Risk of Infection from Exposure to Waterborne *Helicobacter pylori*?

By

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Dedication

For Liz and Rebecca

"Thinking a lot about less and less…
And forgetting, the love we bring." *Althea*, Jerry Garcia and Robert Hunter

"Well I heard there was a secret chord
That David played and it pleased the Lord
But you don't really care for music, do you?
Well it goes like this: the fourth, the fifth
The minor fall and the major lift
The baffled king composing Hallelujah" *Hallelujah,* Leonard Cohen

"The capital-T Truth is about life before death…. It is about simple awareness — awareness of what is so real and essential, so hidden in plain sight all around us, that we have to keep reminding ourselves, over and over: "This is water, this is water." *This is Water*, David Foster Wallace

"Mama said, Don't go near that river,
Don't be hanging around old Catfish John
Come in the morning I'd always be there,
Walking in his footsteps in the sweet Delta dawn." *Catfish John*, Bob McDill and Allen Reynolds

"Oh, the hours we've spent inside the Coliseum,
Dodging lions and wastin' time.
Oh, those mighty kings of the jungle, I could hardly stand to see 'em,
Yes, it sure has been a long, hard climb.". *When I Paint My Masterpiece*, Bob Dylan

"What you are is what you have been. What you'll be is what you do now." Gautama Buddha

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H. pylori: *Helicobacter pylori*
PCR: Polymerase chain reaction
qPCR: Quantitative polymerase chain reaction
SCID: Severe combined immunodeficient
SES: Socioeconomic status
TSA: Tryptic Soy Agar
VBNC: Viable but non-culturable

Abstract

Helicobacter pylori (*H. pylori*) is a stomach bacterium that, while asymptomatic in most people, can cause a cascade of gastric pathology leading to the development of gastric cancer. For this reason, it has been categorized as a class 1 carcinogen. When outside the human stomach, *H. pylori* undergoes a morphological and metabolic change, transitioning into a viable but nonculturable (VBNC) state. This transition has made it challenging to characterize transmission and the infection risk associated with different routes of *H. pylori* exposure.

In 1991 in Lima, Peru, researchers first associated *H. pylori* infection with water source. Since then, *H. pylori* has been associated with lack of access to clean drinking water and sanitation, and the bacterium has been identified in drinking water sources. Working together with Peruvian stakeholders and scientists at the University of Michigan, this dissertation aims to provide information that will help better characterize the risk posed by waterborne *H. pylori*. Thus, the overarching hypothesis of my dissertation is that water can act as a reservoir for *H. pylori*.

In Chapter 1, we conducted an exposure assessment, quantifying the extent to which citizens of Lima are exposed to *H. pylori* via drinking water. After collecting water from a single location in Lima for one year, we examined the frequency and quantity of *H. pylori* found over that time. We show that drinking water in Lima is consistently contaminated with *H. pylori*, without any patterns related to seasonality.

In Chapter 2, we developed a mouse model to estimate the infectious dose of *H. pylori* in drinking water. As proof of principle, we exposed mice to drinking water contaminated with

viable, culturable *H. pylori* strain SS1 (a strain the is known to colonize mice effectively) in various doses over 4 weeks. We found a clear dose-response relationship: higher doses of *H. pylori* in drinking water cause higher incidence of infection, showing that *H. pylori* in water could be infectious. To gauge the infectivity of the VBNC form, we exposed mice to various exposure scenarios of VBNC *H. pylori* in drinking water, with ingested doses ranging from 1E6 to 2.1E9 VBNC cells. To our surprise, none of the mice were infected. This suggests that strains of *H. pylori* may have variability in infectivity between their viable, culturable and their VBNC form. It also suggests that VBNC *H. pylori* in water may not contribute heavily to the overall burden of *H. pylori* infection seen globally.

In Chapter 3, we examined two different strategies to control *H. pylori* infection in Lima. First, we investigated primary antibiotic resistance of *H. pylori* isolates from a clinical setting in Lima and the success rate of antibiotic therapy for treating *H. pylori* infection in Lima. We found that antibiotic resistance to first-line antibiotics amoxicillin and clarithromycin was fairly high (29.7% and 35%, respectively), but that resistance to the second-line antibiotic tetracycline was very low (3%). We also showed that the success rate of antibiotic therapy 6-8 weeks after the initial therapy was 65% - substantially lower than the standard 80-90% success rate hoped for with *H. pylori* infection. This suggests that while antibiotic therapy is still a viable treatment plan in Lima, more targeted treatment strategies (e.g. testing the antibiotic resistance profile of the infecting strain before treatment) for individuals with *H. pylori* infection could result in better clinical outcomes. Secondly, we tested the *H. pylori* reduction capacities of bleach disinfection and boiling water. We exposed culturable *H. pylori* to bleach for 5, 15, or 30 minutes, or brought water inoculated with *H. pylori* to a boil for 0, 1, or 5 minutes. We found that both bleach and

boiling disinfection results in total loss of culturability in *H. pylori* in water. This suggests that both water treatment options are likely useful for eradicating *H. pylori* in drinking water.

This research helps shed light on several knowledge gaps. First, in the longest sampling campaign conducted to date, we show that water in Lima is consistently contaminated with *H. pylori*. Second, we proved that *H. pylori* in drinking water can be infectious in mice. Third, we highlight the differences in infectivity between the VBNC and culturable forms of *H. pylori*, which may cast doubts on the importance of drinking water as a primary source of *H. pylori* infection. Fourth, we show that antibiotic resistance among *H. pylori* is high in Lima, Peru, and provided the most thorough characterization of antibiotic resistance in Lima in the published literature. Fifth, we show that boiling and bleach disinfection may be effective water treatment strategies for waterborne *H. pylori.*

Our exposure assessment is congruent with the hypothesis that *H. pylori* can be transmitted in water, but a better understanding of the population dynamics of *H. pylori* in water is necessary to characterize the risk of infection. Given that *H. pylori* typically exists in the VBNC form in water, our mouse data cast doubt on the importance of water as a reservoir of infection. However, more studies are needed that both examine the infectiousness of multiple strains of *H. pylori* as well as characterize factors – such as pH, the presence of other pathogens – that contribute to more or less efficient infection. With regards to infection control, given the uncertainty associated with *H. pylori* transmission in water, focusing on better and more targeted treatment of infection is a more important in the short term. More research is needed to evaluate the importance of preventing *H. pylori* transmission via water treatment.

Chapter 1 : Introduction

Helicobacter pylori, a gram-negative spiral bacillus bacterium that colonizes the human stomach, has been a mysterious microbiological mover and shaker since its discovery. First isolated in the 1980s, *H. pylori* overturned the idea that the stomach was sterile [1]. Soon after, Robin Warren and Barry Marshall identified *H. pylori* in the stomachs of patients with active gastritis and peptic ulcer [1], and Barry Marshall showed that *H. pylori* could cause gastritis by drinking a live culture of the bacterium [2]. Following this self-experimentation, research in the field has expanded rapidly, and *H. pylori* has now been consistently linked to peptic ulcer disease and categorized as a class 1 carcinogen, causing ~90% of the nearly 720,000 deaths each year from gastric cancer [3,4]. Infection with *H. pylori* is associated with a 3- to 6-fold increased risk of gastric cancer [5], though this burden falls unevenly upon low- and middle-income nations (such as Peru), where the rates of *H. pylori* infection are higher [6]. Indeed, while an estimated 50% of the world's population harbors *H. pylori*, only 20-30% of people in high-income nations carry the bacterium compared with up to 90% of people in low- and middle-income nations [6–9].

As ever, though, the picture is more complicated than a simple "*H. pylori* bad" paradigm. Only 10-20% of people who are infected with *H. pylori* ever develop negative symptoms [5], and early-life *H. pylori* infection is associated with protective effects against asthma and allergies, as well as against esophageal cancer later in life [10–12]. The mechanistic underpinnings of this protective effect have begun to be supported by animal studies [13–15]. To further complicate the picture, *H.*

pylori is difficult to isolate from the environment. When outside the human stomach, *H. pylori* typically changes morphology from spiral bacillus to a coccoid form. When in this coccoid state, *H. pylori* is challenging to culture, though multiple studies have shown that coccoid *H. pylori* is membrane-intact [16], metabolically active, and, in mouse studies, infectious in very high doses [17–19].

The dominant route of *H. pylori* transmission is unclear. Epidemiologically, *H. pylori* infection is associated with low socioeconomic status (SES) crowded living conditions, and a lack of access to clean water and sanitation – especially in childhood [7,9,20]. Children with an infected sibling have a higher likelihood of becoming infected themselves [21,22]. *H. pylori* DNA has been found in multiple bodily areas and excretions, including saliva, dental plaques, the mouth, tonsils, esophagus, vomit, and feces suggesting the possibility of an oral-oral or fecal-oral route [7,9,23–26]. The presence of *H. pylori* in excreta also points to the possibility of indirect transmission, including through fecal-contaminated reservoirs such as food and water [7]. In this dissertation, the focus is on water as a potential transmission route in Peru, where gastric cancer is the leading cancer killer in men and women combined [27], and where there is a special interest in *H. pylori* contamination of water.

This dissertation encompasses work that is a part of an existing collaborative project that began in 2010 between clinicians and public health officials in Peru and scientists and clinicians at the University of Michigan. This projects falls under the paradigm of an integrated assessment (IA) - an interdisciplinary project that brings together perspectives from government, communities, and industry to address a common goal [28]: in this case, to better understand the role of contaminated

water as it relates to *H. pylori* in Lima, Peru. There has been dynamic interplay between the technical teams. consisting of scientists and physicians at the University of Michigan and the stakeholders, consisting of public health officials at the Peruvian Ministry of Health and local scientists and clinicians in Lima, Peru. The teams work together to collect and analyze data, and evaluate options that can be used to inform decision making (Figure 1). Because of the unique stakeholder perspective, this framework provides responsive feedback to researchers, who can then incorporate that feedback into the next sampling campaigns and planned experiments.

Clinicians, scientists, and public health officials in Peru have been interested in water as a potential source of *H. pylori* infection since 1991, when Klein et al associated *H. pylori* infection with the source of drinking water [30]. Conducted in Lima, Peru, this study linked *H. pylori* infection with the source of drinking water. After adjusting for SES, age, weight, and other demographic information, children with an external water source had ~3 times higher odds of infection than those with an internal water source, and that children from high SES families were 11.4 times more likely to be infected if they consumed municipal tap water rather than private well-water. Since that time, additional studies have supported this finding. In a cross-sectional study in Kazakhstan, Nurgaviela et al found that individuals drinking river water had a 13.6 times higher odds of *H. pylori* infection than those who did not [31], and Baker et al linked contaminated private well-water with higher likelihood of *H. pylori* infection in individuals in Pennsylvania [32]. In a large study of 1,852 people in six Latin American countries, Porras et al found higher odds (OR: 1.3, 95% CI 1.0-1.8) of *H. pylori* infection in those lacking indoor plumbing [33]. In Malaysia, Lee et al found that univariate associations between drinking well water, as well as certain unsanitary practices (including using pit latrines and not washing hands

before eating or after using the toilet) with *H. pylori* infection [34], but did not run further models to see how these associations were affected by adjusting for co-variates. None of these studies had a full medical history of individuals, however, and it is possible that infected individuals had acquired *H. pylori* from an infected family member or an outside reservoir of infection. While these studies do not show causality, they remain suggestive, especially considering the large body of evidence suggesting that *H. pylori* is present and viable in water.

H. pylori can survive in water distribution systems and biofilms – extra-cellular proteinaceous structures that can protect bacteria from external stressors [35–37]. Biofilms also present a potential mechanism through which *H. pylori* can be consistently present in water, as cells may grow in and subsequently shed from biofilms into water. *H. pylori* has now been identified in various aquatic environments (drinking water, well water, wastewater, river water, and marine water) through DNA-based culture-independent methods, including PCR and quantitative PCR (qPCR) [32,38–45]. These methods can detect and quantify the amount of *H. pylori* DNA in water, though they cannot determine the viability of the DNA's source. Other culture-independent methods include microscopy techniques, such as fluorescent in-situ hybridization [16,39,46], which can determine whether *H. pylori* cells have intact membranes and are viable. Recently, several investigators have developed novel methods to culture *H. pylori* from previously inaccessible environments: from drinking water [39,47,48], wastewater [49,50], and a co-habitation with the marine copepod *Tigriopus fulvus* [51], though these studies have not yet produced a standardized method for isolating *H. pylori* from the environment. While these culture-based techniques provide

definitive proof that *H. pylori* is present and viable in these environments, they do not provide insight into how common *H. pylori* is in water, nor to the infection risk it poses.

It remains unknown whether the presence of *H. pylori* in water reflects a high proportion of infection and shedding in the population, or whether it is due to a true infectious risk. A recent quantitative microbial risk assessment (QMRA) assessed the potential for a drinking water guideline for *H. pylori* [52], but the input data were quite limited. At that time, there were no quantitative reports of drinking water contamination with *H. pylori*, so the authors had to estimate exposure based on measures of *H. pylori* in surface and recreational water [43,44]. Further, while there were two reports in the literature relating ingested dose to infection, both studies were conducted using the viable, culturable state of *H. pylori* (in the spiral bacillus form), and neither of them was performed in the matrix of drinking water [53,54]. Though some studies have investigated the infectivity of the VBNC form, they were done using gavage – a technique that directly inoculates the stomach with a large bolus of bacteria – and thus are not representative of drinking water exposure. Further, no studies have examined the infectious dose of viable, culturable or VBNC *H. pylori* in water, so it is unknown whether these forms are equally infectious in this exposure route. Finally, there is a paucity of evidence about the effectiveness of point-of-use water treatment options for removing or eradicating *H. pylori*, though some studies have shown that it may remain viable after exposure to chlorine in drinking water [35,46].

With these gaps in the literature and the historical context of gastric cancer being the leading cause of cancer mortality among men and women combined in Peru, there has been an interest and a pressing public health need to better understand how *H. pylori* transmitted and whether

treatment options (both clinically and environmentally) are effective for controlling the spread of *H. pylori* infection. The epidemiological associations and findings of *H. pylori* in water suggest that contaminated water could be a reservoir for infection. Thus, the overarching hypothesis of this dissertation is that drinking water may act as a reservoir for *H. pylori* infection. The objective was to collect data under the integrated assessment approach to ultimately provide data driven advice to policy makers in Lima, Peru.

In the first aim, we conducted an exposure assessment of the *H. pylori* in drinking water in Lima, Peru, examining both the presence/absence and quantity of *H. pylori* found, as well as water characteristics (e.g. pH and temperature) that may contribute to water contamination with *H. pylori.* In the second aim, we investigated the infectiousness of *H. pylori* in drinking water using mice as a model organisms, examining the infectivity of both the viable, culturable form and the viable but non-culturable form of *H. pylori.* In the third aim, we evaluated two control approaches for *H. pylori* infection. First, we assessed the success of antibiotic treatment for *H. pylori* infection, and determined primary antibiotic resistance profiles among clinical isolates of *H. pylori*. Second, we evaluated the *H. pylori* eradication capacities of inexpensive water treatment options (bleach and boiling water) available in Peru.

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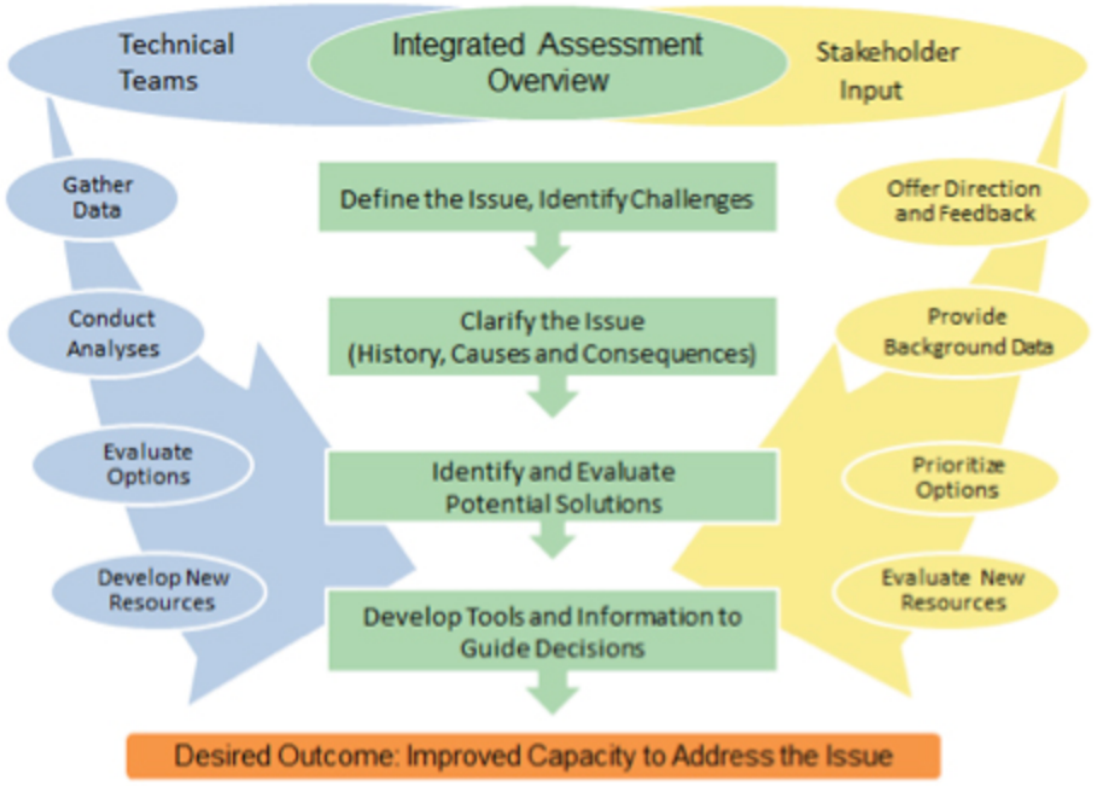

Figure 1.1. The integrated assessment framework. Adapted from Grace et al [29].

Chapter 2 : Examination of Drinking Water Contamination with *Helicobacter pylori* in Lima, Peru

Chapter 2.1: An Assessment of Drinking Water Contamination with *Helicobacter pylori* in Lima, Peru

Introduction: *Helicobacter pylori* (*H. pylori*) is a stomach bacterium that, while asymptomatic in most people, can cause a cascade of gastric pathology leading to the development of gastric adenocarcinoma [1,2]. For this reason, it is categorized as a class 1 carcinogen [1,3]. *H. pylori* infection is hypothesized to be transmitted directly through fecal-oral, oral-oral, or gastro-oral routes, or indirectly through reservoirs, including food and water. In 1991, Klein et al. found higher odds of *H. pylori* infection among study participants in Lima, Peru with municipal drinking water compared to those using private wells [4]. Since that time, lack of access to clean drinking water and proper sanitation has been identified in epidemiological studies as a risk factor for *H. pylori* infection [5–13]. *H. pylori* rapidly changes morphology from a spiral bacillus to a coccoid form in water, entering a viable-but-not-culturable (VBNC) state that makes it challenging to culture [14–16].

Recently, however, five independent studies have isolated and cultured *H. pylori* in wastewater and drinking water [17–21]. In addition, *H. pylori* has been reliably detected in recreational and drinking water using molecular biology techniques such as PCR and fluorescent in-situ hybridization [4,14,15,22–24], and can survive in water distribution systems, likely through protection from biofilms [25,26]. Finally, the VBNC form of *H. pylori* has been shown to be

infectious in mice via gavage [27,28], and we previously showed that waterborne *H. pylori* is infectious in mice [29].

While it is plausible that water contaminated by *H. pylori* is a route for the transmission of *H. pylori* infection, the quantities of *H. pylori* found in drinking water (and thus the risk of infection from such sources) remain poorly characterized. Existing studies have measured the quantities of *H. pylori* in wastewater [23], surface water [30], and recreational water [31], and several studies have measured the presence/absence of *H. pylori* in drinking water using PCR [4,21,32,33]. To our knowledge, only two studies have quantitatively measured *H. pylori* in municipal drinking water: one by our group in Lima, Peru [24], and the other in Spain [34]. Both studies had limited sample sizes (n=87 and n=24, respectively), and were conducted in a variety of locations. In Peru, the highest quantity of *H. pylori* found in drinking water was 1.6E6 genome copies/L [24], while in Spain, it was 1.59E3 genome copies/mL [34].

Our goal in this study was to better characterize the quantities and variation of *H. pylori* in drinking water over time, so we conducted a quantitative assessment of *H. pylori* in water in Lima, Peru, both cross-sectionally from several wells as well as repeated sampling from a single sink in the Lince district. We hypothesized that drinking water in Lima was contaminated with *H. pylori*, and that the quantities of *H. pylori* would vary seasonally.

Materials and Methods

Locations and sample numbers

Three separate water sampling studies were conducted in Lima, Peru. First, in 2013, five water samples were collected from wells, which are used to supplement the drinking water supply downstream of the municipal treatment plant. These wells were located in the districts of Surco, El Agustino, Puente Piedra, and Comas. Second, 17 drinking water samples were collected

between June 19th and July 18th of 2014 from a single sink in the Lince district in Lima. Samples were collected once to twice per week, except during the week of June 30th-July 4th, when samples were collected twice per day from Monday-Friday. Finally, 241 total drinking water samples were collected once a day, five days per week from a single sink in the Lince district in Lima from June 2015 through the end of May 2016.

Water sample collection

Sterile bottles with sodium thiosulfate were prepared prior to sampling. 1L aliquots of drinking water were collected from the faucet after allowing the water to run for at least one minute, and then concentrated by vacuum filtration onto 0.22μM filter membranes [24]. Water quality parameters including pH, temperature, and conductivity were monitored, and free available chlorine was measured using DPD among samples collected from June 2015-May 2016. Samples were handled per the US Geological Society guidelines [35]. All membranes were stored at -80°C until processing and analysis at the University of Michigan.

DNA extraction from membranes

Well-water and drinking water samples from 2014: Membranes were thoroughly scraped in 1x PBS (0.2% Tween 20). After scraping, the eluant was transferred to a 1.5mL tube and centrifuged for 5 minutes at 16,000g. The supernatant was removed, and DNA was extracted from the resulting pellet using the QiaAMP DNA Mini Kit (Qiagen, Hilden, Germany) per the manufacturer's instructions. DNA was eluted in 100uL of buffer AE.

Time-series sampling from June 2015- May 2016: The following phenol chloroform protocol for DNA extraction was adapted from Holinger et al. 2014 [36]. Each 0.22 μM filter (EMD Millapore, Ontario, Canada) was cut into >20 pieces using sterilized scissors, and placed into 2mL tubes holding ~0.5 g of 0.1 mm silica/zirconium beads (Biospec Products, OK, USA), 500 μL of phenol/chloroform/isoamyl (25:24:1) and 500 μL of lysis buffer (75mM NaCl, 75 mM TRIS pH 8.0, 7.5mM EDTA, 2.85% SDS). Samples were mechanically bead beaten for 2.5 minutes at high speed to separate cells from the membrane and lyse the cells. To separate phases, tubes were centrifuged for 7 minutes at 16,000g. Following centrifugation, ~450 μL of aqueous phase was transferred to a new 1.5 mL tube. 10 μL of glycogen (10 mg/mL), 200 μL of 7.5M-ammonium acetate and 650 μL of isopropanol were then added to precipitate the DNA. The samples were then centrifuged for 25 minutes at 16,000x g to pellet the DNA. Afterwards, the supernatant was removed, and pellets were washed with 1 mL of cold 70% ethanol. The samples were then inverted 15-30 times and centrifuged for 10 minutes at 16,000x g. After removal of ethanol, the pellets were dried at 35°C for 1-2 hours using a vacuum spinner. DNA Pellets were suspended in 40 μL of 10mM TRIS with 1mM EDTA, pH 8.0. Following DNA extraction, samples were purified by washing in 1mL of 4°C 70% ethanol and 10 μL of 3M-sodium acetate. After inverting the sample tubes 15-30 times, they were centrifuged for 10 min at 16,000x g. Ethanol was removed, and pellets were dried at 35°C for 1-2 hours using a vacuum spinner. Pellets were again suspended in 40 μL of 10mM TRIS and 1mM EDTA, pH 8.0.

qPCR (quantitative polymerase chain reaction)

qPCR was performed on extracted DNA using a highly sensitive, previously established method [37]. Briefly, quantities of *H. pylori* in drinking water were quantified using a reaction mixture

containing 10 μL 2×SYBR GREEN PCR Master Mix (Applied Biosystems, Grand Island, NY, USA), 0.3 μL of each 20 μM primers HpA-F (ACTTTCTCGCTAGCTGGATGGTA) and HpA-R (GCGAGCGTGGTGGCTTT), 8.9μL of sterile PCR water, and 0.5 μL of DNA template. Plates also included negative controls (no DNA added) and positive controls (*H. pylori* strain SS1 DNA), and a standard curve made with 0.5E1 to 5E5 genome copies of *H. pylori* strain SS1 DNA. qPCR was run at the following conditions: 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds and 60°C for 1 minute, followed by a melting curve analysis, ramping from 60°C to 95°.

Statistical analysis

All statistical analyses were conducted in R Studio. Due to limited sample size, no statistical analyses other than descriptive statistics were examined among water samples from 2014 (drinking water and well-water).

The following statistical analyses were run only on samples from the June 2015-May 2016 sampling period. Descriptive statistics were used to examine the distribution of *H. pylori* contamination, pH, temperature, conductivity, and free available chlorine. Time-series plots were constructed to examine potential seasonal patterns in water characteristics.

Due the large number of negative samples (~80%), two models were run to account for zero-inflated data. The first was a logistic regression that modeled the presence/absence of *H. pylori*, based on all samples. The second was a linear regression modeling the quantity of *H. pylori* as an outcome, run on non-zero samples only. Put together, these regressions model the presence or absence of *H. pylori* in the sample, and, conditional on being a positive sample, the quantity present. Both models were adjusted for all water characteristic co-variates. Since the data arise as a time series because sampling occurred from a single location over the course of one year, two approaches were used to account for the possibility of autocorrelation in the

samples (i.e., non-independence from day-to-day). First, the prior day's presence/absence of *H. pylori* contamination was included as a predictor in both models. Second, the autocorrelative effects of date on the presence/absence of *H. pylori* was measured in the logistic regression by incorporating a smooth function of date of sample using the R 'gam' package. The smooth function adjusts for autocorrelation by modeling the potential long-term calendar trends in the presence/absence of *H. pylori*. Smoothing functions were also used to investigate potential non-linearity in the effect of the remaining water characteristics on the presence of *H. pylori.*

Model residuals were used to examine model fit and to identify potentially outlying values. The influence of extreme values was examined by removing these values from the model and examining the resulting robustness of the models. Due to the highly-skewed distribution of residuals, the quantities of *H. pylori* were log-transformed in the linear regression model.

Results

H. pylori in drinking water

3/5 samples collected from wells and 9/17 drinking water samples collected in the summer of 2014 showed contamination with *H. pylori* (Table 2.1). There was consistent contamination of drinking water with *H. pylori* throughout the sampling period from June 2015-May 2016. About 20% of samples were positive (49/241), every month had at least one day with contaminated *H. pylori*, and the longest stretch of time without a positive sample was 25 days (Figure 2.1).

Water characteristics

There were some missing measurements in the water characteristics data due to lack of reagent or instrumentation on that sampling day. Temperature was measured in 240/241 samples, pH in 238/241, conductivity in 232/241, and free available chlorine in 209/241. The World Health Organization recommends that the free chlorine residual available in drinking water should be

between 0.2 and 0.5mg/L [38]. 189 of 209 samples were at or below 0.5 mg/L and 17/209 samples were below the minimum recommended residual of 0.2mg/L of Free chlorine (Table 2.2). While there is no official standard for pH in drinking water, the EPA recommends that the pH of drinking water should be above 6.5 and below 8.5 to avoid corrosion. 96 of the 238 samples were below the minimum recommended guideline of 6.5 [39]. Temperatures ranged from 19.7-27.7°C, and conductivity ranged from 326-616 µS/cm.

Associations between water characteristics and H. pylori

We found a significant negative association between temperature and presence of *H. pylori*, regardless of the method of analysis. Accounting for date using a smoothing function and all other co-variates, we found that the log odds of the presence of *H. pylori* was 37% lower (ß=-0.46, SE=0.18, $p<0.05$) per degree higher temperature (Table 2.3, 1a). When accounting for autocorrelation with the previous date (Table 2.3, 1b), the log odds of the presence of *H. pylori* was 21% lower per degree higher temperature (ß=-0.24, SE=0.13, $p<0.1$) (Table 3, 1b). Temperature remained statistically significant in the smoothed model, even when the most influential point was removed ($p<0.05$).

In the log-transformed linear regression models incorporating only positive samples, we found a significant positive association between pH and the quantity of *H. pylori* and a marginally significant negative association between conductivity and quantity of *H. pylori.* The quantity of *H. pylori* was 139% higher for each unit higher in pH, and 0.62% lower per µS/cm higher of conductivity (ß=-0.0027, SE=0.0015, $p<0.1$). After removing the most influential data point, the quantity of *H. pylori* 95% higher for each unit higher in pH (ß=0.29, SE=0.14, $p<0.1$, Table 2.3, 2b).

We did not find a statistically significant association between calendar time (i.e., long-term seasonal trends) and the presence of *H. pylori* (figure not shown). Similarly, we did not find association between the presence of *H. pylori* in a given sample and presence of *H. pylori* in the prior day's sample.

Discussion

To our knowledge, this is the longest time-series sampling study of drinking water in Lima, Peru, as well as the most thorough examination of drinking water contamination with *H. pylori* in the scientific literature. Based on our sampling, it appears that drinking water in Lima is consistently contaminated with *H. pylori* about 20% of the time (49/241 positive samples), in both the drinking water collected in Lince and in the wells used to supplement the treated drinking water supply. Based on the null associations found between presence/absence of *H. pylori* and the prior day's sample, there is no strong autocorrelation to indicate any seasonal trends, suggesting that contamination occurs randomly over time in this location.

While we found statistically significant relationships between temperature and the presence/absence of *H. pylori* and between pH, conductivity, and the quantity of *H. pylori*, inference from our data is somewhat difficult. In a laboratory study, the optimal pH for *H. pylori* survival in water was found to be 5.8-6.9, but that doesn't account for other relevant factors, such as co-exposure with chlorine [40]. Adams et al. (2003) found that *H. pylori* goes into a VBNC state more quickly at higher temperatures [14]. However, this study used microscopy rather than culture-based methods, so could not comment on persistence or reproduction at these temperatures. The inconsistency of the relationship between temperature and *H. pylori*, as well as a lack of explanatory mechanism for how pH might be positively associated with the quantity of *H. pylori* makes it uncertain whether these relationships are meaningful. Thus, it seems likely that other,

unaccounted-for factors might be important to presence or quantity of *H. pylori* contamination, such as the frequency of infusions of well-water from contaminated wells, contamination from leaks in the distribution, and the stochastic shedding of cells from biofilms in the pipes.

However, the fact remains that our investigation shows that there is consistent contamination of drinking water with *H. pylori* in Lima, Peru. Since 1996, there have been mixed reports of the presence of *H. pylori* in drinking water. Investigations in Peru [4,24], Sweden [41], Pakistan [32], Iraq [17], Iran [20,42], Costa Rica [21], and Spain [34] showed contamination of drinking water with *H. pylori* using PCR, culture, and microscopy techniques such as fluorescent in-situ hybridization. Studies in Bangladesh [37] and Japan [33] failed to detect *H. pylori* in treated drinking water, though the study in Japan and a further study in Scandinavia detected *H. pylori* via PCR in untreated well-water being used as drinking water [6,33]. The results from our study are consistent with those that found contamination, though the scope of our sampling, in terms of length of time and number of samples, was wider than any previously reported study. The quantities of *H. pylori* we found were also substantially lower than those reported in Spain and Peru, with our highest value being 2.5E3 genome copies/L, compared to 1.59 and 1.6E6 genome copies/L.

Limitations

Because we used a DNA-based method of detection, we could not determine between viable and non-viable *H. pylori* cells in drinking water, so we are unable to infer whether the DNA amplified was from culturable, viable but non-culturable, or non-viable *H. pylori* cells. Thus, examining our results in a risk assessment format would be problematic, as the relative infectiousness of the VBNC compared to the culturable form is not well characterized. However, Sen et al. (2011) found that *H. pylori* DNA cannot be amplified after exposure to chlorine in tap water for 2-3 days; a period of time that it typically takes for water to go from the treatment plant

to a household [43]. This suggests that the DNA detected in our and other studies in chlorinated drinking water may have come from VBNC or viable, culturable *H. pylori* cells.

Further, it is uncertain whether water in the Lince district is representative of water elsewhere in Lima. Based on our previous cross-sectional sampling through the city of Lima, there appeared to be a wide spread of contamination, though this did not appear to be linked to a specific district [24]. Given that there are leaks in the distribution system and the large amount of unaccounted-for water [44], it is possible that some areas of Lima might have more contamination than others. Further studies are needed throughout the city to examine whether such contamination is systemic.

Public Health Implications

Other studies in the literature provide more of a snapshot of water contamination with *H. pylori*, collecting samples either once or a handful of times from multiple locations [6,24,34]. By collecting water from a single location over a year, we better characterized the annual body burden of *H. pylori* from drinking water in Lima, which can be used to more accurately assess risk of infection from this exposure route.

In the only quantitative microbial risk assessment performed thus far for *H. pylori* in drinking water, Ryan et al. (2014) recommended that the maximum contaminant level goal for *H. pylori* be set at <1 organism/L in drinking based on the downstream risk of infection and gastric cancer [47]. That study used quantities of *H. pylori* found in surface and recreational water [30,31], which, when accounting for the efficiency of municipal water treatment in the USA, were substantially lower than those found in our study. However, the dose-response used in that study was performed with viable, culturable cells administered via gavage rather than via drinking water. Our and other quantitative surveys of *H. pylori* contamination of drinking water cannot

distinguish between VBNC, viable culturable, and non-viable *H. pylori* cells. Thus, although several samples in our and other studies [24,34] had quantities of *H. pylori* found to be infectious in either mice, humans, or monkeys [29,45,46], it is unclear whether the sampled water poses the same infectious risk found in dosing trials since these trials used the viable, culturable state of *H. pylori*. Further dosing studies are needed to better characterize the infectivity of viable but non-culturable *H. pylori* in drinking water to develop more accurate risk assessments.

Finally, the contamination of finished water from La Atarjea [4,24] and the consistent contamination of well-water used to supplement the finished drinking water highlights the need for point of use water treatment options and long term investment in water treatment infrastructure to provide safe, potable water to the populace of Lima.

Conclusions

Over a three-year sampling period, we consistently detected *H. pylori* in drinking water from Lima, Peru using qPCR, suggesting that there is continued contamination of the water supply. More work is needed to identify the potential sources for contamination and better characterize the risk of *H. pylori* in drinking water, especially in distinguishing between viable, culturable and VBNC cells . Given that gastric cancer is the most common cause of cancer mortality in Peru, these findings highlight the need for effective point-of-use household water treatment in the short term, and long-term investment in infrastructure to provide high quality drinking water for the citizens of Lima, Peru.

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Table 2.1: Distribution of *H. pylori* in drinking water samples from Lima, Peru.

	Positive (n/N)	Range of genome copies/L in positive samples (above detectable limit only)
June 2015 – May 2016	49/241	4.12E2 – 2.56E3
Summer 2014	9/17	8.8E2 – 1.95E4
Well-water samples: 2013	3/5	1.46E3 – 2.32E3

Table 2.1. Distribution of H. pylori in drinking water samples from Lima, Peru.

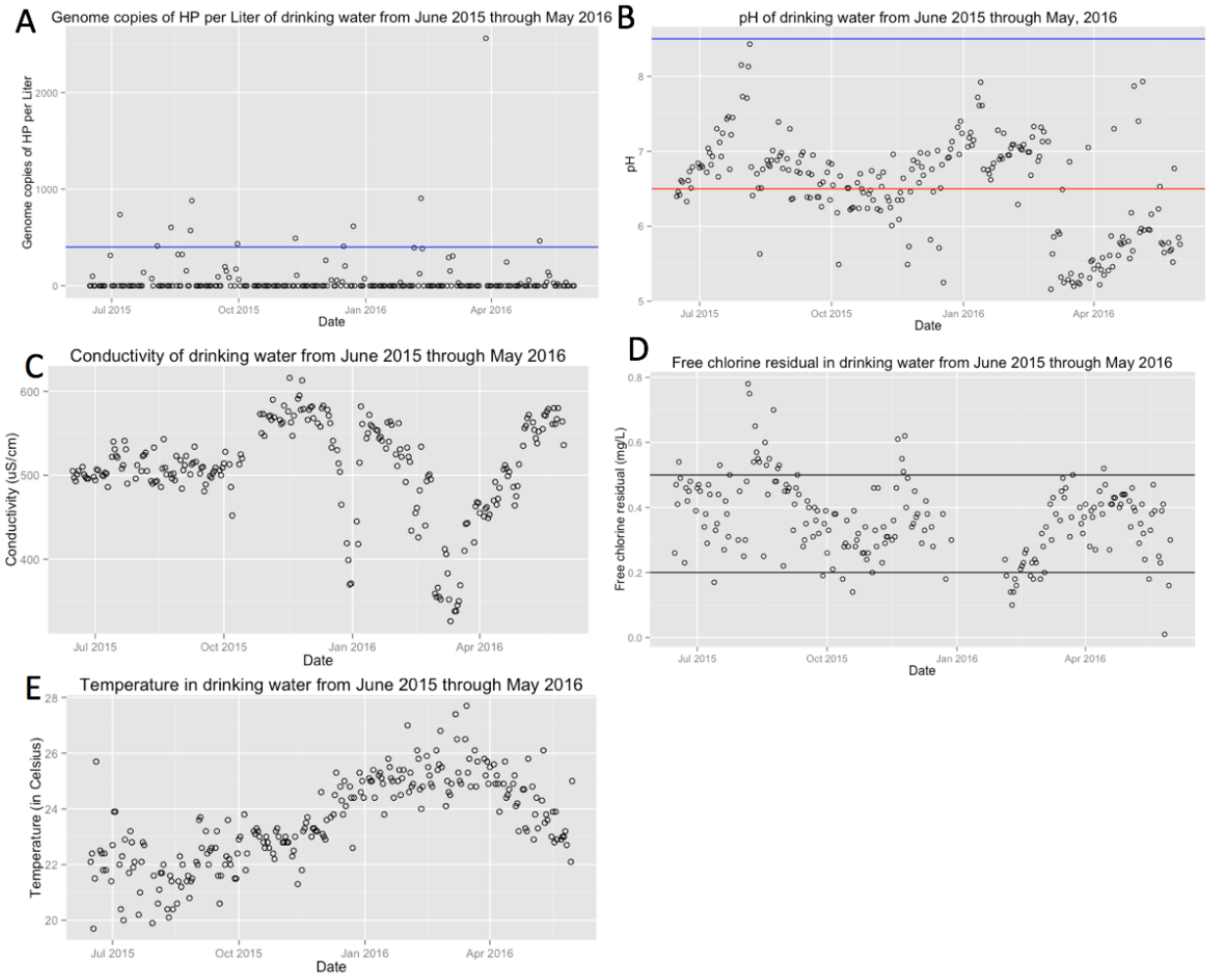

Figure 2.1. Genome copies/Liter of H. pylori *(A), pH (B), Conductivity (C), free chlorine residual (D), and Temperature (E) in drinking water collected from June 2015 through May 2016. A) The blue line represents the limit of detection for genome copies/L of* H. pylori. *B) The blue line represents the upper limit of the EPA secondary standard for pH (8.5), while the red line represents the lower secondary standard for pH (6.5). D) The upper line in the free chlorine residual graph represents the upper limit of the WHO recommendation for FAC (0.5) and the lower line represents the minimum recommended FAC residual.*

Table 2.2. Water characteristics from Jun 2015-May 2016 sampling campaign

	N	Range	Median	Mean	Notes
pH	238	5.16-8.43	6.68	6.54	96 samples below the EPA recommendation of 6.5 (too acidic)
Temperature in Celsius	240	19.7-27.7	23.3	23.6	Fairly high temperatures, good conditions for bacterial growth
Conductivity in	232	326-616	512	504.8	
Free chlorine residual in µS/cm	209	0.01-0.78	0.37	0.3658	17/209 samples below WHO recommendation of 0.2-0.5 mg/L of FAC

Table 2.2. Summary of water characteristics from sampling campaign in Lima from June 2015 through May 2016. Note: Not all water characteristics were measured in all samples due to lack of reagent or instrumentation on that sampling day.

Table 2.3: Statistical models examining the relationship of pH, free available chlorine residual, conductivity, temperature, and the presence/absence or quantity of *H. pylori*.

Variables	Model 1a: Logistic Regression with smoothing (ß, SE).	Model 1b: Logistic Regression - autocorrelation adjustment (ß, SE).	Model 2a: Log-transformed Simple Linear Regression (ß, SE).	Model 2b: Log-transformed Simple linear Regression (ß, SE).^
Intercept	9.73 (5.42)	5.41 (5.12)	0.29 (2.25)	1.54 (2.31)
Prior day presence/absence of *H. pylori*	N/A	0.27 (0.43)	0.03 (0.18)	0.034 (0.18)
Cl2 residual	-0.33 (1.6)	-0.99 (1.58)	0.56 (0.67)	0.28 (0.67)
pH	0.23 (0.33)	-0.04 (0.31)	0.38 (0.14)**	0.29 (0.14)*
Conductivity	-0.003 (0.004)	-0.001 (0.004)	-0.0027 (0.0015)*	-0.002 (0.0015)
Temperature	-0.46 (0.18) **	-0.24 (0.13)*	0.025 (0.57)	-0.017 (0.06)

*Table 2.3. *=p<0.1, **=p<0.05, ^Influential outlying value removed.*

Chapter 2.2: Examination of drinking water from patient homes in Lima, Peru

Note: The text below is excerpted from the results section of Appendix A due to its relevance to this chapter. For the full text, context, and discussion of the entire study from which these results were drawn, please refer to Appendix A.

Cross-sectional investigation of water contamination with H. pylori from homes and the main municipal plant (La Atarjea) in Lima, Peru

Forty-two of 87 filtered water specimens were positive by qPCR (48.3%), with median 931.5 (range 8.5-1,682,500) CN/mg. Residual chlorine was measured in 80 of 83 filtered water samples (96.3%), with median 0.7 (range, 0.1-1.25) mg/L.

The physical properties of the filtered drinking water specimens were as follows: median pH 7.0 (range, 6.5-8.5); median conductivity 53.2 μmhos (microohms per centimeter; range, 40-939);

median Celsius temperature 22.6 (range, 18.8-27.4) and median turbidity 0.1 NTU (nephelometric turbidity unit; range, 0.0-27.4).

Attempts to culture *H. pylori* from patient's drinking water samples and from four samples taken from the main water plant in Lima (La Atarjea) were unsuccessful. However, all four La Atarjea samples, including two samples from the river intake (Rímac River) and two from two different reservoirs of treated water ready for public consumption, tested positive by qPCR: 1378.34, 2520.00, 3275.00, and 3388.00 CN/L, respectively.

Chapter 3 : Dosing Studies with Waterborne *Helicobacter pylori*

Chapter 3.1: Dosing Studies with the Viable, Culturable Form of *Helicobacter pylori* in Drinking Water.[1]

Introduction: *Helicobacter pylori* (*H. pylori*) is a gut bacterium that, while asymptomatic in most people, can cause peptic ulcers and has been categorized as a class 1 carcinogen, causing gastric adenocarcinoma [1,2]. *H. pylori* infection is hypothesized to be transmitted directly through fecal-oral, oral-oral, or gastro-oral routes, or indirectly through reservoirs, such as food and water [3,4]. Since the landmark study by Klein et al in 1991 [5], lack of access to clean drinking water and proper sanitation has been consistently identified in epidemiological studies as a risk factor for *H. pylori* infection [3–10]. Moreover, *H. pylori* has been detected in water using molecular biology techniques such as PCR and fluorescent in-situ hybridization [11–14]. When exposed to water, *H. pylori* rapidly enters a viable-but-not-culturable (VBNC) state [15–17]. This change may be accompanied by a changed morphology (from spiral bacillus to a U-shaped or coccus form), although it survives in the VBNC state in all morphologies in the natural environment [16]. Historically, this conversion to a VBNC state has made *H. pylori* difficult to culture, and has raised skepticism about whether *H. pylori* is viable and infectious in water. However, four independent studies have now isolated and cultured *H. pylori* in wastewater and drinking water using different methods [18–21]. In spite of this evidence that viable *H. pylori* can be

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isolated from drinking water, to our knowledge, there are still no studies demonstrating that drinking water contaminated with *H. pylori* can cause infection in humans or animals. In fact, a recent review by Aziz et al (2013) called for animal models to study the transmission of *H. pylori* in water [17].

Mice are commonly used as a model animal to study different aspects of *H. pylori* infection, including development of gastric inflammation and genes related to successful host colonization [15,22]. These studies typically use oral gavage to infect mice, directly inoculating their stomachs with doses ranging from 10^6-10^9 CFU of *H. pylori*. She et al. (2003) inoculated 16 BALB/C mice over 12 days with 4 doses of ~$4*10^8$ CFU coccoid *H. pylori* by oral gavage [15]. Following exposure, 11 of the 16 mice developed *H. pylori* infection, and culturable *H. pylori* was recovered from their stomachs (compared to 14 of 16 mice dosed with spiral *H. pylori*). This study supports our hypothesis that *H. pylori* can be infectious in the VNBC state and thus transmitted in drinking water. However, the doses in these studies were applied directly to the stomach via gavage, and were higher than those reported in surface water and wastewater, which range from 0-594 cells/mL [14,23,24]. Thus, our goal was to develop a mouse model to demonstrate a dose-response relationship for transmission of *H. pylori* infection in drinking water.

Previously, we conducted pilot studies demonstrating that high concentrations of *H. pylori* strain Sydney Strain 1 in drinking water can infect mice. We carried out two experiments: a one week exposure of Severe Combined Immunodeficient (SCID) C57/BL6 mice (001913 from Jackson Labs) to 10^9 CFU/L of *H. pylori* and a two-week exposure of immunocompetent 16 C57/BL6 mice (000664 Jackson Labs) to varying static concentrations of *H. pylori* (10^5, 10^7, 10^9 CFU/L). These concentrations were chosen for three reasons: 1) the amount of *H. pylori* in

water would be consistent with the highest values found in the literature [23,24], 2) the actual dose in mice would be similar to the dose in humans, and 3) to include a 'worst case' scenario (10^9). The mice were allowed to drink ad libitum from water bottles containing sterilized de-ionized water contaminated with *H. pylori*. Their water was changed twice per week (Tuesday and Friday). After exposure, 5 of 5 SCID mice were infected, and 1 of the C57/BL6 mice exposed to 10^9 CFU/L was infected (confirmed by quantitative culture and histology, unpublished data). Thus, for our current study, we decided to increase the sample size and the exposure period length, and to keep similar concentrations for consistency with our previous study. We chose four weeks as an exposure length since that was used in the only dosing experiment of *H. pylori* in humans [25]. We hypothesized that mice exposed to variable concentrations of *H. pylori* in drinking water would display differing incidences of infection in a dose-dependent manner.

Materials and Methods:

Bacterial strain

H. pylori is not a normal mouse inhabitant, and therefore, most strains of *H. pylori* colonize mice poorly. SS1 (Sydney Strain 1) was selected for this study since it colonizes mice more successfully than other *H. pylori* strains (with infectious doses as low as 200 CFU) [26], and thus would better mimic the success of *H. pylori* infecting humans [22].

H. pylori cultivation, counting, and inoculation

The SS1 strain was grown in microaerobic conditions at 37°C on 5% Sheep Blood Tryptic Soy Agar II plates (BBL). After 3 days of growth, colonies were collected and used to inoculate

Brucella broth (Remel) supplemented with 10% heat-inactivated fetal bovine serum (Fisher Scientific). After shaking overnight at 40 RPM in microaerobic conditions at 37°C, the broth was centrifuged for 20 minutes at 3500 RPM, 4°C to gently pellet the bacteria. The supernatant was removed, and the pellet was suspended in 1mL of 1x PBS. To estimate the total number of bacteria, 10 µL of the *H. pylori* suspension was added to 890 µL of 1x PBS and 100 µL of Buffered Formalin Phosphate. 10 µL of this solution was then pipetted onto a hemacytometer, covered with a cover slip, and cells counted at 40x magnification. Based on the hemacytometer estimate, sterilized water was inoculated with the appropriate amounts of *H. pylori*. To confirm the concentration of *H. pylori* in the water, the stock suspension was serially diluted onto 5% Sheep Blood Tryptic Soy Agar II plates (BBL). After 3 days of growth, the number of *H. pylori* colonies was counted and the stock solution concentration was back-calculated.

H. pylori viability in water

Sterilized water was inoculated in triplicate with 10^{10} CFU/L of *H. pylori* grown and counted using the method above. The water was stored at room temperature for 3 days. *H. pylori* in water was checked for culturability by quantitative plating on 5% Sheep Blood Tryptic Soy Agar II plates (BBL) 1 hour, 2 hours, 4 hours, 1 day, 2 days, and 3 days after inoculation. At these same time points, *H. pylori* cells were checked for viability and morphology using microscopy at 40x magnification and Live/Dead staining (BacLight). Briefly, 6mL of water was centrifuged at 10,000 RPM for 3 minutes. The water was removed, and cell pellets were resuspended in BacLight Live/Dead dye. After incubating for 15 minutes in the dark, the cell suspensions were examined under a microscope using red and green fluorescence. Both live and dead cells were counted, and morphology of each cell type (spiral bacillus, coccus, or U- shape) was recorded.

Staining was used only to estimate the percentage of cells in each state, rather than to quantify cell number.

Transmission and Exposure groups

Fecal-oral transmission is posited as one of the main forms of *H. pylori* transmission [3, 4], and mice consume their own feces. Since the mice were *H. pylori* free, the only way that a mouse could be infected was through drinking infected water. However, once a mouse became infected, it would no longer be possible to determine whether other mice in the cage were infected from drinking contaminated water or from eating *H. pylori* contaminated feces. To account for this, each cage was used as an experimental unit rather than each mouse.

Groups of 4-week old C57/BL6 *Helicobacter*-free mice (Jackson Labs 000664, maximum barrier) were exposed to five different concentrations of *H. pylori* in sterilized, filtered tap water ($1.29x10^5$, 10^6, 10^7, 10^8, and 10^9 CFU/L) and uncontaminated sterilized, filtered tap water (negative control). We conducted two sets of exposures: the first set with positive and negative controls and the 10^5, 10^7, and 10^9 groups, and the second set with the 10^6 and 10^8 groups. All procedures were carried out identically throughout both experiments. The drinking water was changed twice weekly and replaced with fresh water containing *H. pylori*. Each exposure group had 20 cages, with two mice per cage per the Animal Care and Use Committee regulations. The negative control group had 10 cages, with two mice per cage. Unfortunately, one cage of 10^7 CFU/L mice perished in the first week of the experiment after a water bottle leaked, leaving only 19 cages in that exposure group. As a positive control, one cage of two C57/BL6 *Helicobacter*-free severe combined immunodeficient mice (Jackson Labs 001913) was

exposed to 10^9 CFU/L. All mice were housed at ULAM facilities at the University of Michigan Medical School, and all experiments were approved by the Animal Care and Use Committee.

Mouse sacrifice, verification and quantification infection

After 28 days of exposure, the inoculated water was removed, and mice were given autoclaved filtered tap water without *H. pylori*. The negative control mice, 10^5, and 10^6 CFU/L mice were sacrificed 1 day after this final water change, and the 10^7, 10^8, and 10^9 CFU/L mice were sacrificed 2-3 days after the final water change. After sacrifice, mouse stomachs were collected. Two strips of stomach were cut from the greater curvature and fixed by immersion in 10% neutral buffered formalin. Sections were then paraffin-embedded, cut in 5 micron sections, and stained with Warthin-Starry silver stain to detect the presence of *H. pylori.* Histologic scoring was performed as previously described [27]. Briefly, sections were examined in their entirety and the percent of the mucosa containing neutrophilic inflammation; mononuclear cell inflammation, and mucosal metaplasia was quantified. The total score was the sum of the percentages in each lesion category. The remaining portion of the stomach was weighed, homogenized in 1x PBS, and serial dilutions of the homogenate were plated on *H. pylori* selective media (Columbia blood agar base with 10% horse blood, Dent Supplement, 300mg/L urea, and 3500U polymyxin B/L) [28]. Presumptive *H. pylori* isolates were counted and then checked for urease activity using a urease indicator broth (0.33M urea, 0.2% Phenol Red, 0.02% NaN_3, 0.01M pH 6.5 $NaPO_4$ buffer). DNA was then extracted from presumptive colonies using the QiaAMP DNA Mini Kit (Qiagen). The extracted DNA was tested for the presence of the *H. pylori* 16s rRNA gene by PCR using the Takara PCR kit (Fisher, TAK RR001A) and primers HP1 (5′GCAATCAGCGTCAGTAATGTTC3′) and HP2 (5′GCTAAGAGATCAGCCTATGTCC3′),

which are specific to the 16s rRNA gene of *H. pylori* [20]. For PCR, we used an initial denaturation at 95°C for 2 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 minute, and a final extension at 72°C for 7 minutes [20]. PCR products were visualized on a 1.5% agarose gel.

Statistical methods

To gauge the effect of waterborne concentration of *H. pylori* on infection rate and estimate a dose-response trend, a logistic regression model was constructed with infection status (of cages) as the outcome and waterborne concentration of *H. pylori* (log-transformed) as the predictor. Deviations from the linear relationships between log odds of infection and log concentration of waterborne *H. pylori* was investigated by fitting a model including a quadratic version of the log-transformed concentration of *H. pylori* in water. Crude associations between predictors (sex, infection status, CFU/gram stomach tissue, log-transformed waterborne concentration) and gastric inflammation score (range 0-300%) were explored using mixed effects linear models taking into account cage effects were constructed. Since only infected mice had quantitative culture results greater than 0, models were also run on infected mice only. All analyses were run in SAS version 9.4.

Based on our previous experiments, we expected that at least 80% of cages in the 10^9 group would be infected with *H. pylori*. With 20 exposure cages and 10 control cages, this would provide >80% power at α=0.05 significance level to detect differences in infection rates between the exposure groups. The SCID mice were excluded from statistical analyses.

Results

Morphology and culturability of H. pylori in water

Culturability of *H. pylori* dropped consistently at each time point, with a ~50% loss from baseline to hour 1, steady declines in culturability in hours 2 and 4, a log reduction from hour 4 to day 1, and complete loss of culturability 2 days after initial exposure to water (figure 3.1). The results of the morphology experiment are summarized in figure 3.2. At baseline, about 90% of *H. pylori* cells were spiral bacillus. This percentage dropped fairly consistently over time after exposure to water, with a higher percentage of cells manifesting coccus or U-shape forms in later time points.

Exposure to waterborne H. pylori

A cage was counted as infected if the following conditions were met: the quantitative culture plates had colonies with correct *H. pylori* morphology (small, round, and translucent), were positive for the rapid urease test, and were positive for PCR targeting the 16s rRNA gene. If one or both mice in a cage were infected, then we counted that cage as positive. If no mice were infected, then we counted that cage as negative. The results of the experiment are summarized in Table 3.1 and Figure 3.3. None of the cages of negative control or 10^5 mice (0/10 and 0/20, respectively) were infected with *H. pylori*. 1 of 20 cages of the 10^6 group (5%), 3 of 19 cages of the 10^7 group (15.7%), 19 of 20 of the 10^8 group cages, 20 of 20 of the 10^9 group cages had infected mice (100%), and 1 of 1 cages of the SCID mice (positive control) were infected with *H. pylori.* The quantities and range of *H. pylori* recovered from infected stomachs are shown in Table 3.2, and the evidence of *H. pylori* colonization is also shown via histology imaging in Figure 3.4. In the logistic regression model, the log odds of infection increased by 3.57 per 10-

fold increase of waterborne *H. pylori* concentration (p>0.0001) (see Figure 3). Deviations from linearity were not significant (p=0.1021).

Inflammation scoring

The range of inflammation scores of infected mouse stomachs are found in Table 3.2. Crude associations in the complete data set showed significant increased relationships between inflammation and infection (27.86%, p<0.0001), and 10-fold increase in waterborne concentration of *H. pylori* (8.32, p=0.0003) (see Table 3.3). Interestingly, increasing waterborne concentration of *H. pylori* was also associated with a decrease CFU/gram tissue ($4.48*10^6$ fewer CFU/gram tissue per 10-fold increase in waterborne concentration of *H. pylori*). However, this last result is likely spurious, as the only 10^6 mouse to be infected had a relatively high quantity of infection and we did not have sufficient power to accurately gauge the effects of waterborne concentration of *H. pylori* on colonization density. Since only infected mice had positive quantitative culture results and infection status mediates the effect of waterborne concentration on inflammation, we stratified the data by infection status and ran our full model on the infected mice only. Among infected mice, we found that the effect of waterborne concentration is no longer significant (p=0.9572), suggesting that infection status was driving the previous association between waterborne concentration and inflammation. Interesting, among infected mice there was an associated decrease of -1.41 % in inflammation score (p=0.0055) per 10^6 CFU increase in quantitative culture results (see Table 3).

Discussion

To our knowledge, this is the first published study demonstrating the transmission of *H. pylori* in drinking water. We found that contaminated drinking water can be a reservoir of *H. pylori* infection, lending credence to the epidemiological associations in the literature. We also showed that under the tested exposure conditions: 10^9 CFU/L is more than sufficient to infect mice, 10^5 CFU/L is insufficient to infect mice, and that the minimum infectious concentration of *H. pylori* in water for this paradigm falls around 10^6 CFU/L.

Our results demonstrate that *H. pylori* infection via drinking water is possible, but much work remains to better characterize this relationship. For example, our successful infectious concentrations were refreshed twice weekly and were much higher than the concentrations of *H. pylori* described in naturally contaminated drinking water. While SS1 is well adapted to colonizing mice, *H. pylori* is not a normal mouse inhabitant, and the infectious dose in humans may be lower than for mice. We also did not determine the concentration of *H. pylori* in the mice feces using qPCR or other quantitative methods, so the average daily exposure may be higher than the amount in the water.

Gastric Inflammation

While crude associations suggested that waterborne concentration of *H. pylori* affected inflammation status, further analyses showed that this was likely due to the increased infection levels at increased concentrations of waterborne *H. pylori.* While we had insufficient power to accurately gauge this association, this suggests that once infection occurred, it likely progressed in a similar way in all infected animals. The association of increased infection density with

decreased inflammation was surprising, but consistent with other findings in the literature, suggests inflammation suppresses colonization, and that as inflammation is reduced, *H. pylori* continues to colonize more densely within already infected tissue [29]. It is also possible that four weeks is simply not long enough to result in consistent gastric inflammation in infected mice [30].

Public Health Implications

The minimum infectious dose for *H. pylori* in humans is not established. A study by Graham et al found that humans given a single oral dose of 10^4-10^{10} CFU of *H. pylori* resulted in infection, but failed to determine a minimum infectious dose since the study participants at all doses got infected [25]. Mice typically drink around 7mL of water per day [30], so mice in the infected groups consumed between ~$7*10^3$ CFU per day (10^6 group) and $7*10^6$ CFU/day (10^9 group). These amounts (excepting the 10^9 group) are not dissimilar to those that humans would be consuming based on concentrations found in drinking and recreational water in the literature. However, the mice did not drink the daily amount in a single dose; rather, they drank that amount throughout an entire day, meaning that the minimum infectious dose could be smaller than the above numbers since each sip of water may have been the infectious dose. Our results show that *H. pylori* in water is infectious, and thus may be a risk to human health. A recent quantitative microbial risk assessment (QMRA) of *H. pylori* in water agreed, suggesting a Maximum Contaminant Level at 1 organism per liter [31].

Limitations

As noted above, the concentrations to which we exposed mice were higher than those found in the environment [14,23,24], and likely do not accuracy reflect the environmental concentrations. Also, since we used sterilized, filtered tap water, we may be missing water characteristics that potentially aid or inhibit *H. pylori* infection and survival in water, for example lowered pH, presence/absence of other organisms, and presence of particulate matter or metals. Further, the water used in this study contained static concentrations of *H. pylori*, which does not reflect the reality of drinking water contamination, especially in places that lack water treatment like developing nations [32]. Precipitation frequency and seasonal differences likely affect how much *H. pylori* is in the water through sources of contamination including sewage overflows and run-off from farms. Thus, we cannot make predictions as to the potential frequency of water-borne infection; only that it is possible.

Future directions

Continued mouse experiments could be done to start teasing out the importance of these variables by exposing mice to *H. pylori*-contaminated water for a single day (or other relevant periods of time). Changing the water characteristics to reflect those found in municipal or well water would make future studies more representative of actual drinking water conditions. Continued surveys of *H. pylori* in drinking and recreational water using quantitative techniques like qPCR could be done to better gauge the amount of *H. pylori* to which humans are exposed, and thus determine more appropriate doses to test in mice. Better characterizing the infectious dose in humans using carefully planned clinical studies would be the best way (although challenging ethically) to determine the infectivity of *H. pylori* in water. A similar protocol to the one used by Graham et al could be adapted for waterborne exposure [25]. The data from these

combined efforts could be used to continue to update existing QMRAs on waterborne pathogens and provide evidence for the implementation of a drinking water quality standard for *H. pylori.*

Conclusions

In conclusion, our findings could aid quantitative microbial risk assessments for *H. pylori* in drinking water. While much research remains to be done, we have demonstrated that mice can be infected by drinking water contaminated with *H. pylori*.

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Table 3.1: An overview of the experimental results

Exposure group	Average CFU/L of H. pylori in drinking water	Range of waterborne concentrations (CFU/L)	Number of infected cages n/N (x%) by quantitative culture	Total number of infected mice
Negative control	0	0	0/10 (0%)	0/20 (0%)
Positive Control	$1.29*10^9$	$6.50*10^8$ - $2.16*10^9$	1/1 (100%)	2/2 (100%) 2 males
10^5 CFU/L	$1.29*10^5$	$6.50*10^4$ - $2.16*10^5$	0/20 (0%)	0/40 (0%)
10^6 CFU/L	$1.29*10^6$	$5.67*10^4$ - $2.43*10^6$	1/20 (5%)	1/40 (2.5%) 1 female
10^7 CFU/L	$1.29*10^7$	$6.50*10^6$ - $2.16*10^7$	3/19 (15.7%)	4/38 (10.7%) 3 males, 1 female
10^8 CFU/L	$1.29*10^8$	$5.67*10^6$ - $2.43*10^8$	19/20 (95%)	33/40 (82.5%) 18 males, 15 females
10^9 CFU/L	$1.29*10^9$	$6.50*10^8$ - $2.16*10^9$	20/20 (100%)	39/40 (97.5%) 19 males, 20 females

Table 3.1. Overview of experimental results by infection status. All infected mice were confirmed by the rapid urease test, morphology, and PCR.

Table 3.2: Amounts of *H. pylori* recovered from quantitative culture of stomach tissue and inflammation scoring of stomach

Exposure group	Average CFU/gram of stomach (range)	Mean Inflammation Score	Standard Error (inflammation score)
Positive Control (n=2 mice)	$1.41*10^7$ ($9.82*10^6$ - $1.83*10^7$)	N/A	N/A
10^6 CFU/L (n=1 mouse)	$1.64*10^7$	70.97%	N/A
10^7 CFU/L (n=4 mice)	$1.14*10^7$ ($8.47*10^6$ - $1.36*10^7$)	39.3% (9.1%-75%)	28.69%
10^8 CFU/L (n=33 mice)	$1.85*10^7$ ($6.23*10^6$ - $6.86*10^7$)	61.01% (0%-224%)	61.19%
10^9 CFU/L (n=39 mice)	$9.46*10^6$ ($4.1*10^5$ - $2.39*10^7$)	42.7% (0%-116.7%)	32.14%

Table 3.2. Quantitative culture and histological inflammation results from infected mice.

Table 3.3: Associations between gastric inflammation and predictors

Predictor	All mice β(SE)	p-value	Infected mice β(SE)	p-value	Uninfected mice β(SE)	p-value
Infection status	27.86% (6.21%)	<0.0001	N/A	N/A	N/A	N/A
Sex	2.67% (6.71%)	0.6912	11.26% (11.41%)	0.3306	-3.73% (7.06%)	0.5992
Waterborne concentration of *H. pylori*	8.32% (2.20%)	0.0003	-0.55% (10.21%)	0.9572	4.71% (3.62%)	0.1985
CFU/gram tissue (divided by 10^6)	0.18% (0.33%)	0.5864	-1.41% (0.47%)	0.0055	N/A	N/A

Table 3.3. Results from linear mixed models examining the associations between infection status, date of experiment, sex, waterborne concentration of H. pylori, and quantitative culture results on gastric inflammation taking into account cage effects. Associations were performed on all mice, then separately on infected and uninfected mice.

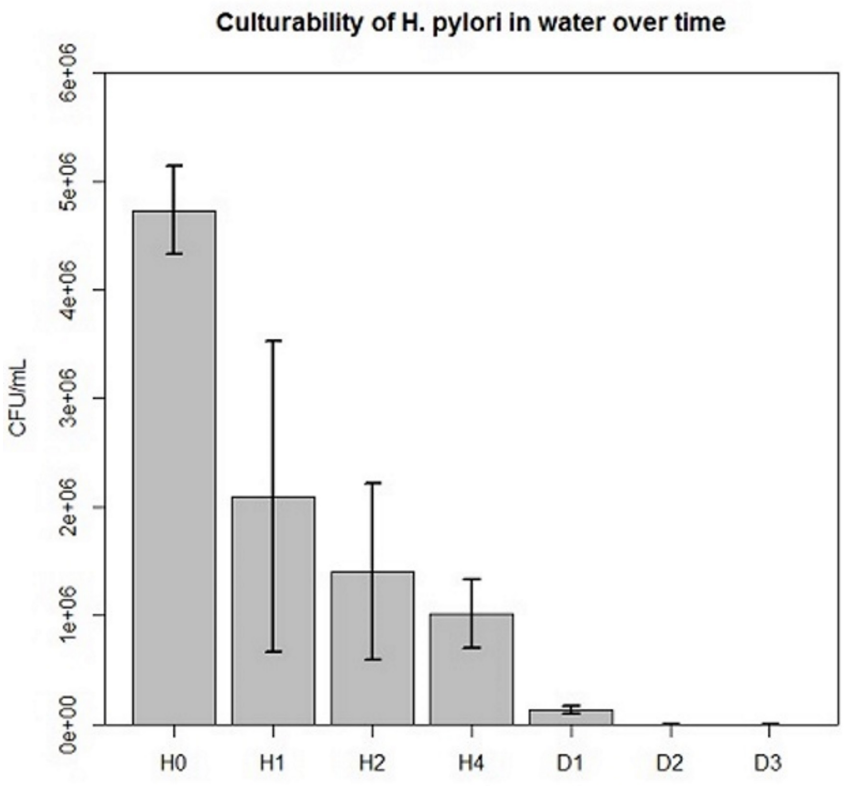

Figure 3.1. Consistent with other results in the literature, culturability of Helicobacter pylori in water decreased steadily over time when kept at room temperature, with complete loss of culturability after 2 days.

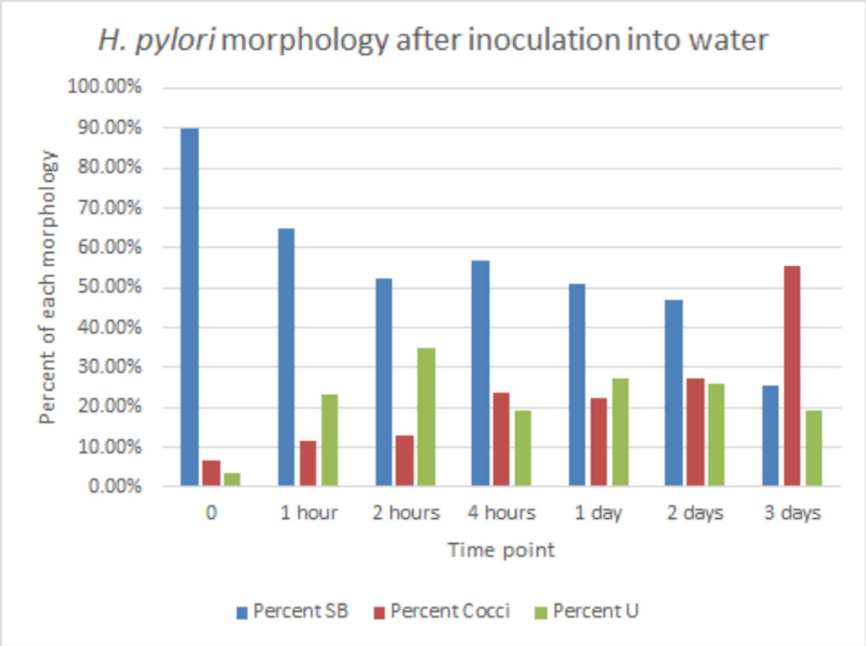

Figure 3.2. Helicobacter pylori morphology in water: results from Bac- Light Live/Dead staining and microscopy. SB, Spiral bacillus morphology; Cocci, O-shaped/coccoid morphology; U, U-shape morphology. At time 0, about 90% of the H. pylori was spiral bacillus morphology. Over time, the percentage of spiral bacilli decreased and the percent of cocci and U-shaped morphologies increased.

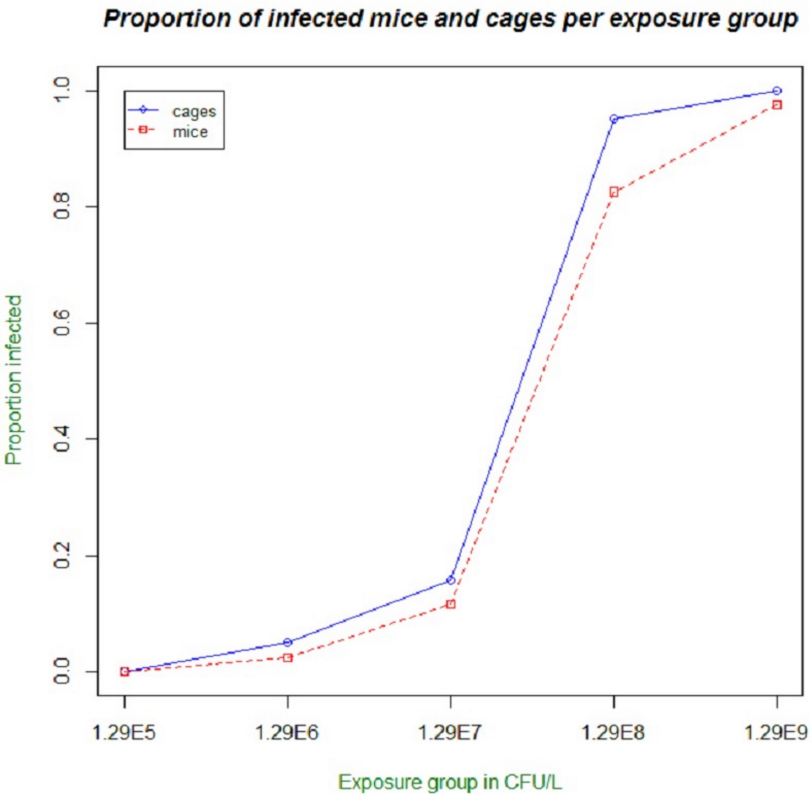

Figure 3.3. Percentage of infected mice and cages per exposure group. The infectious dose for this exposure paradigm appears to fall around 1.29E6 CFU/L in water.

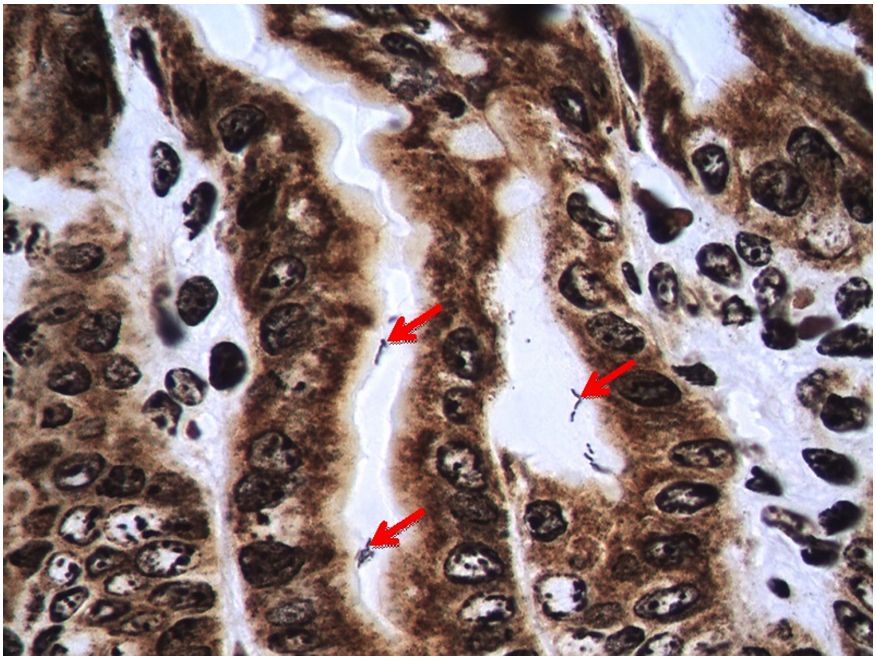

Figure 3.4. Silver stained tissue section of mouse stomach from an infected mouse. Arrows indicate Helicobacter pylori in the gastric mucosa.

Chapter 3.2: Dosing Studies with the Viable-but-not-Culturable Form of *H. pylori* in Drinking Water[2]

Introduction: *Helicobacter pylori* (*H. pylori*) is a gastrointestinal bacterium that causes gastritis, peptic ulcers and, over time, gastric adenocarcinoma[1,2]. *H. pylori* infection is hypothesized to be transmitted through multiple routes, including vertically from mother to child and through contaminated reservoirs like food and water[3,4]. A body of evidence suggests that contaminated water may be a source of *H. pylori* infection, with epidemiological studies consistently associating *H. pylori* infection with lack of access to potable drinking water and proper sanitation[3,5–9]. Furthermore, *H. pylori* has been detected in water using various molecular biology techniques, such as quantitative polymerase chain reaction (qPCR) and microscopy methods[5,10–

[2] This chapter was previously published in the journal *Helicobacter*: Boehnke, Kevin F., et al. "Reduced infectivity of waterborne viable but nonculturable Helicobacter pylori strain SS1 in mice." *Helicobacter* (2017).

[13], and there are reports that it has been cultured from water[14–17]. *H. pylori* enters a viable-but-not-culturable (VBNC) state within a few days after inoculation into water[18–20]. This change is often accompanied by a morphological change from a spiral bacillus to a U-shaped or coccoid form, and *H. pylori* has been found in the VBNC state in all these morphologies in the natural environment[18,21]. However, though *H. pylori* has been cultured from wastewater and drinking water, it is unclear whether this was due to the culturable form being present in the water or investigators being able to revert the VBNC form back to a culturable form using appropriate media.

The fact that *H. pylori* is present in both a culturable and VBNC state has not been accounted for when assessing risk associated with waterborne *H. pylori*. For example, a risk model of waterborne *H. pylori* infection using a quantitative microbial risk assessment methodology[22] did not consider the VBNC form of *H. pylori*. Likewise, our recent study showing that constant exposure to the viable, cultural form of *H. pylori* in drinking water can infect mice did not account for exposure to the VBNC form[20]. While previous studies found that VBNC *H. pylori* administered via gavage could cause infection in mice[19,23], the gavage exposure method is not representative of exposure to drinking water. To fill this gap in the literature, we examined the infectivity of the VBNC form of *H. pylori* in water.

Materials and Methods:

Transmission and Exposure groups

Our studies were carried out sequentially following our initial dosing experiments that examined the infectious dose of viable, culturable *H. pylori* in water[20]. We performed four mouse experiments to assess the infectivity of VBNC *H. pylori* in various different exposure scenarios

(Table 1). Concentrations of VBNC *H. pylori* were chosen based on previous studies[19,20,23] and on the amounts of *H. pylori* found in sources of recreational and drinking water worldwide[24,25]. We first employed a classic single-hit exposure model with waterborne VBNC *H. pylori*, examining whether a single day of water with a high dose of *H. pylori* could cause infection, choosing the high end of waterborne concentrations to test a 'worst-case' scenario and to try to ensure a higher chance of experimental infection. 4 weeks was chosen as the time to wait until euthanasia, given that She et al had found slightly increased colonization rates at 4 weeks compared to 3 weeks[19]. The sample size of 40 mice was chosen for consistency with our previous dosing experiments, in which each exposure group had 40 mice. When this failed to induce infection, we did two follow up experiments (Table 1, experiments 2 and 3). We increased the number of days of exposure (six instead of one), and also exposed severe combined immunodeficient mice to a single day of waterborne *H. pylori*, hypothesizing that more doses and immunocompromised hosts would be more likely to increase infection based on the results of our previous experiments[20]. When these also failed to induce infection, we increased the exposure length again and increased the number of mice to 100 to increase the likelihood of seeing infection. In these experiments, we used a similar experimental design to our original dosing studies[20], exposing the mice to 56 days of contaminated water (experiment 4), and further decreasing the time until euthanasia. When this also failed to induce infection, we did a final follow-up study in which we gavaged mice with 4 doses of ~$2*10^8$ cells of VBNC SS1 over 2 weeks. This, too, failed to induce infection.

The mice were exposed to water contaminated with ~10^9 cells/L VBNC *H. pylori* (See table 1). In experiments 1-3, contaminated water was removed after 24 hours and replaced with either a bottle of freshly contaminated water or (when appropriate) sterilized, filtered tap water.

Each exposure group had 20 cages, with two mice per cage per the Animal Care and Use Committee regulations. In experiment 4, water was changed twice per week, every 3-4 days. As a negative control, 10 mice (5 cages) were given sterile, filtered tap water for 9 weeks. As a positive control, 10 mice (5 cages) were given sterile, filtered tap water inoculated with viable, culturable *H. pylori* for 9 weeks. All mice were housed at University Laboratory Animal Medicine facilities at the University of Michigan Medical School, and all experiments were approved by the Animal Care and Use Committee.

Bacterial strain

SS1 (Sydney Strain 1) was selected for this study for consistency with our previous studies[20], and because it colonizes mice more successfully than other *H. pylori* strains[26].

H. pylori cultivation, counting, and inoculation

H. pylori cultivation was carried out as previously described[20]. Briefly, SS1 was plated from stocks and grown at 37°C on 5% Sheep Blood Tryptic Soy Agar II plates (BBL, Franklin Lakes, New Jersey, USA) in microaerobic conditions. After 3 days, colonies were collected and suspended in plates of Brucella broth (Remel, Columbus, Ohio, USA) supplemented with 10% heat-inactivated fetal bovine serum (Fisher Scientific, Waltham, Massachussetts, USA). After shaking overnight in microaerobic conditions at 37°C, the broth was centrifuged at 1917g and 4°C for 20 minutes. The supernatant was removed, and the pellet was suspended in 1x PBS. To confirm the concentration of *H. pylori*, the stock suspension was serially diluted onto 5% Sheep Blood Tryptic Soy Agar II plates (BBL). Sterilized, filtered tap water was then inoculated with

the stock suspension. After 4-7 days of growth, the number of *H. pylori* colonies was counted and the stock solution concentration was back-calculated.

H. pylori viability in water

Previous methodologies for inducing the VBNC state have differed across studies. She et al inoculated sterilized tap water with live *H. pylori* and stored it at 4°C for 40 days, defining cells as VBNC when they were in the coccoid state and did not grow when plated[19]. Wang et al incubated fresh *H. pylori* colonies in Ham's F12 medium with 10% calf serum for 3 days, then stored them at 4°C, defining cells as VBNC once they stopped growing[23]. Cellini et al inoculated a Brucella broth/2% fetal calf serum solution with fresh *H. pylori* and incubated it for 20 days until the cells no longer grew when plated[27]. As we wanted to examine the infectivity of VBNC *H. pylori* in water, we chose to incubate *H. pylori* in water for the VBNC conversion.

Inoculated water was held for 2-4 days at room temperature to ensure that the VBNC conversion had occurred before giving water to the mice. To check culturability, inoculated water was plated on 5% Sheep Blood Tryptic Soy Agar II media and incubated for 7 days in microaerobic conditions at 37°C. *H. pylori* cells were checked for viability and morphology using microscopy at 60x magnification and LIVE/DEAD BacLight Bacterial Viability Kit (Life Technologies, Eugene, Oregon, USA). To undertake viability and morphological analyses, 50mL of water was centrifuged at 10,400g for 3 minutes, the supernatant was removed, and cell pellets were suspended in BacLight Live/Dead dye. After incubating for a minimum of 15 minutes in the dark, the cell suspensions were examined in triplicate per the manufacturer's instructions.

Metabolic activity of VBNC *H. pylori* cells was examined using Biolog Phenotypic Microarray plates PM1, which contain 95 separate carbon sources which are commonly utilized

by a variety of microbial species. All necessary reagents were purchased from Biolog (Hayward, CA). *H. pylori* cells were grown on 5% Sheep blood Tryptic Soy Agar II media, then collected from the plates and suspended in sterile, autoclaved water. Cell suspensions were stored at room temperature for 0, 3, 4, 7, or 8 days. At each respective time point, cell suspensions were spun down at 1917g for 20 minutes. The supernatant was discarded, and the resulting pellets were checked for metabolic activity using the PM1 plate. Briefly, pellets were re-suspended in inoculating fluid IF-0a GN/GP (1.2x), and then supplemented to a final concentration of 0.05% Bovine Serum Albumin (BSA) and 1.25 mmol/L $NaHCO_3$. (*H. pylori* has been shown to use more carbon sources and grow successfully in media containing BSA[28,29], so it was included to ensure better visualization of metabolic activity in the VBNC state.) Dye mix D (Biolog, Hayward CA) was then added to achieve a final concentration of 0.01%. 100uL of this solution was pipetted into each well of the PM1 plate, which was then incubated in microaerobic conditions for 48 hours. Cells were considered to be metabolically active if they induced a color change in any of the wells containing nutrient sources, and the negative control had no color change. This was not measured in a quantitative way, but checked visually, as the purpose of this experiment was to examine *H. pylori* VBNC cell viability rather than examine the carbon sources used.

Dose estimation

To estimate the doses consumed by the mice, water bottles were weighed before being placed in cages and immediately after their removal. Since water drips out of water bottles when they are placed in the cage and when the cages are moved, 'dummy' bottles were filled with water and treated in the exact same way as experimental bottles. The amount of water lost from dummy

bottles was averaged, and that average was subtracted from the total water lost from each bottle. As mice were housed two per cage, the adjusted total per cage was then halved to provide the individual dose per mouse.

Mouse euthanasia, verification and quantification of infection

After exposure, the mice were euthanized and their stomachs were collected. The stomach was weighed, homogenized in 1x PBS, and serial dilutions of the homogenate were plated on *H. pylori* selective media (Columbia blood agar base with 10% horse blood, Dent Supplement, 300mg/L urea, and 3500U polymyxin B/L)[30]. Presumptive *H. pylori* isolates were counted and then checked for urease activity using urease indicator broth (0.33M urea, 0.2% Phenol Red, 0.02% NaN_3, 0.01M pH 6.5 $NaPO_4$ buffer). DNA was extracted from stomach homogenate using the QiaAMP DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) per the manufacturer's instructions. Extracted DNA was tested for the presence of the *H. pylori* VacA gene by PCR using the Takara PCR kit (Fisher, TAK RR001A) and primers VagA-F (5-CAATCTGTCCAATCAAGCGAG) and VagA-R (5-GCGTCAAAATAATTCCAAGG)[31]. PCR was run with an initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 1 minute, and a final extension at 72°C for 5 minutes. PCR products were visualized on a 1.5% agarose gel.

Results

Morphology, culturability, and metabolic activity of H. pylori in water

In all experiments, there was a complete loss of culturability of *H. pylori* 2-3 days after initial inoculation into water. Despite being non-culturable, cells were still found to be membrane intact via Live/Dead staining 8 days after inoculation into water (Figure 3.5).

The VBNC *H. pylori* cells also induced color changes in the Biolog PM1 panels at each time point, respectively (Figure 3.6). This suggests that the cells were metabolically active, as they were metabolizing the carbon sources in each well. The cells in the viable, culturable state utilized many more carbon sources than any of the cells in the VBNC state. No differences in metabolic activity were seen between VBNC cells on days 3, 4, 7, and 8 (data not shown).

Exposure to waterborne H. pylori

A cage was counted as infected if the following conditions were met: the quantitative culture plates had colonies with correct *H. pylori* morphology (small, round, and translucent), were positive for the rapid urease test, and were positive for PCR targeting the VacA gene. Cages were counted as positive if one or both mice in a cage were infected. If no mice were infected, then that cage was counted as negative. The results of the five exposure scenarios and the positive and negative controls are summarized in Table 3.5. Further, the mice dosed with SS1 via gavage were also not infected.

The negative controls showed no signs of infection, and confirmed *H. pylori* cultures were recovered from 8/10 positive controls. None of the mice exposed to VBNC *H. pylori* showed any sign of infection, either via culture or PCR.

Discussion

We were unable to cause infection in mice with the VBNC form of SS1, either in drinking water or via gavage. Our inability to cause infection was surprising, given the known capacity of this strain to successfully infect mice[26,32], our wide range of exposure scenarios, and our previously published study that showed that SS1 in water could infect mice in a dose-dependent manner[20]. In our previous study, 4 weeks of exposure to water spiked with 10^9 CFU/L, 10^8 CFU/L 10^7 CFU/L, and 10^6 CFU/L of *H. pylori* caused infection in 39/40, 33/40, 4/38, and 1/40 mice, respectively. The ingested cumulative doses are 2-2000 fold lower than those used in this current experiment, showing that SS1 is less infectious (or completely non-infectious) in the VBNC state than when viable and culturable. This suggests that *H. pylori* strains may be less infectious than when viable and culturable.

However, there are few dosing experiments in the literature that examine this phenomenon. She et al found that 11/16 mice gavaged with VBNC *H. pylori* were infected compared to 14/16 gavaged with the same dose of viable, culturable *H. pylori*[19]. Also using gavage to administer doses, Cellini et al showed that 8/20 mice were infected from VBNC *H. pylori* compared to 9/20 with viable, culturable *H. pylori* [27]. Both studies used strains that were recently isolated from clinical biopsies of patients with ulcers. Combined with our results from drinking water and gavage exposure to SS1, this suggests that different strains may differ in their ability to infect mice when in the VBNC state.

Our inability to cause infection could be due in part to the drinking water exposure route, which may affect the dose that reaches the stomach compared to gavage methods. Gavage directly inoculates the stomach with a large bolus of bacteria, while drinking water contains comparatively lower doses and must go through the mouth and esophagus before reaching the

stomach, which may result in bacterial losses along the way. While this may affect our results, our total cumulative doses – especially in experiment 4 – were comparable to (or higher than) the doses reported in previous studies (10^8-$4*10^8$ CFU/dose). Further, our gavage experiments showed no signs of infection either. Finally, our previous study in which we administered viable, culturable *H. pylori* to mice in drinking water found relatively similar dose/response rates as other studies that were done with gavage[20].

Limitations and public health implications

As with any animal study, we cannot be certain that our results accurately reflect what would occur with human exposure. Since *H. pylori* is a human pathogen, it is possible that the VBNC form is more infectious in humans than in mice. Further, we only exposed mice to one strain of *H. pylori*, and it is possible that other strains would be more infectious in the VBNC state than SS1, as has been seen in other published papers in the literature[19,27]. Despite our large sample sizes and high doses, our inability to infect mice with VBNC *H. pylori* via drinking water suggests that VBNC SS1 in water is not infectious in mice. This may reflect the strain that we used, the route of exposure, or may simply mean that we did not account for some crucial piece of the puzzle that is yet unknown about the transmission of *H. pylori* via water. The genetic variability of *H. pylori* strains is vast[33], so it may be possible that some strains lack the capability to persist in water, but instead are transmitted only via other exposures, such as person-to-person or fecal-oral routes[4]. However, if our results are true, this could imply that, since much *H. pylori* in water is in the VBNC state, that the route of drinking water does not contribute significantly to the transmission of *H. pylori* infection.

Future Directions

Examining different strains of VBNC *H. pylori* in these exposure scenarios would give insight into the trade-offs of survival and infectivity associated with the VBNC state. Further, investigating the distributions of VBNC vs. viable, culturable *H. pylori* populations in the natural environment would provide a better understanding of the infectivity of the various forms of *H. pylori*. Such experiments would allow for more accurate risk assessments of *H. pylori* in water, as it is very likely that multiple strains and forms of *H. pylori* are present in contaminated drinking or surface water sources.

Conclusions

We found that mice exposed to VBNC SS1 *H. pylori* via drinking water were not infected, despite the various exposure scenarios (immunocompromised, high doses) that might have promoted infection with VBNC bacteria. While other studies that have used viable, culturable SS1 to successfully infect mice via gavage and drinking water, our results suggest that VBNC SS1 is either not infectious (or potentially has greatly reduced infectivity). Future studies could examine different *H. pylori* strains in similar exposure scenarios to compare the relative infectivity of the VBNC versus the viable, culturable state, which would help inform future risk assessments of *H. pylori* in water.

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Table 3.4. Experimental overview of exposure scenarios.

Experiment number	Exposure groups	Time to VBNC Conversion	Exposure	Euthanized
1	40 C57/BL6 mice (20 male, 20 female)	2 days	Exposure to 1 day of 10^9 cells/L of VBNC *H. pylori*.	4 weeks after final exposure
2	40 C57/BL6 mice (20 male, 20 female)	2 days	Exposure to 6 days of 10^9 cells/L of VBNC *H. pylori*^	2 weeks after final exposure
3	10 C57/BL6 Severe Combined Immunodeficient mice (4 male, 6 female)	2 days	Exposure to 1 day of 10^9 cells/L of VBNC *H. pylori*	1 week after final exposure
4	100 C57/BL6 mice (50 male, 50 female)	4 days	Consistent exposure to $>10^9$ cells/L of VBNC *H. pylori* over 56 days	4 days after final exposure
Negative Control	10 C57/BL6 mice (4 male, 6 female)	N/A	Sterile, filtered tap water for 60 days	Day 60
Positive Control	10 C57/BL6 mice (4 male, 6 female)	N/A	Consistent exposure to $>10^9$ cells/L of viable, culturable *H. pylori* over 56 days	4 days after final exposure

Table 3.4. Table 1. Experimental overview of various drinking water exposure scenarios.^Mice were exposed to contaminated drinking water for 3 days, followed by 11 days of sterile water, and then another 3 days of contaminated water.

Table 3.5: Overview of experimental results

Experiment number	Average number of VBNC *H. pylori* cells per Liter drinking water (Range)	Average cumulative ingested dose per mouse (Range)	Number of infected cages n/N (%)	Total number of infected mice n/N (%)	VacA PCR positive results n/N (%)
Experiment 1	10^9	10^6	0/20 (0%)	0/40 (0%)	0/40
Experiment 2	2.14E9 (1.15E9-3.42E9)	5.33E7 (4.09E7 – 6.91E7)	0/20 (0%)	0/40 (0%)	0/40
Experiment 3	2.22E9	5.44E6 (4.20E6 – 6.19E6)	0/5 (0%)	0/10 (0%)	0/10
Experiment 4	7.49E9 (9.30E8 – 2.04E10)	2.30E9 (1.75E9 – 3.83E9)	0/50 (0%)	0/100 (0%)	0/100
Negative control	0	0	0/5 (0%)	0/10 (0%)	0/10
Positive Control	4.80E9 (2.42E8 – 2.04E10)	1.07E9 (8.45E8 – 1.64E9)	5/5 (100%)	8/10 (80%)	8/10 (80%)

Table 3.5. Results from viable but nonculturable H. pylori dosing experiments. While the positive control showed consistent levels of infection with previous studies, mice exposed to VBNC H. pylori showed no signs of infection.

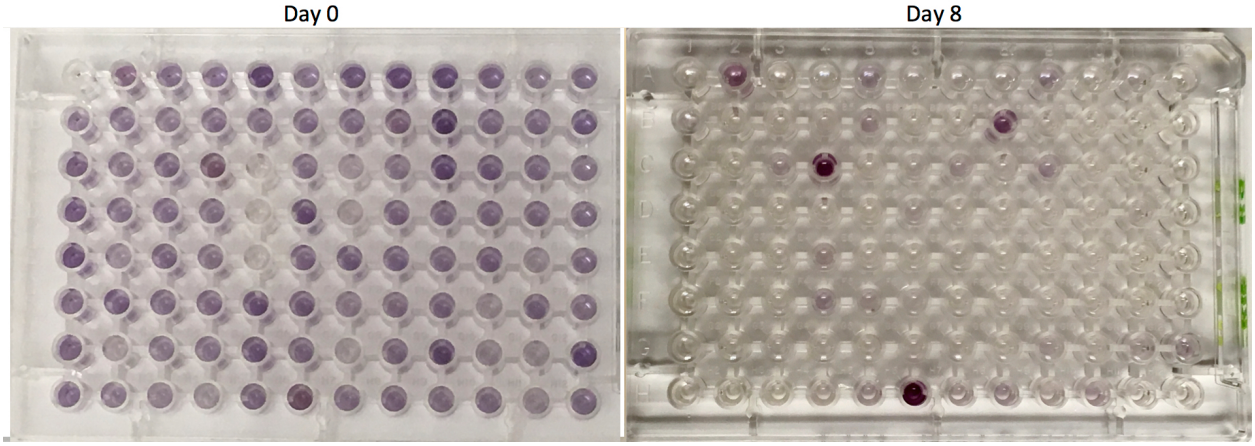

Figure 3.5. PM1 plates of Day 0 (viable and culturable) and Day 8 Helicobacter pylori cells (VBNC). Each well contains a different carbon source, and wells with a purple color change indicate that the carbon source was being used. Viable culturable H. pylori utilized a much wider variety of carbon sources than the VBNC H. pylori.

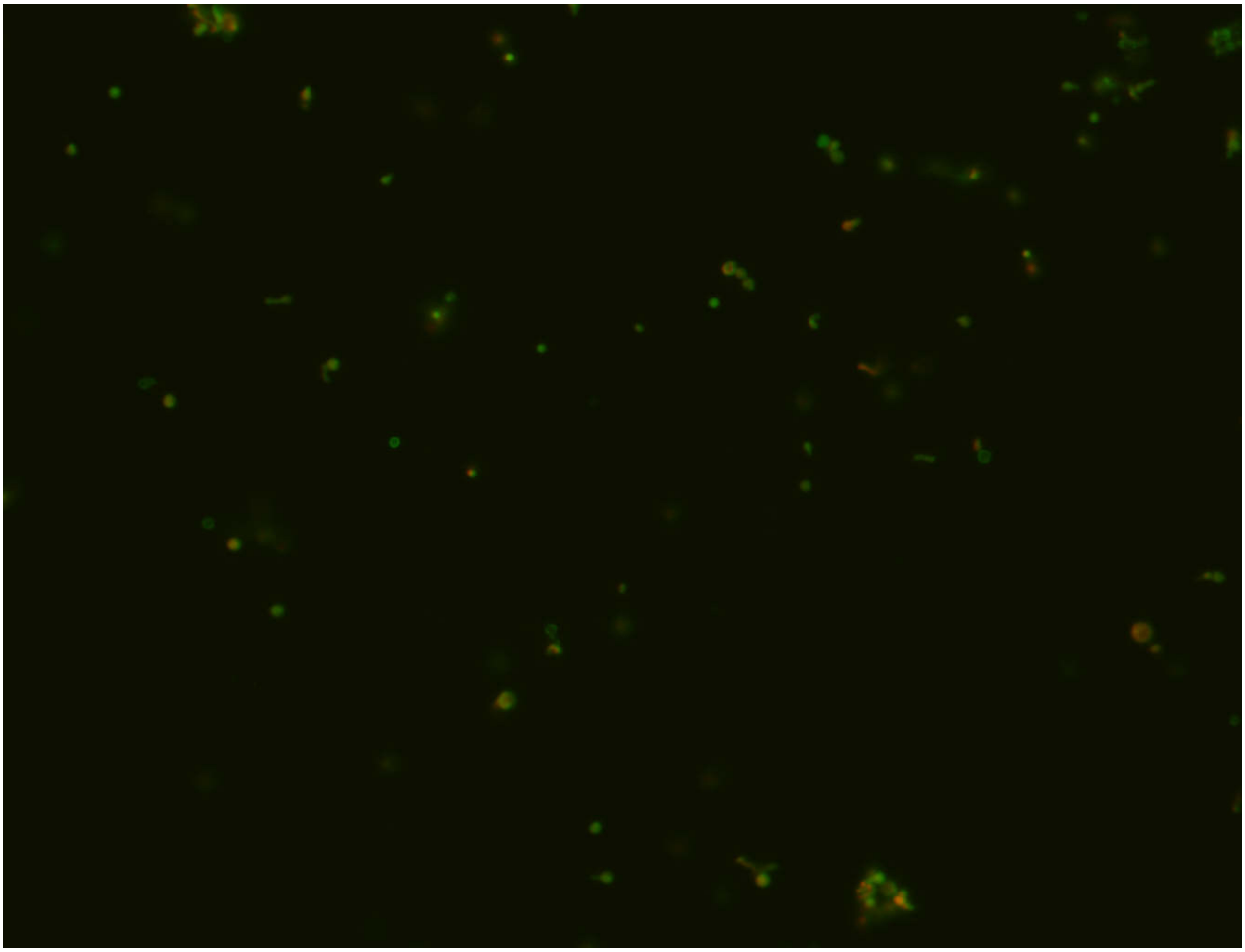

Figure 3.6. 60x magnification of Helicobacter pylori suspension in water after 8 d of incubation at room temperature. Green cells are membrane intact, and red cells have membrane damage. The predominant form was coccoid.

Chapter 4 : Methods for Controlling the Spread of *H. pylori* Infection: Environmental Eradication and Clinical Treatment

Chapter 4.1: Antibiotic Resistance among *Helicobacter pylori* Clinical Isolates in Lima, Peru.

Introduction: *Helicobacter pylori* is a stomach bacterium that colonizes ~50% of people globally.[1] *H. pylori* is the primary risk factor for gastric cancer - the third highest cause of global cancer morbidity.[2] *H. pylori* infection rates are highly dependent on socioeconomic status; ~80% of those living in low socioeconomic areas of Latin America, Asia, and Eastern Europe are infected, compared to <20% of asymptomatic Caucasians in the US.[3] *H. pylori* infection is treatable with different regimens of antibiotics,[4] and eradication of *H. pylori* is a recognized way to lower incidence of gastric cancer.[5] However, recurrence of infection is variable,[6,7] and the emergence of antibiotic resistance compromises treatment efficacy. Thus, determining the best course of treatment is important to improve treatment efficacy and reduce recurrence of *H. pylori* infection.

Unfortunately, there is no broad consensus about an optimal antibiotic therapy for treatment of *H. pylori*. For example, meta-analyses of European and Asian clinical data compared the standard triple therapy (amoxicillin, clarithromycin, and a proton pump inhibitor for 7-14 days) with 5 or 10 day quadruple therapy regimens (adding metronidazole or tinidazole to the triple therapy), and found that quadruple therapies are both significantly more effective and cheaper than the triple therapy.[8–10] However, we previously published a study comparing eradication therapies in 7 sites of 6 Latin American countries that showed the 14-day triple

therapy was superior to the 5-day concomitant quadruple therapy, and no different than the 10-day sequential quadruple therapy.[11] These inconsistencies reflect localized differences in antibiotic use practices, such as the use of clarithromycin for upper respiratory infections, which can drive antibiotic resistance patterns in other organisms like *H. pylori*.[12]

The differences in efficacy of antibiotic therapy are supported by primary antibiotic resistance data. For example, *H. pylori* resistance to amoxicillin varied widely between Africa (65.6%), Europe (0.5%), Asia (11.6%), and the Americas (2.2%).[12] Even in the same region, patterns of resistance differ: within Central and Latin America, reported average metronidazole resistance varies from 30% in Argentina to 83% in Columbia, and tetracycline resistance varies from 2% in Brazil to 33% in Columbia.[13] As such, characterizing local resistance patterns is important for selecting therapies with the highest likelihood of success.

Our research focused on Peru, where gastric cancer is the leading cancer killer in men and women combined.[14] Thus, we searched the literature for reports of primary antibiotic resistance to *H. pylori* in Peru. Three studies were identified, which reported 36.9% resistance to levofloxacin,[15] an average of 66% resistance to metronidazole,[16,17] and 50% resistance to clarithromycin,[16] and 0% resistance to tetracycline.[16] There were no data on amoxicillin, and the reported results of other antibiotics were based on small sample sizes, so whether their results are generalizable is unknown.

As successful eradication of *H. pylori* infection is an important step towards prevention of gastric carcinoma,[5,18] our objective was to assess primary *H. pylori* antibiotic resistance among 76 clinical isolates from Lima, Peru. We hypothesized that we would find similar rates of antibiotic resistance seen in other studies in Peru. We collected clinical isolates from a cohort of patients recruited in Lima, Peru, measuring resistance to metronidazole, amoxicillin, tetracycline,

clarithromycin, levofloxacin, and rifampicin to cover the gamut of antibiotics used from initial through 2nd and 3rd line therapies.[11,19] Our data found significant primary antibiotic resistance to first- and second-line antibiotics among *H. pylori* isolates from a clinical setting in Lima, Peru.[20]

Methods

Patient recruitment, treatment, and sample collection

The study protocol was approved by the Ethics Committee of the Universidad Peruana Cayetano Heredia in Lima, Peru, and the Institutional Review Board of the University of Michigan in Ann Arbor, Michigan. The cohort of patients from whom *H. pylori* isolates were obtained has been previously described.[20] All experiments were conducted under the registered Clinical Trial Gob NCT015128, and SWOG clinical trial S1119. Briefly, patient recruitment occurred between September 2011 and August 2013 at the clinical facilities of the Universidad Peruana Cayetano Heredia Hospital in Metropolitan Lima. Signed, informed patient consent for procedures, antibiotic treatment, follow-up, and downstream molecular analyses was obtained prior to enrollment in the trial. Study participants were ages 20-70 and had symptoms of dyspepsia for at least six months. Patients with gastric cancer or peptic ulcer disease were excluded from this study. Stomach biopsies were obtained via endoscopy under sedation from 109 adults symptomatic patients. The diagnosis of *H. pylori* infection was done histologically. Six biopsies per patient were obtained: four for histologic studies and two for culture, which were stored in 1.5mL of 1x PBS with 20% glycerol at -80ºC until processing. Following endoscopy, infected patients were treated with twice a day esomeprazole, amoxicillin, and clarithromycin for 14 days. Indigent patients received treatment free of charge. Patients were followed up one year after treatment to check for *H. pylori* infection status via the Urea Breath Test.[20]

H. pylori isolation

Gastric biopsy samples thawed on ice and then homogenized using OMNI probes at maximum speed. (International, Kennesaw, Georgia, USA). 50µl of the homogenized sample was plated onto both 5% Sheep's Blood Tryptic Soy Agar plates (Remel, Columbus, Ohio, USA) and on *H. pylori* selective media (Columbia blood agar base with 10% horse blood, 10mg/L vancomycin, 5mg/L trimethoprim, 5mg/L cefsulodin, 5mg/L amphotericin B, 300mg/L urea, and 3500U polymyxin B/L).[21] Plates were incubated at 37°C in microaerobic conditions for 3-7 days. Presumptive *H. pylori* isolates were subcultured, then confirmed by morphology and checked for urease activity using a urease indicator broth (0.33M urea, 0.2% Phenol Red, 0.02% NaN_3, 0.01M pH 6.5 $NaPO_4$ buffer). Glycerol stocks of each isolate were prepared in Brucella Broth (Remel, Columbus, Ohio, USA) with 15% glycerol.

Detecting vacA and cagA with PCR

Extracted DNA from biopsy samples was tested for the presence of *H. pylori cagA* and *vacA* genes by PCR using previously described primers and the Takara PCR kit (Clontech, Mountain View, CA, USA). For *cagA*, previously described conditions and primers F1 (5′ GATAACAGGCAAGCTTTTGAGG 3′) and B1 (5′ CTGCAAAAGATTGTTTGGCAGA 3′) were used to amplify a 349 base pair product.[22] Previously described primers VAG-F (5′-CAATCTGTCCAATCAAGCGAG) and VAG-R (5′-GCGTCAAAATAATTCCAAGG) were used under the following conditions to amplify the m1/m2 subunits of the *vacA* gene: initial denaturation at 95°C for 2 min followed by 35 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 1 minute, completed with a final extension at 72°C for 5 minutes to amplify a 570 or 645 base pair product.[23] PCR products were visualized on a 1.5% agarose gel.

Antibiotic resistance and breakpoints

Using a protocol adapted from the University of Michigan Health System Clinical Microbiology laboratory and bioMérieux's instructions, *H. pylori* isolates were tested for susceptibility to amoxicillin, clarithromycin, levofloxacin, metronidazole, rifampicin, and tetracycline using E-test® (bioMérieux, Durham, North Carolina, USA). Isolates were subcultured, then grown on Mueller Hinton agar supplemented with 5% sheep's blood (Remel, Columbus, Ohio, USA). Colonies were collected and suspended in 1x phosphate buffered saline and visually compared to a 3.0 McFarland Turbidity Standard. Cell suspensions were then spread on Mueller Hinton agar (5% sheep's blood) and stored for 15 minutes in microaerobic conditions, allowing the suspension to dry on the plate. Then, E-test® strips were placed on the plates with sterile forceps, and the plates were incubated for 72 hours at 37ºC in microaerobic conditions. Results were interpreted per the European Committee on Antimicrobial Susceptibility Testing. [24] The minimum inhibitory concentrations (MIC) of amoxicillin, clarithromycin, levofloxacin, metronidazole, rifampicin and tetracycline were measured according to the manufacturer's instructions.

Quality control

ATCC strain 43504 (*H. pylori*) and ATCC 25922 (*E. coli*) were used as quality control strains. See table 4.1 for expected QC minimum inhibitory concentrations (MICs). ATCC 43504 was prepared and treated in the same way as unknown isolates, and was run simultaneously with each batch of isolates against clarithromycin, amoxicillin, metronidazole, and tetracycline. ATCC 25922 was plated from a glycerol stock 48 hours onto an MH plate before testing, and sub-cultured onto an MH plate 24 hours before testing. Colonies were suspended in 1x PBS to a

visual density of a 0.5 McFarland standard, and was run simultaneously with each batch of isolates against levofloxacin and rifampicin. QC results were typically within range, though interestingly, our reference strain of ATCC 43504 was completely resistant to metronidazole, and consistently had tetracycline MICs between 0.047 and 0.25 (slightly lower than usual).

Breakpoints and interpretation of results

H. pylori plates were read after 72 hours of incubation in microaerobic conditions. *E. coli* QC plates were checked after 24 hours of incubation. MICs of strains were interpreted according to EUCAST (European Committee on Antimicrobial Susceptibility Testing) standards, which are "based on epidemiological cut-off values, which distinguish wild-type isolates from those with reduced susceptibility".[24]

Statistical analysis

Antibiotic resistance MICs were examined using descriptive statistics. Student's *t*-tests were used to examine whether isolates with the m1 vs. m2 subunit of the *vacA* gene were resistant to different numbers of antibiotics.

Results

Seventy-six *H. pylori* strains were isolated from the gastric biopsies and were tested for primary antibiotic resistance (Table 4.2). About one third of isolates were resistant to either clarithromycin or amoxicillin, which are typically used for the standard triple therapy. 10.5% were resistant to both amoxicillin and clarithromycin, and 40.1% of strains were resistant to >3 of the tested antibiotics (Table 4.3). Metronidazole was the antibiotic to which isolates were most commonly resistant (61.8%), while isolates showed least resistance to tetracycline (3.9%).

Resistance to levofloxacin and rifampicin among the clinical isolates was 53.9 and 46.1%, respectively.

PCR (polymerase chain reaction)

By PCR, all 76 strains were positive for the *cagA* pathogenicity island, 57/76 (75%) were positive for *vacA* m1 and 19/76 (25%) were positive for *vacA* m2. No differences were seen between the presence of *vacA* m1/m2 and the mean number of antibiotics to which isolates were resistant.

Discussion

To our knowledge, this is the first study characterizing *H. pylori* primary antibiotic resistance to amoxicillin and rifampicin in Peru. When comparing our results to published studies, we found that the MIC cutoffs used were inconsistent between studies. Using a mini-well agar dilution method to determine antibiotic resistance, Vasquez et al. used a clarithromycin MIC of 0.125 and a metronidazole MIC of 4 mg/L,[16], rather than the EUCAST cutoffs of 0.5 and 8, respectively. Our study showed comparable primary antibiotic resistance among *H. pylori* isolates to metronidazole, and slightly higher resistance to clarithromycin and levofloxacin.[13,15] We conducted a brief meta-analysis compiling all primary antibiotic resistance data in Peru from ours and other reports from the literature (Table 4.4).

This study demonstrates a high incidence of primary *H. pylori* antibiotic resistance in Lima, Peru to antibiotics used in the standard triple therapy. This suggests that there is a need to either develop new antibiotics for *H. pylori* eradication therapy, or to reduce the use of these antibiotics

for treating other infections to help protect their efficacy against *H. pylori.* Our study results also highlight the importance of continuing to characterize regional antibiotic resistance patterns to determine the best course of treatment While inference from our results for clinical practice is limited due to our small sample size, we noted some important trends in our data and resulting meta-analysis. First, the small percentage of isolates resistant to tetracycline is worth examining in future studies to see if this trend holds. If it does, tetracycline could be of potential clinical use for *H. pylori* eradication in Lima. Second, virtually all clinical isolates tested were resistant to one or more of the antibiotics commonly used to treat this infection, including amoxicillin, clarithromycin, levofloxacin and metronidazole. This may contribute to the lower than anticipated response to *H. pylori* therapy observed in other parts of Latin America.[11,25] This overall pattern of antibiotic resistance suggests that it may be worth considering treatment alternatives for *H. pylori* infection in Lima, Peru. We suggest that clinicians consider testing the antibiotic resistance profile of clinical isolates from patients with treatment-resistant infection as a way to guide their treatment decisions.

An emerging appearance of *H. pylori* antibiotic resistance has also been reported from other parts of the world, including Asia, Europe and the Americas.[12,13,15–17,19,26,27] This observation, coupled with reports of *H. pylori* reinfection after successful antibiotic treatment,[6,7] makes *H. pylori* treatment more challenging. Meanwhile, gastric cancer remains one of the most common and most lethal cancers in men and women combined in Peru.[2,14] After accounting for emerging patterns of antibiotic resistance of *H. pylori*, it might be useful to reconsider present treatment practices while investigating new therapies and considering testing of *H. pylori* clinical isolates for antibiotic sensitivity in certain regions of the world, such as Peru.

Conclusion

We show high rates of primary antibiotic resistance to among *H. pylori* clinical isolates in Lima, Peru. More studies are needed to confirm this finding to optimize clinical treatment of *H. pylori* infection in Peru.

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Table 4.1. Quality Control ranges for ATCC 43504 and 25922

Antibiotic	***H. pylori* strain 43504 QC Ranges**	***E. coli* strain 25922 QC Ranges**
Tetracycline	0.12-1 mg/L	----
Amoxicillin	0.015-0.12 mg/L	----
Metronidazole	64-256 mg/L	----

Clarithromycin	0.015-0.12 mg/L	----
Levofloxacin	----	0.008-0.06 mg/L
Rifampicin	----	4-16 mg/L

Table 4.1. E-test strip quality control ranges for antibiotics used in this study.

Table 4.2. Primary antibiotic resistance of clinical isolates from Lima, Peru.

	Amoxicillin	Clarithromycin	Tetracycline	Levofloxacin	Metronidazole	Rifampicin
Resistance cut-off (mg/L)	>0.125	≥0.5	>1	>1	>8	>1
Isolates Susceptible	51/76 (67.1%)	49/76 (64.5%)	73/76 (96.1%)	35/76 (46.1%)	29/76 (38.2%)	41/76 (53.9%)
Isolates Resistant	25/76 (32.9%)	27/76 (35.5%)*	3/76 (3.9%)	41/76 (53.9%)	47/76 (61.8%)	35/76 (46.1%)
MIC50 (mg/L)	0.0555	0.094	3	0.048	256	0.875
MIC90 (mg/L)	256	256	32	0.125	256	32
MIC Range (mg/L)	0-256	0-256	0.25-32	0.032-32	0.25-256	0-256

*Table 4.2. Results of MIC testing using E-test strips from Biomerieux. Susceptibility and resistance was determined using EUCAST standards. *5 isolates had a 0.5 ug/mL MIC for clarithromycin, which falls between the susceptible and resistant cutoffs for clarithromycin. For the purposes of this analysis, they were considered resistant.*

Table 4.3. Primary resistance to >1 antibiotic among clinical isolates.

	No resistance	**1**	**2**	**3+**	**Resistant to Clarithromycin and Amoxicillin**
Number of isolates (n/N)	2/76 (2.6%)	21/76 (27.6%)	22/76 (28.9%)	31/76 (40.8%)	8/76 (10.5%)

Table 4.3. Nearly all isolates were resistant to at least one antibiotic, and 70% were resistant to 2 or more. 8/76 isolates were resistant to both clarithromycin and amoxicillin, which are both used in the triple therapy.

Table 4.4. Reported primary antibiotic resistance among *H. pylori* isolates from Peru using EUCAST guidelines.

	Amoxicillin	Clarithromycin*	Tetracycline	Levofloxacin	Metronidazole	Rifampicin
Vasquez et al, 1996[16]	N/A	38.9% (7/18)	0% (0/5)	N/A	27.8% (5/18)	N/A
Berg et al, 1997[17]	N/A	4.2% (1/24)	N/A	N/A	63.6% (49/77)	N/A
Mochizuki et al, 2011[15]	N/A	N/A	N/A	36.8% (35/95)	N/A	N/A

Totals, including present study	32.9% (25/76)	29.7% (34/118)	3.7% (3/81)	44.4% (76/171)	59.1% (101/171)	46.1% (35/76)

*Table 4.4. Meta-analysis of primary antibiotic resistance among isolates from all studies in Peru. *6 isolates had a clarithromycin MIC of 0.5mg/L. For the purposes of this meta-analysis, we counted then as resistant.*

Chapter 4.2: Examination of Water Treatment Options for *H. pylori* Eradication

Introduction: *Helicobacter pylori* is a bacterium that colonizes the human stomach. While *H. pylori* is asymptomatic in most people, it can cause peptic ulcers and has been categorized as a class 1 carcinogen, causing gastric adenocarcinoma [1,2]. *H. pylori* is hypothesized to be transmitted directly through fecal-oral, gastro-oral, or oral-oral routes, or indirectly through reservoirs such as animals, contaminated food, or water [3,4]. Lack of access to clean drinking water and proper sanitation has been consistently identified in epidemiological studies as a risk factor for *H. pylori* infection[5–9]. *H. pylori* has cultured from both wastewater and drinking water[10–14], and has been reliably detected in recreational and drinking water using DNA-based molecular biology techniques such as PCR, and microscopy methods such as fluorescent in-situ hybridization [15–20]. Further, *H. pylori* can survive in water distribution systems and incorporate into biofilms [21,22], suggesting a mechanism for sustained contamination of water supplies with *H. pylori*.

To complicate matters, however, *H. pylori* rapidly enters a viable-but-not-culturable (VBNC) state when exposed to water, making it challenging to culture[15,16,23]. *H. pylori* in this VBNC state maintains metabolic activity[24,25] and is membrane intact[15]. However, amongst the studies that have cultured *H. pylori* from water, it is uncertain whether they caused *H. pylori* to revert from the VBNC state to a viable, culturable state, or if they simply captured *H. pylori* in the viable culturable state in the environment. Since *H. pylori* remains difficult to culture and culture methods do not necessarily accurately quantify the number of viable cells, studies have begun to examine the use of alternative methods of quantifying viable *H. pylori* cells in the

VBNC state. One such method treats cells with propidium monoazide (PMA) upstream of quantitative polymerase chain reaction [26]. PMA is a photoreactive dye that binds with high affinity and intercalates into double stranded DNA, preventing downstream amplification. While it can penetrate damaged cell membranes, intact cell membranes are impermeable to PMA, making it a useful agent for determining between viable and non-viable cells. This method has been used successfully to examine dynamics of *H. pylori* survival in room temperature water, in drinking water distribution systems, in cohabitation with amoebas, and against treatment with ozone[26–30].

Despite these unknowns, *H. pylori* in water – be it VBNC or viable and culturable – may pose a risk to human health. The afore-mentioned epidemiological associations provide a link between contaminated water and *H. pylori* infection, several studies have shown that VBNC *H. pylori* is infectious in mice via gavage[31–33], and we previously showed that waterborne viable culturable *H. pylori* is infectious in mice[34]. This evidence provides motivation for examining potential interventions that could eradicate or remove *H. pylori* from drinking water, both in municipal treatment processes as well as with point-of-use options when municipal water is not available or is of poor quality.

However, there remains a dearth of literature around water treatment options for *H. pylori*. *H. pylori* can survive in this VBNC state even when exposed to common disinfectants; after exposure to 0.8mg/L chlorine in drinking water for 24 hours, *H. pylori* still maintained gene expression of the toxin VacA during that time[16]. *H. pylori* is also more resistant to low levels of chlorine (0.1-0.3 mg/L) and ozone (0.05-0.125 mg/L) than *E. coli*, but similarly susceptible to monochloramine. However, the evaluation of other cost-effective water treatment methods, like boiling, have not been examined.

Thus, the objective of our study was to use PMA-qPCR and culture techniques to examine the effectiveness of boiling water and bleach disinfection for *H. pylori* eradication in drinking water. Since user compliance of household drinking water treatment techniques varies substantially[35], we tested the efficacy of the treatment methods in multiple different exposure scenarios. We hypothesized that longer exposure to bleach or boiling disinfection would result in effective eradication of *H. pylori* by both culture-dependent and –independent methods. We further hypothesized that boiling disinfection would be more effective than bleach disinfection, given that *H. pylori* appears to be able to retain some metabolic function even after longer exposures to chlorine in water[6].

Methods

Culture of H. pylori and inoculation

H. pylori strain SS1 was used in all experiments. Tryptic Soy Agar II plates with 5% sheep's blood (Remel) was inoculated with glycerol stocks of SS1 and incubated in microaerobic conditions at 37°C for 3 days. The resulting colonies were collected and suspended in 1x PBS, and this solution was serially diluted and plated on TSA II plates (5% sheep's blood) and incubated in microaerobic conditions at 37°C for 7 days to calculate the quantity of input cells. The remaining cell suspension was used to inoculate sterile filtered tap water to examine the effectiveness of the various treatment scenarios.

Boiling

H. pylori was added to an Erlenmeyer flask containing 250mL of room temperature sterile filtered tap water, and then heated until it had just begun to boil, boiled for 1 minute, or boiled

for 5 minutes. The flask was then cooled to room temperature, and then vacuum concentrated as described below.

Chlorine exposure

Sodium hypochlorite solution was added to room temperature sterile filtered tap water, then allowed to equilibrate in solution for 5 minutes. After 5 minutes, the free available chlorine concentration was measured with a Hach Spectrophotometer (average concentrations of 3.06-3.46mg/L). Then, *H. pylori* stock solution was added to the chlorinated water and held at room temperature for 5, 15, and 30 minutes. After exposure to chlorine, several grains of sodium thiosulfate were added to remove any residual chlorine, which was confirmed by a Hach Spectrophotometer. The cells were then vacuum concentrated as described below.

Concentration of cells

Water was vacuum concentrated onto 0.22μM membranes. Membranes were gently scraped into 1x PBS to remove the bacteria. This solution was transferred to 1.5mL microcentrifuge tubes and centrifuged for 6 minutes at 3000 rpm. The supernatant was removed and pellet was re-suspended in 1100 μL of 1xPBS. To test for culturability of treated cells, 100 μL of this new cell suspension was plated onto TSA II 5% Sheep's Blood agar (Remel) and incubated in microaerobic conditions at 37°C for 14 days. The remaining sample was split into 2 microcentrifuge tubes containing 500 μL of treated cells.

PMA Treatment

The following method for PMA treatment was adapted from Augusti et al. (2010). 1.25 µL of Propidium monoazide (PMA) was added to one duplicate of each 500 µL sample. The other duplicate was used as a control: this sample represented the total number of cells recovered after treatment, as downstream qPCR would measure DNA from all cells in this suspension, be they viable, VBNC, or dead. After the PMA addition, samples inverted >25 times to mix, and then were kept in the dark for 5 minutes. Next, samples were placed on ice and positioned 20cm away from 600W high intensity light for 15 minutes, turning and inverting every few minutes to ensure suspension homogeneity and to avoid overheating. After exposure to light, the tubes were centrifuged for 5 minutes at 14,000g. DNA extraction was then carried out using the Qiagen QiaAmp Mini Kit according to the manufacturer's instructions.

Control cells: not boiled or exposed to bleach

To examine if any cell damage or loss of culturability occurred in untreated cells, suspensions of freshly grown *H. pylori* were inoculated into sterilized tap water for 0, 1, 5, 15, and 30 minutes. After the requisite waiting period, the cells were concentrated by vacuum filtration, and then plated and treated with PMA in the same way as samples exposed to either chlorine or boiling.

Percent recovery calculations

In the treatment and control trials, percent recovery was calculated by dividing the total number of input cells (culturable) by the total number of cells measured by qPCR in aliquots that were not PMA treated (See PMA Treatment).

qPCR (quantitative polymerase chain reaction)

qPCR was performed on extracted DNA using the method from Janzon et al. (2009)[36]. Briefly, quantities of *H. pylori* were quantified using a reaction mixture containing 10ul 2×SYBR GREEN PCR Master Mix (Applied Biosystems, Grand Island, NY, USA), 0.3 µL of each 20 µM primers HpA-F (ACTTTCTCGCTAGCTGGATGGTA) and HpA-R (GCGAGCGTGGTGGCTTT), 8.4µL of sterile PCR water, and 1 µL of DNA template. Plates also included negative controls (no DNA added) and positive controls (*H. pylori* DNA), and a standard curve made with 10E2 to 10E7 genome copies of *H. pylori* strain SS1 DNA. qPCR was run at the following conditions: 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds and 60°C for 1 minute, followed by a melting curve analysis, ramping from 60°C to 95°.

Statistical analysis

Student's t-tests were used to compare differences in the mean log-reduction between boiling and bleach trials. One-way ANOVAs were used to compare the log-reduction measured by PMA-qPCR between 5, 15, and 30 minutes of exposure to bleach, and between 0, 1, and 5 minutes of exposure to boiling water.

Results

The results of the bleach and boiling trials are summarized in tables 4.5 and 4.6, respectively. Figure 4.1 shows the compiled PMA-qPCR results of boiling, bleach, and controls trials. Culturability was completely lost in all bleach and boiling trials, resulting in a ~8 log reduction in culturable cells. This result was seen regardless of the exposure length. Surprisingly, we saw a much lower log-reduction (1.32-1.49) using the PMA-qPCR method in both boiling and bleach trials. No significant differences were seen between the PMA-qPCR results for bleach trials (F-

statistic=0.12, p=0.88) or for the boiling trials (F-statistic=0.28, p=0.76), regardless of the length of exposure to each respective disinfection technique. This was also surprising, as we expected to see that longer exposure to disinfection would result in increased eradication of *H. pylori* cells. No significant differences were seen between the mean PMA-qPCR results between bleach and boiling trials (0.03, 95% CI [-0.21, 0.27], p=0.8). There were statistically significant differences between the average log-reduction by PMA-qPCR of control trials vs. exposure trials (0.65, 95% CI: [0.44, 0.86], p<0.0005).

The results of the control trials are summarized in table 4.7. As expected, culturable cells were recovered at each time point, with a general trend of a slightly decreased number of cells recovered with increasing time. Culturability decreased by 1.56-2 log, with a general increasing trend as the length of exposure to drinking water increased. Given the ~8 log-reduction seen in the disinfection trials, this suggests that both bleach and boiling disinfection result in ~6 log-reduction of *H. pylori* cells by the culture-based method.

However, no such trend among controls was seen with the PMA-qPCR method: cells exposed to water for 5 minutes showed the greatest log-reduction by qPCR (0.92), while cells exposed for 30 minutes only showed a log-reduction of 0.71. In both the boiling trials and the control trials, there was one instance in which the total recovered cells exceed 100%. This may be because there were some dead or VBNC cells in the initial inoculum, which resulted in a higher quantity of genomic DNA than the total number of culturable cells.

Discussion:

In our present study, we found that boiling and bleach disinfection treatments, regardless of exposure length, reduced culturability in *H. pylori* cells, by ~6 log. However, *H. pylori*'s

conversion to a VBNC state reflects a limitation of culture-based methodology. When using the culture-independent technique of PMA qPCR, we did not see a consistent or significant dose-response in our exposure methods between *H. pylori* exposed to a minimal level of disinfection (e.g. 0 minutes of boiling or 5 minutes of chlorine disinfection) vs. a longer time exposure (i.e. 5 minutes of boiling or 30 minutes of bleach exposure). Nor did we see consistency between culture-based and the culture-independent methods, as there was a >6-log difference between them. Based on the culture methods, these study results were somewhat consistent with our hypothesis that boiling and bleach disinfection could successfully remove *H. pylori*. However, we saw no support for our hypothesis that boiling disinfection would be more effective than bleach disinfection, and the PMA-qPCR results cast doubt on the efficacy of either of these methods for successful eradication of *H. pylori* from drinking water due to the conversion of *H. pylori* .

This leads to two potential inferences. First, that PMA-qPCR may not be appropriate for use as a culture-independent technique for testing the *H. pylori* reduction capacities of water treatment devices. A remarkably similar pattern was observed when using PMA-qPCR vs. culture methods to investigate monochloramine disinfection of bacteria in the effluent of biologically active carbon[37]: there was an ~1.5 log reduction by PMA-qPCR, and ~4-log reduction in culturability using the heterotrophic plate count method. While another recent study showed a 4-5 log decrease in coccoid *H. pylori* using PMA qPCR following ozone disinfection[30], the authors calculated their log-reduction by comparing their disinfected, PMA-treated cells to cells that were neither disinfected nor PMA-treated, which over-estimates the effectiveness of their technique. The cells in that experiment were held at 10 days at room temperature, which,

based on viability estimates from other experiments[26], would have already undergone at least ~2 log reduction by that time, potentially compromising the validity of those results.

Second, this could suggest that *H. pylori* is in some way uniquely resistant to boiling and bleach disinfection. While little data exists on the efficacy of boiling to remove *H. pylori*, Moreno et al. (2007) found that *H. pylori* can survive in the VBNC state for 3 hours in chlorinated water (0.96mg/L – 1.16mg/L Cl_2), and can continue producing the toxin VacA in the presence of chlorine for over 24 hours[16]. Further, Baker et al found that *H. pylori* is more resistant to chlorine disinfection than *E. coli*, having a slower decrease in loss of culturability at low doses of chlorine (0.1-0.3mg/L)[38]. While this is intriguing, more research is needed to tease out whether this holds in other scenarios as well.

However, our study does have important limitations. First, and most importantly, we cannot determine whether the *H. pylori* cells in our PMA-qPCR assays were in VBNC state, or dead/dying but still membrane-intact (rendering them viable by PMA-qPCR). Until more reliable methods are developed to quantitatively differentiate between cells in these different states, accurately measuring the ability of water treatment systems to remove *H. pylori* will remain difficult, as we found in our current study. Second, we are uncertain that loss of culturability actually results in cell death, since, as mentioned above, *H. pylori* can still produce its primary toxin after 24 hours of exposure to chlorine[16]. Finally, we used sterilized, filtered tap water in our study, which is not representative of drinking water in nearly any setting. Thus, it is possible that other water characteristics or co-contamination with other bacteria or toxicants may affect the disinfection capabilities of our treatment methods.

In conclusion, we found that both boiling and chlorine disinfection were capable of quickly reducing culturability in *H. pylori* strain SS1. However, PMA-qPCR, while it may be

useful in some contexts, does not appear to be a good way of measuring the efficiency of boiling and bleach disinfection for eradicating *H. pylori*. More research is needed to further examine this relationship and optimize culture-independent methods to gauge the relative population proportions of *H. pylori* in drinking water.

Conclusions: *H. pylori* transmission in water seems plausible given the strong epidemiological associations and consistent findings of water contaminated with *H. pylori*. Based on the culture-dependent eradication rates, both boiling and bleach disinfection appear to be relatively suitable for treating *H. pylori*-contaminated water. More research is needed to develop better culture-independent techniques that quantify the presence of VBNC *H. pylori* in water.

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Table 4.5. Overview of Bleach Exposure trials.

Bleach Trial	n	Mean Chlorine mg/L (mean, sd)	Mean chlorine after mg/L (mean, sd)	Total number of culturable input cells (mean, sd)	Average Percent Recovery by qPCR (mean, sd)	Log-reduction by PMA (mean, sd)	Culturable after treatment?
5 minutes	5	3.46 (0.48)	2.46 (0.58)	2.40E9 (7.53E8)	8.7 (10.3)	1.32 (0.18)	NO
15 minutes	5	3.39 (0.60)	1.89 (0.53)	2.40E9 (7.53E8)	14.6 (13.8)	1.38 (0.29)	NO
30 minutes	4	3.06 (0.31)	1.10 (0.57)	1.54E9 (5.02E8)	21.6 (17.1)	1.40 (0.29)	NO

Table 4.5. No differences were seen between the different time trials with regards to culturability. With regards to PMA-treated cells, there were no differences seen between the log-reduction in cells based on treatment length.

Table 4.6: Overview of Boiling trials.

Boiling Trials	n	Total number of culturable input cells (mean, sd)	Average Percent Recovery by qPCR (mean, sd)	Log-reduction by PMA-qPCR (mean, sd)	Culturable after treatment?
0 minutes (water was brought to a boil and then removed from heat)	5	1.94E9 (1.13E9)	69.6 (63.7)	1.36 (0.55)	NO

1 minute	6	1.43E9 (8.11E8)	30.9 (41.2)	1.49 (0.32)	NO
5 minutes	5	1.94E9 (1.13E9)	123.1 (122.4)*	1.33 (0.18)	NO

*Table 4.6. Boiling trial results. No differences were seen between the different time trials with regards to culturability. With regards to PMA-treated cells, there were no differences seen between the log-reduction in cells based on treatment length. *: Percent recovery is higher than 100% due to the denominator being the total number of input culturable cells. qPCR captures all cells, including non-viable cells and VBNC cells, which is why this value is higher.*

Table 4.7: Overview of control trials.

Control Trials	**n**	**Average Percent Recovery by qPCR (mean, sd)**	**Log-reduction by PMA-qPCR (mean, sd)**	**Log-reduction: culturability (mean, sd)**
0 minutes: added to water then immediately vacuum concentrated	2	113 (71.8)*	0.67 (0.43)	1.56 (0.26)
1 minute	2	48 (35.3)	0.63 (0.20)	1.78 (0.46)
5 minutes	2	94.9 (37.6)	0.92 (0.37)	1.86 (1.07)
15 minutes	2	59.4 (8.6)	0.72 (0.34)	1.83 (0.46)
30 minutes	2	88.9 (41.3)	0.71 (0.59)	2.0 (0.79)
Average	2	80.86 (38.9)	0.73 (0.10)	1.81 (0.61)

*Table 4.7. Results from the control trials. *: Percent recovery is higher than 100% due to the denominator being the total number of input culturable cells. qPCR captures all cells, including non-viable cells and VBNC cells, which is why this value is higher.*

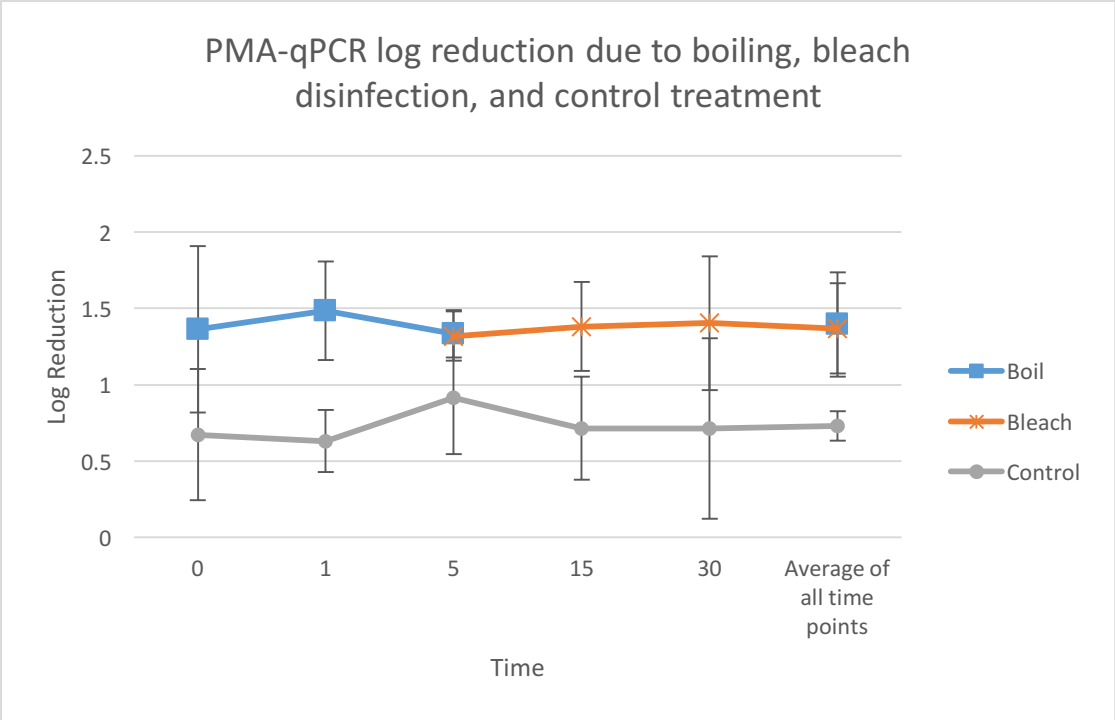

Figure 4.1. Comparison of log reduction due to boiling, bleach disinfection, and control treatment in exposed cells. No significant differences were seen between boiling and bleach disinfection. Control cells showed consistently lower log-reduction of viable cell counts by PMA-qPCR.

Chapter 5 : Conclusions

This dissertation investigated the role of contaminated water as a potential transmission route for *H. pylori* infection in Lima, Peru. The main contributions of this dissertation to our understanding of the risk of *H. pylori* in drinking water relate to potential differences in infectivity between the viable culture and VBNC state and the importance of exposure route (drinking water versus gavage).

In Chapter 2, we conducted an exposure assessment of *H. pylori* in drinking water in Lima, Peru, sampling water from: the homes of patients in a clinical cohort, wells that supplement the municipal drinking water treatment system, finished water from the treatment plant, and a sink in the Lince district. We found that water in Lima was consistently contaminated with *H. pylori*. Of the water collected from patient homes, 48% (n=42/87) of drinking water samples were positive. All samples of source water (n=2) and finished water directly from the municipal plant (n=2) were positive for *H. pylori*. 60% (n=3/5) well-water samples, and 22.5% (58/258) of samples collected from the Lince district were positive for *H. pylori*. While there were some statistically significant associations between pH and the quantity of *H. pylori* as well as between temperature and the presence of *H. pylori*, these associations were not in the expected direction. Thus, they may reflect stochastic events (such as shedding from biofilm or infusions of contaminated water from leaks in the distribution system) that were not captured in the variables used as model inputs. Taken together, these results show that water in Lima is regularly contaminated with *H. pylori*, which is consistent with results seen in many other developing countries throughout the world[1–9]. This suggests that *H. pylori* can either

propagate or survive in drinking water in Peru. However, the major limitation of this study was our inability to determine the relative population proportions of *H. pylori* in water, i.e. viable and culturable, VBNC, or dead/dying. Future studies and methods are needed to quantitatively examine the proportions of these cell populations to better understand the risk of waterborne *H. pylori*.

In Chapter 3, we investigated the infectious potential of *H. pylori* in drinking water using a mouse model. First, we exposed mice to various doses of viable, culturable *H. pylori* strain SS1 in drinking water, and found that they were infected in a dose-dependent manner[10]. Next, we exposed mice to various exposure scenarios of VBNC *H. pylori* strain SS1 in drinking water. Surprisingly, we were unable to induce infection using VBNC *H. pylori*, even after 56 days of exposure to $>10^9$ cells/L[11]. We further investigated the infectious potential of VBNC SS1 using gavage, giving 4 doses of $\sim 2.5*10^8$ cells/dose to mice over 2 weeks. This also failed to induce infection. Given these results, we concluded that while viable, culturable *H. pylori* appears to be infectious in water, the infectivity of VBNC *H. pylori* is less certain. Other investigators have been able to cause infection in mouse models via gavage using *H. pylori* strains recently isolated from human gastric biopsy samples[12–14], suggesting that there may be variability in infectivity between strains. Since we only used one strain of bacteria, and the genetic diversity of *H. pylori* is vast [15], it is possible that there could be some strains of *H. pylori* that are better suited to infect hosts via water compared to other routes. Finally, this could imply that, since much *H. pylori* in water is in the VBNC state, that the route of drinking water does not contribute significantly to the transmission of *H. pylori* infection.

In Chapter 4, we examined clinical and environmental methods for controlling *H. pylori* infection. Clinically, we found high rates of primary antibiotic resistance to amoxicillin and

clarithromycin, which are typically used as first-line treatment of *H. pylori* in Lima[16]. After including our results in a meta-analysis of primary antibiotic resistance in Peru[17–19], we found that overall rates of antibiotic resistance are quite high, including in antibiotics used in the standard triple therapy (amoxicillin: 32.9%, clarithromycin 29.7%, metronidazole 59.1%) and in second- and third-line treatments (rifampicin 46.1%, levofloxacin 44.4%). However, we found that primary resistance to tetracycline is low, suggesting the clinical potential for tetracycline in the future. Due to these high rates of antibiotic resistance, we conclude that approaches that help prevent infection would be useful to pursue. Further, to help quell antibiotic resistance, we suggest that clinicians should perform antibiotic resistance profiling of clinical isolates before starting antibiotic treatment. However, given the low number of studies and overall isolates that have examined primary antibiotic resistance and treatment success in Peru, more studies are needed to properly inform clinical practice. These results also highlight the importance of characterizing regional antibiotic resistance patterns, as the compiled results for antibiotic resistance in Peru are quite different from those in other areas of the world[20,21].

We then investigated the disinfection potential of bleach and boiling water for eradicating *H. pylori*, using both culture-dependent (plating) and -independent (PMA-qPCR) methods to estimate disinfection efficiency. After exposure to either 0, 1, or 5 minutes of boiling or 5, 15, or 30 minutes of bleach, all culturability of exposed cells was lost – a >6 log reduction. When using culture-independent methodology, these disinfection methods caused an average of 1.3-1.5 log-reduction in the number of membrane intact cells, with no dose-dependent effects. Though this mismatch was puzzling, this has also been observed elsewhere in the literature. After exposure to monochloramine, the heterotrophic plate count of effluent from water filters showed a 4.5 log-reduction, while the same cells measured by PMA-qPCR showed an ~1.5 log-reduction[22]. This

suggests that PMA-qPCR may not be an appropriate method for measuring water-treatment efficacy, and further reinforces the need to develop new methods to differentiate between VBNC, viable culturable, and dead *H. pylori* cells to accurately estimate the effectiveness of these different treatment options.

Risk of infection from waterborne H. pylori?

In reviewing the literature, we found only one published quantitative microbial risk assessment of *H. pylori* in drinking water[23]. The exposure assessment was based on quantities of *H. pylori* found in surface water in the United States, and assumed that "1 gene copy/L is equivalent to… 1 CFU/L or 1 organism/L". The dose-response examination evaluated the risk of infection based on studies that used the infectious dose of viable, culturable *H. pylori* in monkeys and humans administered via gavage or oral routes. The authors concluded that a maximum contaminant level of <1 organism/L was necessary to meet a goal of 1/10000 annual infection risk. However, this risk assessment could be updated based the findings from this dissertation, which suggest that more nuanced approaches are needed.

In the exposure assessment in chapter 2, we conducted year-long time-series sampling campaign of *H. pylori* in drinking water, which could be used as a more accurate input for Lima than surface water found in the USA. However, the quantities of *H. pylori* in finished drinking water that were far higher than those used in the model inputs, and contamination ~20% of the time in our time-series exposure assessment. Had these data been used as inputs in the model, the calculated risk of infection would have likely been much higher, but would have failed to incorporate the important nuanced difference between viable culturable, VBNC, and dead cells – something that we could not capture in our DNA-based method. This highlights the need to

develop risk scenarios that account for variable proportions of VBNC, viable culturable, and dead *H. pylori* cells in drinking water.

The dosing study in chapter 3 that used viable, culturable *H. pylori* had exposure groups with much larger sample sizes than those used in the published QMRA (n=38-40 per exposure group vs. n=2-3), and used waterborne *H. pylori* opposed to oral or gavage exposure. Thus, validating whether *H. pylori* infectivity in mice is representative of that in humans, and potentially updating the dose-response of viable, culturable *H. pylori* with the mouse data from this aim could strengthen further risk assessments. Next, the second set of dosing experiments in chapter 3 suggest variability in infectivity between VBNC and viable, culturable cells of strain SS1. Given that *H. pylori* typically exists in the VBNC form in water, this suggests that using a dose-response that relies on viable, culturable data may not be reliable. Given that gavage studies with different strains of VBNC *H. pylori* show that it can be infectious in mice[12,14], this highlights the need for studies that better elucidate the infectious dose of various VBNC *H. pylori* strains in drinking water, as well as coordinating dose-response models with exposure assessments appropriately to estimate the risk of waterborne *H. pylori*.

Future directions

While our results show contamination of drinking water in Lima with *H. pylori*, our mouse studies cast doubt on the infectious potential of VBNC *H. pylori* in drinking water. However, our single study is not sufficient to fully validate this claim. There remain many unknowns that must be better understood. To perform accurate risk assessments, new methods are needed to tease out population dynamics of *H. pylori* in water, which would provide better estimates of the number of cells that are VBNC, dead/dying, or viable and culturable. Such studies should also seek to

understand the relationship between water characteristics, such as pH and temperature, and the relative frequency of each form of *H. pylori.* Further, as culture-based methods become more standardized and accessible, it may become easier to examine the infectivity of different strains of *H. pylori*, especially those that seem to propagate most successfully in water. Different strains of *H. pylori* may be differently infectious in the VBNC state, as is suggested by the successful dosing studies (albeit via gavage rather than waterborne transmission) performed with various strains isolated from peptic ulcer patients[12–14]. Additional investigations of such strains would increase the precision of dose-response estimation that gauge risk of infection. Finally, *H. pylori* can cohabitate with amoebae and zooplankton, which may protect it from disinfection, help it survive successfully in water, and potentially allow it to be transmitted more effectively to humans[24–26]. Further investigation of interactions between *H. pylori* and such organisms would inform exposure scenarios that might affect the likelihood of infection from waterborne *H. pylori*. Once such data are available, accurately assessing the risk of waterborne *H. pylori* will become more possible.

Contributions of the Integrated Assessment approach

Our Integrated Assessment approach resulted in a dynamic idea exchange between study teams at the University of Michigan and in Peru. After seeing the water contamination results from our initial clinical study (see Appendix A), officials at the Directorate General of Environmental Health and Food Safety (DIGESA) were interested in collecting more data. Based on their suggestions, and with the assistance of a DIGESA microbiologist, we were able to pursue additional sampling, culminating in the study results shown in Chapter 2.1. Similarly, after we presented our water contamination results at a workshop in Lima, we were asked whether the

quantities of *H. pylori* we found in drinking water were a cause for concern. This question led to the extensive dosing experiments covered in chapter 3, which were the first to examine the infectivity of viable, culturable *H. pylori* and VBNC *H. pylori* in drinking water. Finally, after discussing the importance of developing targeted treatment of *H. pylori* clinical infection, we conducted our antibiotic resistance testing study in Chapter 4.1. The methods used from this study and the results gleaned will help guide future clinical studies at the Universidad Peruana Cayetano Heredia, where our collaborator Dr. Alejandro Bussalleu is now conducting antibiotic resistance testing of clinical isolates from Lima using the same methodology. I am very grateful that I had the opportunity to work with the many dedicated individuals from both Peru and Michigan on this project, as it gave me a greater awareness of the importance of incorporating multiple viewpoints when designing studies to improve public health.

Conclusions

Taken together, our results show that: 1) water in Lima is consistently contaminated with *H. pylori*, 2) VBNC *H. pylori* in drinking water may not be a large contributor to overall *H. pylori* transmission, given our failure to infect mice using VBNC *H. pylori*, 3) antibiotic resistance is high in Lima, and treatment success is lower than expected based on trials in 7 Latin American countries[27,28], and 4) there is uncertainty about the efficacy of existing water treatment technologies for eradicating *H. pylori* in drinking water. Based on these data, we cannot make any recommendations for policy decisions related to drinking water and *H. pylori*, as more studies are needed to better evaluate the risk from waterborne *H. pylori*. However, this does not mean that unimportant to improve access to clean water and sanitation in Lima. Investing in water treatment infrastructure for drinking water and sanitation has been shown to be both

important for saving lives as well as being cost-effective. An estimated 842,000 deaths from diarrheal disease occur each year due to inadequate drinking water, sanitation[29], and water-related hygiene, and investments in water infrastructure can yield up to seven dollars for every dollar invested[30]. For additional control measure, measures should be taken to maintain treatment efficacy in the era of antibiotic resistance. These include phenotyping antibiotic resistance profiles of clinical isolates, and creating a database for clinicians to record treatment success rates of various treatment regimens, as well as antibiotic resistance patterns from their clinics.

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Chapter 6 : Final Thoughts on Science and the PhD Process[3]

Throughout my science education, I have dutifully memorized facts: the stages of photosynthesis, the enzymes involved in the Krebs cycle, how to balance equations in chemical reactions. In contrast, the focus of my ancient history classes was on answering big, open-ended questions: Why did historical figures act in certain ways? How did the assassination of Julius Caesar affect the Roman Empire? Would our world be different had he not been murdered? There were other questions, too, related not just to historical events but to the nature of knowledge, to what we know and how we know it. What's the evidence? How reliable is it? Does the conventional explanation account for all the available information (including competing ideas) and the broader context?

Eventually, I tried applying a similar thought process to my scientific interests. I found that approach to science much more appealing—and also useful. I took it with me as I became a scientist.

Brushes with waterborne illness and professional experiences with water filtration inspired me to pursue a Ph.D. in public health, focusing on the waterborne transmission of *Helicobacter pylori* in Lima. I chose it because it's a big-picture project, a collaboration between public health

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officials, scientists, and doctors with the shared goal of providing data-driven advice to policymakers.

I rapidly discovered that new data, or a new technical approach, won't solve access to clean water. After all, the technology to improve water quality is already available, and water-treatment infrastructure is known to be cost-effective. The problem persists because the challenge of clean water ties into complex political and social issues: culture, economics, science, emotion, ideology. You can't solve such problems without accounting for the bigger picture. Narrow thinking can even lead to strategies that do harm, like privatization efforts in Peru that modestly improved water infrastructure but priced the poor out of the market. As our challenges become more complex, even strictly scientific problems require a broader perspective, akin to that embraced by historians, philosophers, and other humanist scholars.

I have benefited from studying history in other ways. I learned to think critically and to write rigorous, compelling qualitative arguments. Slashed research budgets make writing about broader impacts more crucial than ever. Academic scientists must defend their work against competing political and economic priorities, not just in grant proposals but also in public and political spheres. Scientists are increasingly involved with governments and policymakers: Every year, we've had to justify our research project to a new Peruvian minister of health in order to legally continue.

As stable academic science positions stagnate, a growing proportion of scientists seek employment outside academia. Private-sector and governmental careers usually require thinking that encompasses regulatory and cultural concerns—and pragmatic concerns like profits. The

ability to consider and weigh diverse arguments and to communicate clearly with various stakeholders is essential.

Science's inherently reductive approach and its acute attention to the finest details have yielded great benefits. But the scope of science is changing. In addition to practicing the traditional craft, today's scientists need to be prepared to tackle complex challenges in a globalized (and multidisciplinary) world, to think critically about how we solve problems, and to communicate persuasively with diverse audiences. More than my science classes did, studying history taught me these skills. Scientists can be too eager to write off other disciplines as “soft,” subjective, and therefore inferior to science and its rigorous approach. Those other fields, though, can enhance the practice and understanding of science, among scientists and the public. I encourage my peers to think about science in this larger context, as a liberal art intrinsically tied to its cousins and aimed at illuminating, improving, and adding meaning to the human experience.

Appendix A: Clinical, Epidemiologic, and Genomic Studies (SWOG S1119) of *Helicobacter pylori* in Lima, Peru: Role of Contaminated Water[4]

Introduction

Helicobacter pylori (*H. pylori*) is a bacterium that infects the stomachs of one-half of the world's population, including 80% of those living in low socioeconomic areas of Latin America, Asia, and Eastern Europe. By contrast, less than 20% of asymptomatic Caucasians carry *H. pylori* in the USA[1]. *H. pylori* is a Group 1 carcinogen because of its direct causal relationship to gastric carcinoma[2]. The WHO estimates that for 2008, there were globally over 989,600 new diagnoses and 738,000 deaths from gastric cancer[3]. In Peru, gastric cancer is the most common cancer and cause of cancer mortality in men and women combined[4].

Most accept that fecal-oral, oral-oral, and gastro-oral transmission from mother to child is the principal mechanism of *H. pylori* infection. However, a genotypic study in shantytown households in Lima, Peru, showed 70% discordance between the *H. pylori* strains from mother, children, and others in the family. These results suggest this infection is community acquired and that there may be other sources of infection[5]. Historically, drinking water from the La Atarjea water treatment plant, Lima's primary source of drinking water, has tested positive for *H. pylori* in 50% of 48 samples[6,7]. This information correlated with the frequency of *H. pylori* infection in children, their socioeconomic status, and the type of water they drank. The presence of *H. pylori*

[4] This chapter was previously published in the *Journal of Cancerology*: Valdivieso M, Bussalleu A, Sexton R, Boehnke K, Osorio S, Reyes IN, et al. Clinical, Epidemiologic, and Genomic Studies (SWOG S1119) of Helicobacter Pylori in Lima, Peru: Role of Contaminated Water. J Cancerol [Internet]. 2016;3(2):52–63. Available from: http://www.journalofcancerology.com/resumen.asp?id=55&indice=2016032

in drinking water by polymerase chain reaction (PCR), the high reinfection rate, and the genomic heterogeneity of this organism in Lima suggest that contaminated water may play a role in the transmission of the infection[5–8].

The distribution of gastric carcinoma in Metropolitan Lima, a surrogate of *H. pylori* infection, is highest in the lower socioeconomic areas of Puente Piedra, Lince, Villa El Salvador, El Agustino, Breña, and Rimac (21-25/100,000) and lowest in high socioeconomic areas such as San Isidro and Miraflores (9-13/100,000)[4]. The treatment of *H. pylori* infection is effective in approximately 80-90% of patients, with best results attributed to sequential regimens[9]. There is, however, an increasing rate of treatment failure due to antibiotic resistance, particularly to clarithromycin[10–12]. The rate of annual recurrence is higher in developing countries than in developed countries (13 vs. 2.67%, respectively) and recurrence rates are variable, though high, in Latin America[11–14]. The highest percentage of infection recurrence has been reported in Peru: 73% at eight months and 30% at 18 months[6,8,15]. In other Latin American countries, the annual reported infection recurrence rate is as high as 54% in Chile, 50% in Brazil, and 37% in Mexico[14]. The high recurrence rates indicate that reinfection from environmental sources is possible, leading us to our current study.

This study was conducted based on the hypothesis that the drinking water in Lima was contaminated with *H. pylori*. The presence of *H. pylori* was to be determined by culture and by molecular techniques.

Methods

The study protocol was approved by the Ethics Committee of the Universidad Peruana Cayetano Heredia in Lima, Peru, and the Institutional Review Board of the University of Michigan in Ann Arbor, Michigan. Signed informed consent form was required. The study opened on September 1, 2011 with patient accrual completed on August 5, 2013. The protocol was opened in high- and low-risk areas simultaneously. Patients were followed for a year after therapy. All authors had access to the study data and reviewed and approved the final manuscript.

Patients

We targeted 100 adults between the ages of 20 and 70 with symptoms of dyspepsia for at least six months and with indications for gastroscopy (approximately 80 patients from high-risk areas and 20 from low-risk areas). The presence of peptic ulcer disease and gastric cancer were exclusion criteria for the study. Patients would have a histologic diagnosis of *H. pylori* gastric infection and a Zubrod's performance status of 0 to 2. As a result, there would be gastric biopsy positive and negative patients for *H. pylori* infection. Patients would have resided in the same target districts for at least 10 years. Patients were invited to respond to three questionnaires: (i) risk factors of *H. pylori* infection, (ii) ROME III of general gastrointestinal symptomatology, and (iii) dyspepsia symptoms. Infected patients were treated with triple antibiotic therapy as previously described[16,17].

Patients attended the clinical facilities of the Universidad Peruana Cayetano Heredia Hospital in Metropolitan Lima. Under sedation, six gastric biopsies were obtained. Samples were taken preferentially from the body and antrum of the stomach and always from areas where the most inflammation was present. In the end, half of the biopsies were usually from the antrum and the

other half from the body. We used only one sample from the body and one from the antrum to perform the molecular analysis and culture. The remaining samples were used to perform the histopathological analysis and diagnosis. Research samples were suspended in 1.5 ml of 1x phosphate buffered saline (PBS) with 20% glycerol and frozen at –80 °C until analysis.

Based on our experience in the SWOG/Gates Foundation study of 1,400 patients in seven sites of Latin America, patients were treated with a 14-day triple-standard regimen consisting of twice a day esomeprazole (instead of omeprazole), clarithromycin, and amoxicillin[16,17]. Treatment was provided free of charge to indigent patients. The response to therapy was assessed by the urea breath test (UBT) at 6-8 weeks, and those who did not respond to initial treatment received second- line treatment consisting of tetracycline, furazolidone, bismuth subsalicylate, and pantoprazole. All were evaluated at one year. *H. pylori* isolates from patients who did not respond at 6-8 weeks were tested for susceptibility to amoxicillin, clarithromycin, levofloxacin, metronidazole, rifampicin, and tetracycline using E-test strips from BioMerieux, France, and following the manufacturers' recommendations. Results were interpreted as per the European Committee on Antimicrobial Susceptibility Testing[18].

Drinking water and biofilms

The drinking water of gastric biopsy positive patients for *H. pylori* infection was sampled utilizing autoclaved bottles with sodium thiosulfate and sterile sponge swabs. Biofilm samples were collected from the inside of household faucets with swabs. This approach to obtain biofilms has been successful in our laboratory. Two two-liter aliquots of drinking water were sampled from household faucets after the water ran for at least one minute to assure the water collected

was more representative of drinking water from the distribution system rather than water that had been sitting in the pipes. Water quality parameters including pH, temperature, dissolved oxygen, turbidity, conductivity, and free available chlorine were monitored using a water meter. Samples were handled as per the US Geological Society guidelines[19]. The two-liter aliquots of collected water were concentrated onto 0.22 µm membranes using vacuum filtration. One set of membranes and the biofilm samples were stored at –80 °C until processed. The other set of membranes were plated immediately on selective media for *H. pylori* culture in Lima using the technique of Degnan, et al.[20].

Briefly, special peptone, beef extract, yeast extract, sodium chloride (NaCl), phenol red (100 mg), and agar were dissolved in sterile water and autoclaved for 20 minutes at 121 °C. After tempering the mixture to 50 °C, calf serum with iron (7%), antibiotics (7.5 mg/l amphotericin B, 10 mg vancomycin, 5 mg trimethoprim, 5 mg cefsulodin, 3,500 U/L polymyxin B), and 600 mg/L of urea were added, followed by a drop-wise addition of 0.8 ml of 1 N hydrochloric acid. Water samples were concentrated onto 0.22 µ membranes and placed aseptically onto the plates. Plates were incubated in anaerobic jars with Campylobacter GasPaks™ for seven days at 37 °C. All samples requiring molecular analysis for *H. pylori* were blinded and shipped to the University of Michigan for processing and analysis by PCR.

Biofilm and water sample processing

Membranes with concentrated water samples were scraped in 1 x PBS buffer with 0.2% Tween® 20. Biofilm samples were wrung out in three sequential 10 ml aliquots of the same buffer. Tween® 20 was incorporated into the 1 x PBS solution to help remove cells and particulate

matter from the membranes and biofilm sponges. Suspensions were centrifuged, pelleted, and transferred to 1.5 ml Eppendorf tubes, where they were washed with 800 µl TE buffer. Samples were re-pelleted, the TE buffer removed, and samples were processed using the MoBio UltraClean™ Soil Kit (MO BIO Lab- oratories, Carlsbad, CA, USA), using the maximum yield alternative protocol.

Biopsy sample processing

Biopsy samples were homogenized using Omni Tip™ probes (OMNI International, Kennesaw, GA, USA). Following homogenization, biopsy samples were plated on Columbia Blood Agar (Oxoid, Altrincham, Cheshire, England) containing 10% defibrinated horse blood (Remel, Columbus, Ohio, USA), Dent supplement (Oxoid, Altrincham, Cheshire, England), and 3,500 U/l polymyxin B. Plates were incubated at 37 °C for 3-7 days in microaerophilic conditions. Presumptive colonies were streaked onto 5% sheep blood Tryptic Soy Agar plates (Remel, Columbus, Ohio, USA) and confirmed as *H. pylori* with a rapid urease test and PCR. DNA extraction was performed using the Mastergram™ Gram Positive DNA Purification Kit (Epicentre, Charlotte, NC, USA).

Histologic interpretation of gastric biopsies

Biopsies were interpreted according to the histologic grading of gastritis by the Sidney System[21,22]. All patients had hematoxylin and eosin (H&E) stain as we do not routinely perform other stains such as a modified Giemsa or Warthin-Starry stain. Thus, the different levels of gastritis, whether acute, chronic, superficial, or deep, level of polymorphonuclear cells infiltrate,

H. pylori density, presence of lymphocyte follicles, intestinal metaplasia, dysplasia, or gastric atrophy were described.

Urea breath test

We used the kit of Kimberly-Clark PY Test 14C- Urea Breath Test. Results were reported as disintegrations per minute (DPM). Analysis for accuracy used the 10- minute breath sample. A breath sample DPM < 50 was defined as a negative result; DPM ≥ 200 was defined as a positive result; DPM in the range of 50-199 was classified as in- determinate.

Quantitative polymerase chain reaction with HpF/HpR

H. pylori in water, biofilm, and biopsy samples were quantified using a reaction mixture containing 10 µl 2 × SYBR® Green PCR Master Mix (Applied Biosystems, Grand Island, NY, USA), 0.4 µl of 20 µm primers (HpF: [gcgacctgctggaacattac] and HpR: [cgttagctgcattactggaga]), 0.5-1 µl DNA template, and sterile H2O to bring the reaction volume to 20 µl. The standard curve comprised 101 to 106 cells *H. pylori*/µl. The quantitative PCR (qPCR) cycle included initial denaturation of target DNA at 95 °C for two minutes, followed by 45 cycles of 94 °C for one minute, 60 °C for one minute, and 72 °C for one minute to render a 138-bp product[23]. Efforts were put in place to assure the purity of the qPCR assay with melting curve analyses and the use of positive (*H. pylori*-positive samples) and negative controls (wells without *H. pylori* DNA added). All *H. pylori*-positive samples showed the same melting point as unknown positive samples. All negative controls were negative. Several *H. pylori* samples were sequenced and they always corresponded to *H. pylori*. No other sensitivity or specificity assays were performed.

Polymerase chain reaction with CagA and VacA

Extracted DNA from biopsy samples was tested for the presence of *H. pylori* CagA and VacA genes by PCR using previously described primers and the TaKaRa PCR kit (Clontech, Mountain View, CA, USA). For CagA, previously described conditions and primers F1 (5 '-GATAACAGGCAAGCTTTTGAGG 3) and B1 (5 CTGCAAAAGATTGTTTGGCAGA 3) were used to amplify a 349-base pair product[24]. Previously described primers VAG-F (5'-CAATCTGTCCAATCAAGCGAG) and VAG-R (5-GCGTCAAAATAATTCCAAGG) were used under the following conditions to amplify the m1/m2 subunits of the VacA genes: initial denaturation at 95°C for two minutes followed by 35 cycles of 95 °C for one minute, 52 °C for one minute, 72°C for one minute, completed with a final extension at 72 °C for five minutes to amplify a 570 or 645 base pair product[25]. The PCR products were visualized on a 1.5% agarose gel.

Statistical considerations

Univariate linear regression models were used to evaluate linear associations between levels of *H. pylori* detected in a patient's biopsy specimen and drinking water samples taken from the patient's home, as measured by qPCR. Kruskal-Wallis tests were used to compare median levels of *H. pylori* detected in all three specimen types be- tween high-risk and low-risk patients. Chi-square and Fisher's exact tests were used to assess relationships between baseline biopsy results and patient characteristics. Data were further summarized using descriptive statistics and graphics. All analyses were conducted using R version 3.12 and SAS version 3.

This trial is registered with ClinicalTrials.gov, registration number NCT0151287, and SWOG clinical trial S1119.

Results

A total of 192 patients registered to the trial. Seven patients were excluded for the following reasons: three patients were breast-feeding at time of enrollment, two patients withdrew consent, and two patients were registered in error. Among the remaining 185 patients, 109 had *H. pylori*-positive biopsies and were eligible and analyzable for treatment and water specimen studies.

Seventy-six patients had negative biopsies and served as controls. Among the 109 patients with positive biopsies, 35% resided in low-risk districts and tested positive for *H. pylori*, compared to 69.5% of those residing in high-risk districts ($p = <0.0001$) (Tables 4.1 and 4.2). Patient characteristics and district of residence are shown in tables 4.2 and 4.3, respectively. Most patients were female, had a normal or overweight body mass index (BMI) class, and were over the age year is shown in table 4.4. Among the patients who had a definitive UBT result at the 6-8 week follow-up visit, 61 (66.3%) tested negative for *H. pylori*. Among the 31 patients who tested positive at 6-8 weeks, 23 (74.1%) responded to second-line therapy. Of the 31 patients positive at 6-8 weeks after therapy, 18 tested negative at the one-year follow-up visit, 17 of them having completed second-line treatment. Among those with definitive results, including the administration of second-line therapy, the eradication rate at one year was 85%. Of the 12 patients who tested positive at one year, 11 consented to undergo esophagogastroduodenoscopy and gastric biopsy; three of these patients' biopsies yielded positive results. Five UBT-positive patients at one year were negative at 6-8 weeks, suggesting possible reinfection.

Thirteen of the 31 isolates from patients who did not respond to treatment at 6-8 weeks had studies of in vitro antibiotic sensitivity and two thirds demonstrated resistance to amoxicillin (69.2%), levofloxacin (69.2%), and metronidazole (61.5%). Resistance was less common to clarithromycin (15.4%), tetracycline (7.7%), and rifampicin (38.5%). The minimum inhibitory concentrations (MIC50, MIC90 and ranges) of the resistant strains were as follows in table 4.5. Gastric biopsy findings in biopsy positive patients were as follows: 99 (96%) had superficial chronic gastritis, 54 (53%) had deep or profound chronic gastritis, 93 (90%) had mucinous changes of the gastric mucosa, 91 (84%) had polymorphonuclear leukocytosis, 19 (18%) had intestinal metaplasia, and seven (6%) had gastric atrophy. The density of *H. pylori* present was described as large in 35%, moderate in 45%, and few in 19%.

A summary of specimen data by source of origin is shown in table 4.6. The qPCR results were available for 109 biopsy specimens, 87 filtered water, and 50 biofilm samples. Additionally, residual chlorine content data was available for 83 filtered water samples.

Sixty-one of 76 negative gastric biopsy samples were positive by qPCR (80.3%), with median 123.4 (range, 1.9-538,322) CN/mg tissue. Of 109 positive gastric biopsy samples, 106 were positive by qPCR (97.2%), with median 46,914.3 (range, 5.1- 1,798,528.1) CN/mg tissue. Forty-two of 87 filtered water specimens were positive by qPCR (48.3%), with median 931.5 (range 8.5-1,682,500) CN/mg. Eighteen of 50 biofilm samples were positive by qPCR (36%), with median 1,654 (range, 24-68,600) CN/mg. Thus, the highest concentrations of qPCR *H. pylori* were measured in gastric biopsies and in the filtered water specimens. Residual chlorine was

measured in 80 of 83 filtered water samples (96.3%), with median 0.7 (range, 0.1-1.25) mg/L (Fig. 4.1).

The physical properties of the filtered drinking water specimens were as follows: median pH 7.0 (range, 6.5-8.5); median conductivity 53.2 μmhos (microohms per centimeter; range, 40-939); median Celsius temperature 22.6 (range, 18.8-27.4) and median turbidity 0.1 NTU (nephelometric turbidity unit; range, 0.0-27.4). There was no evidence of an association between the levels of *H. pylori* detected by qPCR in a patient's gastric biopsy and the patient's drinking water, for both filtered water and biofilm specimen.

Furthermore, there was no evidence that the level of *H. pylori* detected in the gastric biopsies of biopsy positive patients differed by gastric cancer risk of patient's district. Finally, no significant correlations were found between the level of *H. pylori* detected by qPCR in baseline gastric biopsy and response to treatment at 6-8 weeks and at one year. Out of the 109 eligible patients with *H. pylori*-positive gastric biopsies, 71 tested positive for CagA (65%), 78 tested positive for VacA (72%), and 15 (14%) tested positive for both. There was no evidence of an association between these results and clinical response at 6-8 weeks or at one year.

Attempts to culture *H. pylori* from patient's drinking water samples and from four samples taken from the main water plant in Lima (La Atarjea) were unsuccessful. However, all four La Atarjea samples, including two samples from the river intake (Rímac River) and two from two different reservoirs of treated water ready for public consumption, tested positive by qPCR: 1378.34, 2520.00, 3275.00, and 3388.00 CN/L, respectively.

Information regarding primary water source for various domestic uses was obtained by patient survey for 74 *H. pylori* biopsy negative and 109 *H. pylori* biopsy positive patients. Most patients (95%) reported having interior plumbing and consuming water derived from the public system. For all intended uses surveyed, there were no differences between *H. pylori*-negative and -positive patients with regards to primary water source.

Patient-reported symptoms as measured by the Rome III questionnaire were similar for biopsy positive and biopsy negative patients. The percent of patients reporting symptoms of primary interest were: chronic dyspepsia (73%), chronic heartburn (35.1%), chronic postprandial distress syndrome (22.2%), and chronic irritable bowel syndrome (18.4%).

Discussion

In a part of the world where gastric carcinoma is the most common form of cancer and cause of cancer death[4], the finding of *H. pylori*-specific DNA in the drinking water is significant. Fifty percent of water samples from homes of *H. pylori*-infected patients in this study showed evidence of *H. pylori*-specific DNA. Due to difficulties culturing *H. pylori* from water, we were unable to obtain any positive *H. pylori* isolates from water in Lima. The reasons for our inability to culture *H. pylori* from drinking water are multiple, including technical difficulties and the possibility that *H. pylori* might be present in its coccoid form that is harder to culture[26].

H. pylori rapidly changes morphology from a spiral bacillus to a coccoid form in water, entering a viable but non-culturable state that makes it challenging to culture[26,27]. Historically, this conversion has raised doubts about whether *H. pylori* is viable and infectious in water. However, several independent studies have isolated and cultured *H. pylori* in wastewater and drinking water[28–31]and *H. pylori* has also been reliably detected in recreational and drinking water using molecular biology techniques[29,32]. Finally, the viable but non-culturable form of *H. pylori* has been shown to be infectious in mice via gavage[33] and we previously showed that waterborne *H. pylori* is infectious in mice as well[34]. In addition to being culturable from water, there is evidence that *H. pylori* can survive or propagate in water in biofilms, extracellular structures that protect bacteria from chlorine, antibiotics, and other features of inhospitable environments, suggesting a mechanism by which *H. pylori* could shed into and contaminate water[26,35].

Our hypothesis that the drinking water of Lima is contaminated by *H. pylori* is corroborated by the large quantities of *H. pylori* we detected in water samples. Some of these quantities are higher than doses required for experimental human infection (ranging from 10^4 to 10^{10} CFU/dose)[36] and are similar to findings in recreational waters[37,38]. A recent risk assessment suggested that the maximum contaminant level goal for *H. pylori* be set at < 1 organism/l based on quantities of *H. pylori* in recreational water, a finding that our current study supports[39ry].

Our observations could be challenged because we were unable to culture *H. pylori* from drinking water in Lima. It could be argued that the presence of *H. pylori*-specific DNA in water does not prove the viability of *H. pylori*. However, the identification of *H. pylori*-specific DNA in the treated water of the water plant and in the homes of patients who are infected with *H. pylori*,

coupled with the observed reinfection after therapy and the frequency of gastric cancer in Lima, reinforce the validity of our findings. We may have detected the non-culturable but viable coccoid form of *H. pylori* in water. As discussed earlier, the infectious viability of this form of *H. pylori* has been demonstrated in mice[33]. The higher detection of *H. pylori*-specific DNA in drinking water of infected patients relative to values found in the main water plant may also reflect an additional contamination factor associated with the water irrigation system in Lima. However, our assertion is limited by our inability to culture *H. pylori* in water and the lack of corresponding fingerprinting for comparison between the *H. pylori* strains present in the water of the water plant, the *H. pylori* present in the water in patient's homes, and the *H. pylori* present in their gastric mucosa.

Gastric biopsies were interpreted according to the histologic grading of gastritis by the Sidney System[21,22] and by H&E stain. Most patients had dif- ferent forms of either superficial (96%) or deep forms of chronic gastritis (53%) with mucinous changes of the gastric mucosa (90%). Intestinal metaplasia (18%) and gastric atrophy (6%) were less common. Even though some would argue that we should have used histochemical stains, such as a modified Giemsa stain or the Warthin-Starry stain, to enhance the detection of *H. pylori* in gastric biopsies, the review by Yantiss, et al. concludes that *H. pylori* is usually detectable in H&E- stained sections and that most ancillary stains show comparably high sensitivities (> 90%) for its detection[40].

In contrast to our previous report on the efficacy of triple therapy for *H. pylori* infection in Latin America, where a response of over 80% was observed, our response of less than 70% in this pilot study in Lima, Peru, raises concern[16,17]. Patients who did not respond to initial therapy responded

favorably to second-line therapy (71.8%). We identified antibiotic resistance to amoxicillin, levofloxacin, and metronidazole in two-thirds of *H. pylori* strains of patients who did not respond at 6-8 weeks from treatment. We also identified five patients who became positive at one year after being negative at 6-8 weeks, suggesting the possibility of *H. pylori* reinfection. Findings of resistance and reinfection would support the failure rate of 15% (12/80 patients) at one year that, when combined with the 12 patients who had inconclusive UBT results, would make the failure rate 26% (24/92 patients). Ramirez-Ramos, et al. had previously reported a failure rate of 73% at eight months after successful treatment for *H. pylori* infection in Lima[15]. Soto, et al. reported a 30% recurrence rate at 18 months after successful treatment of *H. pylori* infection in Lima as well[8]. Soto further reported that, utilizing randomly amplified polymorphic DNA patterns and DNA sequence methodology, most of the episodes of recurrence observed represented reinfection.

By PCR, we identified the presence of *H. pylori* in patient's baseline gastric biopsy at a significantly higher rate in high-risk versus low-risk districts ($p < 0.0001$), likely the result of the presence and virulence of the *H. pylori* strain. The evidence of *H. pylori* in symptomatic patients with negative gastric biopsy for *H. pylori* infection raises the possibility of sub-clinical *H. pylori* infection, previously suspected in Spain[41]. We assured there was no evidence of contamination and ran the appropriate controls in these patients. As a result, one wonders if the use of enhanced narrow band imaging technology can uncover lesions induced by *H. pylori* in symptomatic patients who have negative biopsies. This technology has proven beneficial and superior to white-light imaging in recognizing the microvascular and mucosal surface pattern of patients

with depressed-type early gastric carcinoma lesions in Japan. This common form of gastric cancers in turn would be subject to limited endoscopic curative resections[42].

Neither the presence of pathogenicity markers such as CagA and VacA, nor the physical characteristics of the drinking water and the observations derived from the Rome III questionnaire demonstrated significance in this study.

This study suggests that the drinking water of Metropolitan Lima is contaminated with *H. pylori*. The clinical and epidemiologic implications of this finding are significant not only for Lima, but for other cities of Peru and areas where *H. pylori* and gastric cancer are frequent such as Latin America, Asia, and Eastern Europe. Improvements in the technology of drinking water preparation and its distribution system could result in an effective primary prevention strategy of *H. pylori* infection and gastric carcinoma that will be more effective than massive antibiotic therapy of infected patients.

In Japan, for example, improvements in sanitary conditions and eradication of *H. pylori* infection have reduced the incidence of gastric cancer by one-third[43]. However, these measures and massive screening have their limitations to the point of some advocating moving from secondary prevention to primary prevention of *H. pylori* infection and gastric cancer[44]. The findings of this study support that strategy.

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Table 6.1. Participants

Screened	192
Eligible	185
H. pylori negative	76
H. pylori positive	109

Distribution of patients by risk district		
Risk	***H. pylori* negative**	***H. pylori* positive**
Low	37	20 (35%)
High	39	89 (66.5%)
		P < 0. 0001

Table 0.1. Study participants and distribution of patients by risk district.

Table 6.2. Characteristics of eligible patients

	Overall (n = 185)	Negative (n = 76)	Positive (n = 109)	Fisher's exact p value
District Risk				< 0.0001
Low	57	37	20	
High	128	39	89	
Age			0.31	
20-29	26	13	13	
30-39	24	13	11	
40-49	49	19	30	
50+	86	31	55	
Sex				0.52
Female	129	51	78	
Male	56	25	31	
Body mass index				0.06
Missing	1	1	0	
Underweight	2	0	2	
Normal	93	38	55	
Overweight	71	25	46	
Obese	18	12	6	
Household children				0.15
2 or fewer	170	68		102
3-4	13	8	5	
5 or more	2	0	2	

Table 0.2. Characteristics of eligible patients.

Table 6.3. *H. pylori*-positive findings by patient's district risk

	Negative	Positive	Total
High-risk			
Breña	1	0	1
Comas	2	13	15
El Agustino	0	1	1
Los Olivos	7	21	28
Puente Piedra	3	6	9
Rimac	3	8	11
San Juan de Lurigancho	4	3	7
San Martin de Porres	18	35	53
Villa Maria del Triunfo	1	2	3
Low-risk			
La Molina	4	5	9
Miraflores	10	4	14
San Borja	5	3	8
San Isidro	2	1	3
Surco	6	7	23
TOTAL	76	109	185

Table 0.3. H. pylori-positive findings by patient's district risk

Table 6.4. Treatment results by urea breath test at 6-8 weeks and at one year

Time	Not done	Inconclusive	Negative	Positive
6-8 Weeks	14	3	61 (66.3%)	31 (33.6%)
One year	17	12	68 (85%)*	12 (15%)

Table 0.4. Includes patients receiving second-line therapy

Table 6.5. Antibiotic resistance information on 13 patients who did not respond to initial antibiotic treatment.

MIC	Amoxicillin	Clarithromycin	Levofloxacin	Metronidazole	Rifampin	Tetracycline
MIC50	0.19	0.032	32	48	0.75	0.064
MIC90	256	256	32	256	32	2.45*
MIC range	0-256	0-256	0.25-32.0	0.25-256.0	0.032-32.0	0-4

Table 0.5. One value was >0.125 MIC: minimum inhibitory concentration.

Table 6.6 *H. pylori* biopsy positive patients: Specimen data summary

	All eligible	Biopsy qPCR	Filtered water qPCR	Residual CL filtered water	Water biofilm qPCR
High-risk					
Breña	0	0	0	0	0
Comas	13	13	11	11	7
El Agustino	1	1	0	0	0
Los Olivos	21	21	17	17	8
Puente Piedra	6	6	6	6	3
Rimac	8	8	6	6	5
San Juan de Lurigancho	3	3	3	3	1
San Martin de Porres	35	35	29	25	17
Villa Maria del Triunfo	2	2	2	2	1
Low-risk					
La Molina	5	5	4	3	4
Miraflores	4	4	2	1	0
San Borja	3	3	1	1	0
San Isidro	1	1	1	0	0
Surco	7	7	5	1	4
TOTAL	109	109	87	76	50

Table 0.6. CL: chlorine, qPCR: quantitative polymerase chain reaction

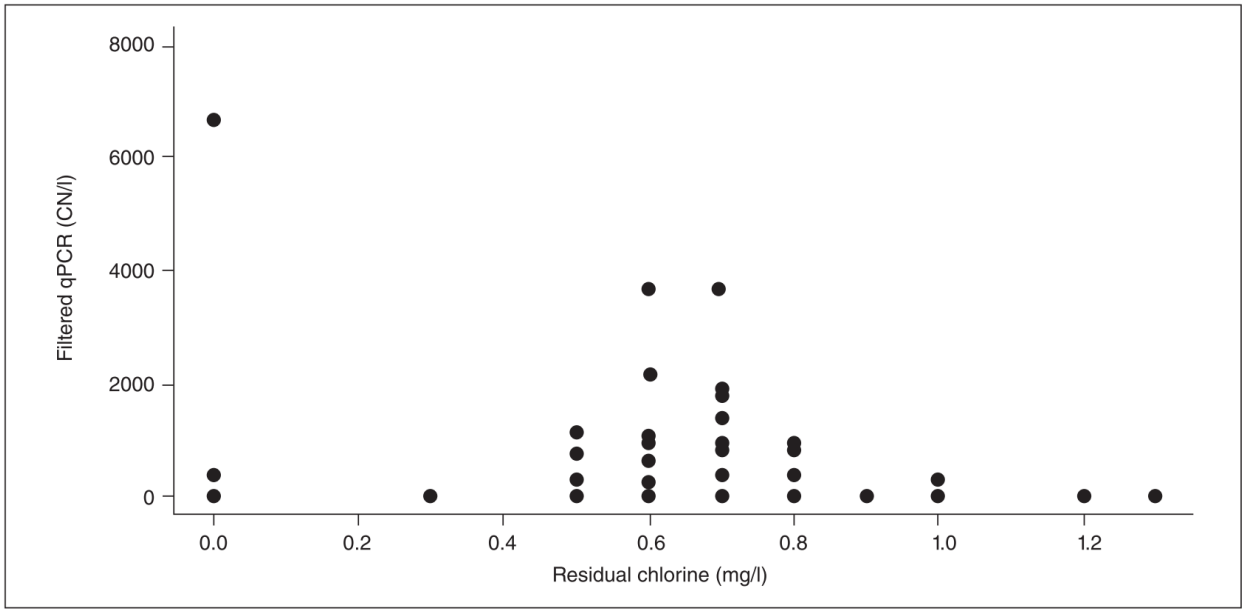

Figure 0.1. Relationship between quantitative polymerase chain reaction in filtered drinking water and residual chlorine. No significant correlations were seen.