Helicobacter

Helicobacter ISSN 1523-5378
doi: 10.1111/hel.12216

Animal Model Reveals Potential Waterborne Transmission of *Helicobacter pylori* Infection

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Keywords
Helicobacter pylori, drinking water, transmission, mouse model.

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Abstract

Background: *Helicobacter pylori* infection has been consistently associated with lack of access to clean water and proper sanitation, but no studies have demonstrated that the transmission of *H. pylori* can occur from drinking contaminated water. In this study, we used a laboratory mouse model to test whether waterborne *H. pylori* could cause gastric infection.

Materials and Methods: Groups of immunocompetent C57/BL6 *Helicobacter*-free mice were exposed to static concentrations (1.29×10^5, 10^6, 10^7, 10^8, and 10^9 CFU/L) of *H. pylori* in their drinking water for 4 weeks. One group of *Helicobacter*-free mice was exposed to uncontaminated water as a negative control. *H. pylori* morphology changes in water were examined using microscopy Live/Dead staining. Following exposure, *H. pylori* infection and inflammation status in the stomach were evaluated using quantitative culture, PCR, the rapid urease test, and histology.

Results: None of the mice in the negative control or 10^5 groups were infected. One of 20 cages (one of 40 mice) of the 10^6 group, three of 19 cages (four of 38 mice) of the 10^7 CFU/L group, 19 of 20 cages (33 of 40 mice) of the 10^8 group, and 20 of 20 cages (39 of 40 mice) of the 10^9 CFU/L group were infected. Infected mice had significantly higher gastric inflammation than uninfected mice (27.86% higher inflammation, $p < .0001$).

Conclusions: We offer proof that *H. pylori* in water is infectious in mice, suggesting that humans drinking contaminated water may be at risk of contracting *H. pylori* infection. Much work needs to be performed to better understand the risk of infection from drinking *H. pylori*-contaminated water.

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]. As the landmark study by Klein et al. in 1991 [3], 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 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. [17] called for animal models to study the transmission of *H. pylori* in water.

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 to 10^9 CFU of *H. pylori*. She et al. [15] inoculated 16 BALB/C mice over 12 days with four doses of ~4×10^8 CFU coccoid *H. pylori* by oral gavage. 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 to 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 (SS1) in drinking water can infect mice. We carried out two experiments: a 1-week exposure of severe combined immunodeficient (SCID) C57/BL6 mice (001913 from Jackson Labs, Bar Harbor, Maine, USA) to 10^9 CFU/L of *H. pylori* and a 2-week exposure of immunocompetent 16 C57/BL6 mice (000664 Jackson Labs, Bar Harbor, Maine, USA) to varying static concentrations of *H. pylori* (10^5, 10^7, and 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, five of five 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 authors Kevin Boehnke (K. Boehnke), Kathryn A. Eaton (K.A. Eaton), Chuanwu Xi (C. Xi)). Thus, for this 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 4 weeks as an exposure length because 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

Helicobacter pylori is not a normal mouse inhabitant, and therefore, most strains of *H. pylori* colonize mice poorly. Sydney Strain 1 was selected for this study because 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, Franklin Lakes, New Jersey, USA). After 3 days of growth, colonies were collected and used to inoculate Brucella broth (Remel, Columbus, Ohio, USA) supplemented with 10% heat-inactivated fetal bovine serum (Fisher Scientific, Waltham, Massachussetts, USA). After shaking overnight at 40 rpm in microaerobic conditions at 37 °C, the broth was centrifuged for 20 minutes at 2200 g, 4 °C to gently pellet the bacteria. The supernatant was removed, and the pellet was suspended in 1 mL of 1× PBS. To estimate the total number of bacteria, 10 μL of the *H. pylori* suspension was added to 890 μL of 1× 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 were counted at 40× 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. 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 1, 2, 4 hours, 1, 2, and 3 days after inoculation. At these same time points, *H. pylori* cells were checked for viability and morphology using microscopy at 40× magnification and LIVE/DEAD BacLight Bacterial Viability Kit (Life Technologies, Eugene, Oregon, USA). Briefly, 6 mL of water was centrifuged at 9,300 g 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. As 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 (1.29×10^5, 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 as 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 μm 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 and homogenized in 1× PBS, and serial dilutions of the homogenate were plated on *H. pylori* selective media (Columbia blood agar base with 10% horse blood, Dent supplement, 300 mg/L urea, and 3500 U polymyxin B/L) [28]. Presumptive *H. pylori* isolates were counted and then checked for urease activity using a urease indicator broth (0.33 mol/L urea, 0.2% phenol red, 0.02% NaN_3, 0.01 mol/L pH 6.5 $NaPO_4$ buffer). DNA was then extracted from presumptive colonies using the QiaAMP DNA Mini Kit (Qiagen, Valencia, California, USA). 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 minutes, followed by 35 cycles of 94 °C for 1 minute, 56 °C for 1 minute, 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* were 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

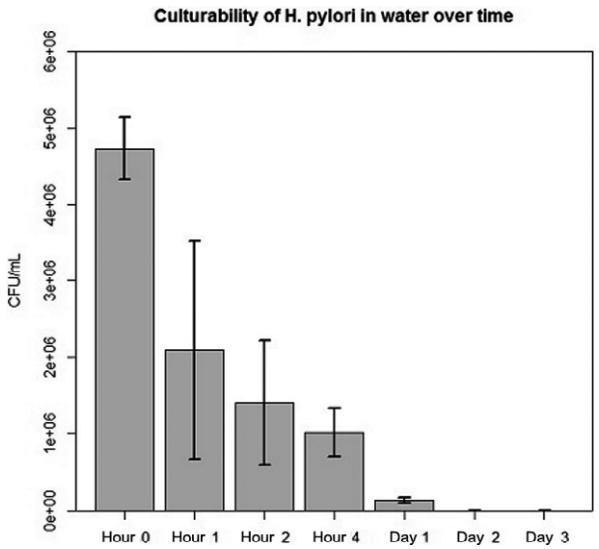

Figure 1 Consistent with other results in the literature, culturability of *Helicobacter pylori* in water decreased steadily over time when kept at room temperature, with complete loss of culturability after 2 days.

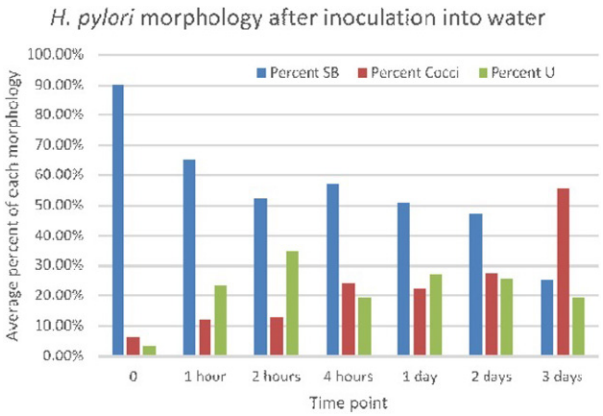

Figure 2 *Helicobacter pylori* morphology in water: results from BacLight Live/Dead staining and microscopy. SB, Spiral bacillus morphology; Cocci, O-shaped/coccoid morphology; U, U-shape morphology. At time 0, about 90% of the *H. pylori* was spiral bacillus morphology. Over time, the percentage of spiral bacilli decreased and the percent of cocci and U-shaped morphologies increased.

concentration) and gastric inflammation score (range 0–300%) were explored using mixed effects linear models taking into account cage effects. As only infected mice had quantitative culture results >0, models were also run on infected mice only. All analyses were run in SAS version 9.4 (SAS Institute, Cary, North Carolina, USA).

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 $\alpha = 0.05$ 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. The results of the morphology experiment are summarized in Fig. 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 1, Fig. 1, and Fig. 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, and 20 of 20 of the 10^9 group cages had infected mice (100%), and 1 of 1 cages of the SCID mice (positive control) was infected with *H. pylori*. The quantities and range of *H. pylori* recovered from infected stomachs are shown in Table 2, and the evidence of *H. pylori* colonization is also shown via histology imaging in Fig. 4. In the logistic regression model, the log odds of infection increased by 3.57 per 10-fold increase of waterborne *H. pylori* concentration ($p > .0001$) (Fig. 3). Deviations from linearity were not significant ($p = .1021$).

Inflammation Scoring

The range of inflammation scores of infected mouse stomachs are found in Table 2. Crude associations in the complete data set showed significant increased relationships between inflammation and infection (27.86%, $p < .0001$) and 10-fold increase in waterborne concen-

Table 1 Overview of experimental results by infection status. All infected mice were confirmed by the rapid urease test, morphology, and PCR

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

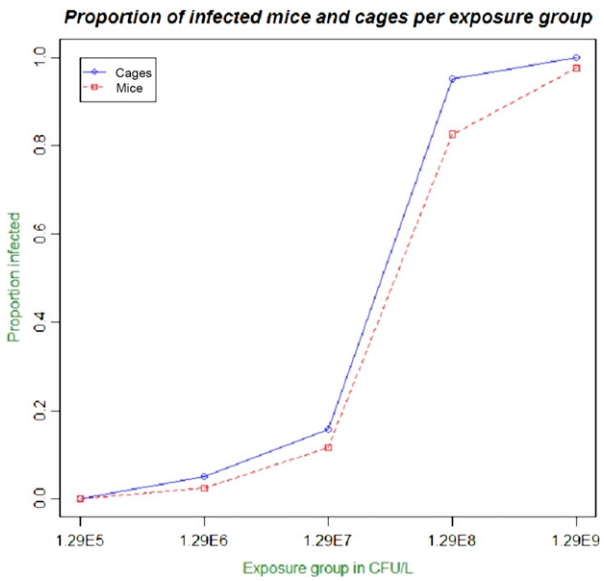

Figure 3 Percentage of infected mice and cages per exposure group. The infectious dose for this exposure paradigm appears to fall around 1.29E6 CFU/L in water.

tration of *H. pylori* (8.32, $p = .0003$) (Table 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. As 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 ($p = .9572$), suggesting that infection status was driving the previous association between waterborne concentration and inflammation. Interestingly, among infected mice, there was an associated decrease of −1.41% in inflammation score ($p = .0055$) per 10^6 CFU increase in quantitative culture results (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 and 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 for 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.

Table 2 Quantitative culture and histologic inflammation results from infected mice. 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%

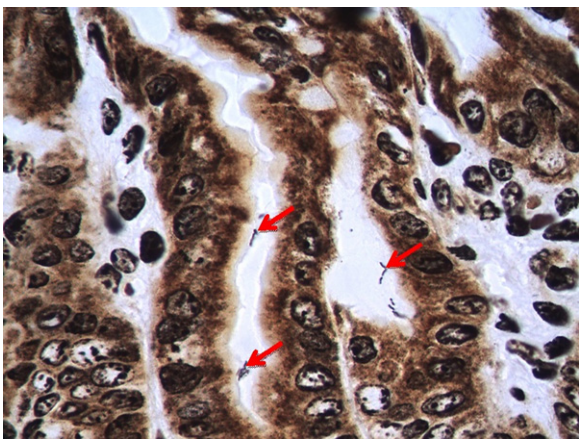

Figure 4 Silver stained tissue section of mouse stomach from an infected mouse. Arrows indicate *Helicobacter pylori* in the gastric mucosa..

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, suggesting that 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 4 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. [25] 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 because the study participants at all doses got infected. Mice typically drink around 7 mL of water per day [31], 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 con-

Table 3 Results from linear mixed models examining the associations between infection status, sex, waterborne concentration of *H. pylori*, and quantitative culture results on gastric inflammation taking into account cage effects. The first columns shows results from crude associations. The second column shows results from the full model run on infected mice, and the third column shows results from the full model run on uninfected mice. Associations were performed on all mice, then separately on infected and uninfected mice. Associations between gastric inflammation and predictors

Predictor	All mice β (SE)	*p*-Value	Infected mice β (SE), taking into account sex, concentration of H. pylori, and CFU/gram tissue.	*p*-Value	Uninfected mice β (SE)	*p*-Value
Infection status	27.86% (6.21%)	<.0001	N/A	N/A	N/A	N/A
Sex	2.67% (6.71%)	.6912	11.26% (11.41%)	.3306	−3.73% (7.06%)	.5992
Waterborne concentration of *H. pylori*	8.32% (2.20%)	.0003	−0.55% (10.21%)	.9572	4.71% (3.62%)	.1985
CFU/gram tissue (divided by 10^6)	0.18% (0.33%)	.5864	−1.41% (0.47%)	.0055	N/A	N/A

centrations 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 because 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 one organism per liter [32].

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 accurately reflect the environmental concentrations. Also, since we used sterilized, filtered tap water as the matrix of exposure, 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 proper water treatment [33]. Precipitation frequency and seasonal differences likely affect how much *H. pylori* is in the water through sources of contamination including sewage overflows and runoff from farms.

Future Directions

Continued mouse experiments could be performed 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 performed 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. [25] could be adapted for waterborne exposure. 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 QMRAs for *H. pylori* in drinking water. While much research remains to be carried out, we have demonstrated that mice can be infected by drinking water contaminated with *H. pylori*.

Acknowledgements and Disclosures

The study was supported partially by an Integrated Assessment grant to C.X. and M. V. from the Graham Sustainability Institute at the University of Michigan and also by a generous donation from Dr. Laurence Baker, D.O. (Collegiate Professor of Cancer Developmental Therapeutics; Professor, Departments of Internal Medicine and Pharmacology; University of Michigan School of Medicine). We thank Clinton Fontaine (Research Laboratory Manager at University of Michigan Medical School) for his assistance with this project, Dr. Brisa Sanchez, PhD, (Associate Professor of Biostatistics at the University of Michigan) for statistical advice, and Dr. Juanita Merchant MD, PhD (Professor in the Departments of Internal Medicine and Integrative and Molecular Physiology at the University of Michigan) for the use of her laboratory space.
Competing interests: the authors have no competing interests.

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