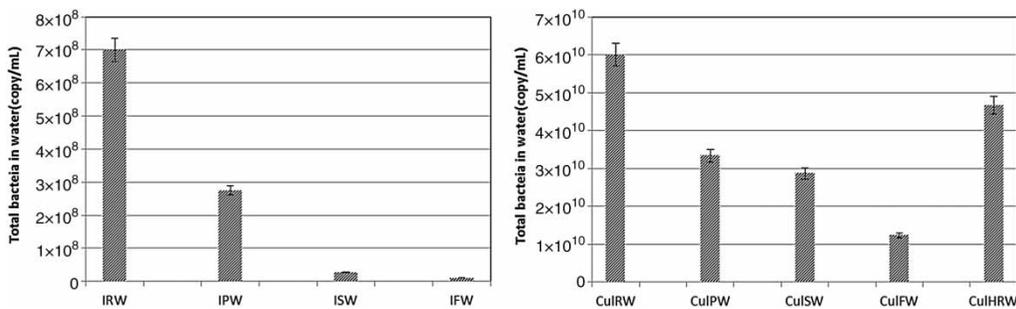
Figure 6 │ *H. pylori* in wastewater by real-time PCR.

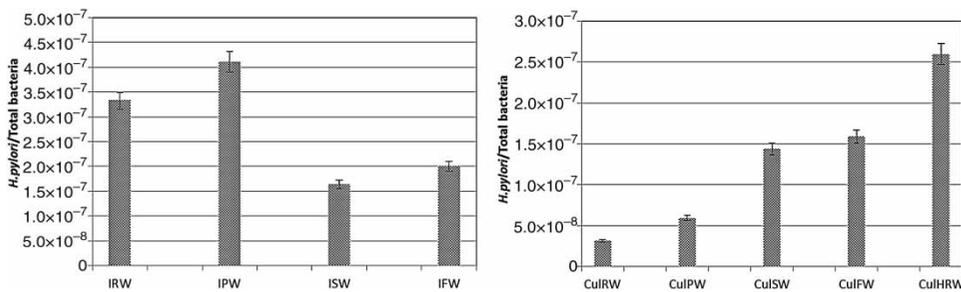**Figure 7** │ Total bacteria in wastewater by real-time PCR.

Figure 8 │ Ratio of *H. pylori* to total bacteria in the wastewater and Huron River.

However, *H. pylori* may be more resistant to antibiotics and antioxidants, as the ratio of *H. pylori*/total bacteria increased through the wastewater treatment process (Figure 8). In the receiving Huron River, *H. pylori* cells were also found, which meant these *H. pylori* cells may pose a potential risk to human health (Figure 6). A high number of *H. pylori* cells at the Huron River sampling site also meant contamination by *H. pylori* from some non-point sources of pollution.

CONCLUSIONS

The real colony morphology of *H. pylori* was confirmed by culture plates with TSA and selective medium. *H. pylori* was found in all processed wastewater samples in Ann Arbor WWTP and the receiving Huron River. There are many antibiotic- and UV-resistant bacteria, including *H. pylori*, surviving in the final effluent of Ann Arbor WWTP and flowing into the Huron River.

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