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An assessment of drinking water contamination with *Helicobacter pylori* in Lima, Peru

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Abstract

Background: *Helicobacter pylori* is a gut bacterium that is the primary cause of gastric cancer. *H. pylori* infection has been consistently associated with lack of access to sanitation and clean drinking water. In this study, we conducted time-series sampling of drinking water in Lima, Peru, to examine trends of *H. pylori* contamination and other water characteristics.

Materials and methods: Drinking water samples were collected from a single faucet in Lima's Lince district 5 days per week from June 2015 to May 2016, and pH, temperature, free available chlorine, and conductivity were measured. Quantities of *H. pylori* in all water samples were measured using quantitative polymerase chain reaction. Relationships between the presence/absence and quantity of *H. pylori* and water characteristics in the 2015-2016 period were examined using regression methods accounting for the time-series design.

Results: Forty-nine of 241 (20.3%) of drinking water samples were contaminated with *H. pylori*. Statistical analyses identified no associations between sampling date and the likelihood of contamination with *H. pylori*. Statistically significant relationships were found between lower temperatures and a lower likelihood of the presence of *H. pylori* ($P < .05$), as well as between higher pH and higher quantities of *H. pylori* ($P < .05$).

Conclusions: This study has provided evidence of the presence of *H. pylori* DNA in the drinking water of a single drinking water faucet in the Lince district of Lima. However, no seasonal trends were observed. Further studies are needed to determine the presence of *H. pylori* in other drinking water sources in other districts in Lima, as well as to determine the viability of *H. pylori* in these water sources. Such studies would potentially allow for better understanding and estimates of the risk of infection due to exposure to *H. pylori* in drinking water.

KEYWORDS
Helicobacter pylori, Lima, Peru, time series, water

1 | 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.[1,3,4] *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.[5–7] Although the global prevalence of *H. pylori* infection is decreasing, it is more prevalent in low-income nations as compared with high-income nations, with estimates in adults as low as 11% in Belgium and up to 93.6% in Nigeria.[8] Lima, Peru, where the first association between water source and *H. pylori* infection was discovered, has an estimated prevalence of 45.5%.[9] In 1991, Klein et al found higher odds of *H. pylori* infection among study participants in Lima 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.[6,7,10–15]

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 and renders cultivation techniques an inadequate stand-alone way of detecting *H. pylori* in water.[16-18] Recently, however, five independent studies have isolated and cultured *H. pylori* in wastewater and drinking water.[19-23] 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,[5,16,17,24–26] and has been shown to survive in water distribution systems, likely through protection from biofilms.[27,28] Further, *H. pylori* may be able to better survive in the presence of certain freshwater amebae[29] and marine zooplankton,[30] suggesting another route of survival in water. While the VBNC form of *H. pylori* has been shown to be infectious in mice via gavage[31,32] (but not in drinking water),[33] and viable, culturable *H. pylori* is infectious through the route of drinking water in mice.[34]

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* reported to be 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,[25] surface water,[35] and recreational water,[36] and several studies have measured the presence/absence of *H. pylori* in drinking water using PCR[5,21,35] (50%, 28.6%, and 4% positive, respectively). To our knowledge, only two studies have quantitatively measured *H. pylori* in municipal drinking water: one by our group in Lima, Peru,[26] and the other in Spain.[37] 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,[26] which was remarkably similar to Spain, where the highest quantity was reported as it was 1.59E3 genome copies/mL—equivalent to 1.59E6 genome copies/L.[37]

To follow-up our initial work in Lima, we performed two small studies. The first of these conducted in 2013 examined five water samples collected from wells used to supplement the drinking water 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 19 and July 18 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 30-July 4, when samples were collected twice per day from Monday to Friday. Three of 5 samples collected from wells and 9 of 17 drinking water samples collected in the summer of 2014 showed contamination with *H. pylori*, with well water samples containing up to 2.3E3 genome copies/L of *H. pylori* and drinking water samples containing up to 1.95E4 genome copies/L of *H. pylori* (Boehnke KF, Brewster RK, Xi C. Unpublished laboratory results. 2017).

To follow-up our studies and better characterize the quantities and variation of *H. pylori* in drinking water over time, we conducted a quantitative assessment of *H. pylori* in water in the Lince district in Lima, Peru.

2 | MATERIALS AND METHODS

2.1 | Water sample collection

A total of 241 total drinking water samples were collected once per day, 5 days per week from a single sink within a government building in the Lince district in Lima from June 2015 through the end of May 2016. Approximately 20 people utilize this sink regularly. Sterile bottles with sodium thiosulfate were prepared prior to sampling. 1 L aliquots of drinking water were collected from the faucet after allowing the water to run for at least 1 minute and then concentrated by vacuum filtration onto 0.22 μmol L^{-1} filter membranes.[26] Water quality parameters including pH, temperature, and conductivity were monitored, and free available chlorine was measured using *N*,*N* Diethyl-1,4Phenylenediamine Sulfate (DPD) among samples collected from June 2015 to May 2016. Samples were handled as per the US Geological Survey guidelines.[38] All membranes were stored at −80°C until processing and analysis at the University of Michigan.

2.2 | DNA extraction from membranes

The following phenol-chloroform protocol for DNA extraction was adapted from Holinger et al.[39] Each 0.22 μmol L^{-1} filter (EMD Millipore, Ontario, Canada) was cut into >20 pieces using sterilized scissors and placed into 2 mL tubes containing ~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 (75 mmol L^{-1} NaCl, 75 mmol L^{-1} TRIS pH 8.0, 7.5 mmol L^{-1} 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 16 000 × *g*. Following centrifugation, ~450 μL of aqueous phase was transferred to a new 1.5 mL tube. About 10 μL of glycogen (10 mg/mL), 200 μL of 7.5 mol L^{-1}-ammonium acetate, and 650 μL of isopropanol were added to precipitate the DNA. The samples were then centrifuged for 25 minutes at 16 000 × *g* to pellet the DNA. Afterward, 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 16 000 × *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 10 mmol L^{-1} TRIS with 1 mmol L^{-1} EDTA, pH 8.0. Following DNA extraction, samples were purified by washing with 1 mL 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 minutes at 16 000 × *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 10 mmol L^{-1} TRIS and 1 mmol L^{-1} EDTA, pH 8.0.

2.3 | Quantitative polymerase chain reaction (qPCR)

qPCR was performed on extracted DNA using a highly sensitive, previously established method.[40] Briefly, the number of genome copies/L of *H. pylori* in drinking water was 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 μmol L^{-1} primers HpA-F (ACTTTCTCGCTAGCTGGATGGTA) and HpA-R (GCGAGCGTGGTGGCTTT), 8.9 μL 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 was constructed with 0.5E1 to 5E5 genome copies of *H. pylori* strain SS1 DNA. Given that each *H. pylori* genome has 1 copy of *hpaA*,[41] we assumed that one genome copy of *H. pylori* was equivalent to 1 genomic unit (GU). The lowest value (0.5E1 genome copies) was used to determine the limit of detection in calculating the quantity of *H. pylori* in each sample. qPCR was run under 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 to 95°C. All drinking water samples, standard curve samples, and controls were run in triplicate.

2.4 | Statistical analysis

All statistical analyses were conducted in R Studio version 0.99.891. 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 to a 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 nonzero 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 covariates. As the data arise as a time series because sampling occurred from a single location over the course of 1 year, two approaches were used to account for the possibility of autocorrelation in the samples (ie, nonindependence 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 nonlinearity 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.

3 | RESULTS

3.1 | *H. pylori* in drinking water

Throughout the sampling period (June 2015 to May 2016), contamination of the drinking water with *H. pylori* was observed, with 20.3% (49/241) of samples being positive for *H. pylori*. Each month *H. pylori* contamination was detected on at least 1 day, with the longest stretch of time without a positive sample being 25 days (Figure 1A). In this figure, the limit of detection is reported as 400 genome copies/L, due to back calculation from the total elution volume and quantity of sample used per reaction [(5 genome copies/well) * (well/0.5 μL sample) * (40 μL of total DNA/L drinking water sample)].

3.2 | Water characteristics

There were some missing measurements in the water characteristics data due to lack of reagent or instrumentation on that sampling day (Table 1; Figure 1B-E). pH was measured in 238 of 241 samples, conductivity in 232 of 241, free available chlorine in 209 of 241, and temperature in 240 of 241. The World Health Organization (WHO) recommends that the free chlorine residual available in drinking water should be between 0.2 and 0.5 mg/L.[42] One hundred and sixty-nine of 209 samples were at or below 0.5 mg/L, 20 of 209 samples were above 0.5 mg/L, and 17 of 209 samples were below the minimum recommended residual of 0.2 mg/L of free chlorine (Table 1). The WHO recommends that the pH of drinking water should be above 6.5 and below 8.5 to avoid corrosion 5.[43] Ninety-six of 238 samples were below the minimum recommended guideline of 6.5. Temperatures ranged from 19.7 to 27.7°C, and conductivity ranged from 326 to 616 μS/cm.

3.3 | Associations between water characteristics and *H. pylori*

We found a significant negative association between temperature and the presence of *H. pylori*, regardless of the method of analysis. Accounting for date using a smoothing function and all other covariates, we found that the log odds of the presence of *H. pylori* were 37% lower ($\beta = -0.46$, SE = 0.18, $P < .05$) per degree higher temperature (Table 2, 1a). When accounting for autocorrelation with the previous date (Table 2, 1b), the log odds of the presence of *H. pylori* were 21% lower per degree higher temperature ($\beta = -0.24$, SE = 0.13,

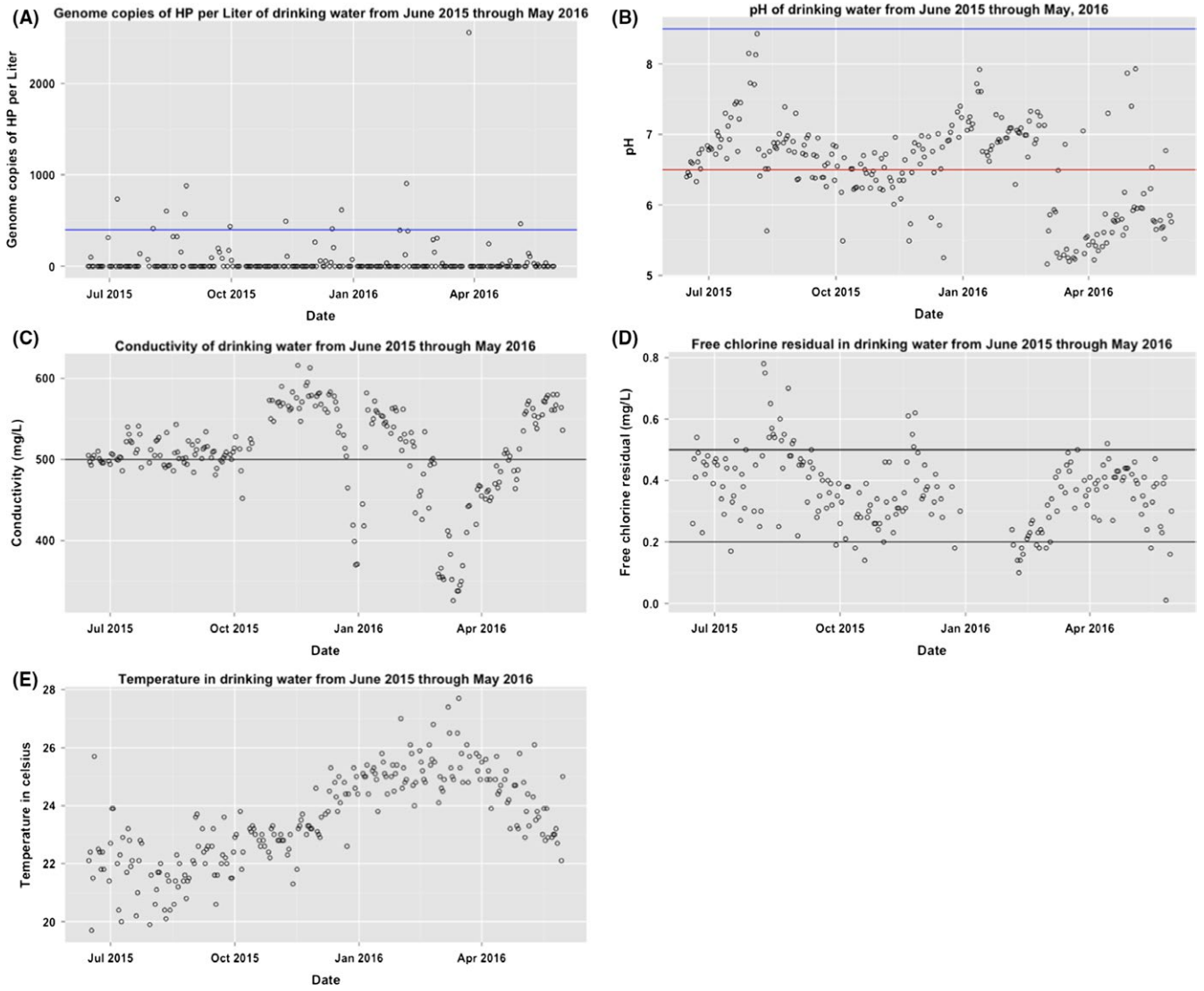

FIGURE 1 Genome copies/liter of *H. pylori* (A), pH (B), conductivity (C), free chlorine residual (D), and temperature (E) in drinking water collected from June 2015 through May 2016. A, The line represents the back-calculated limit of detection for genome copies/L of *H. pylori*. B, The top line represents the upper limit of the EPA secondary standard for pH (8.5), while the lower 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

$P < .1$) (Table 2, 1b). Temperature remained statistically significant in the smoothed model, even when the most influential point was removed ($P < .05$). Although the approach to adjusting for autocorrelation (smoothing vs adjusting for prior day) changes the direction of the association with pH, pH was not significantly related to the presence/absence of *H. pylori* in either model.

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 ($\beta = -0.0027$, SE = 0.0015, $P < .1$). After removing the most influential data point, the quantity of *H. pylori* was 95% higher for each unit higher in pH ($\beta = 0.29$, SE = 0.14, $P < .1$, Table 2, 2b).

We did not find a statistically significant association between calendar time (ie, 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 the presence of *H. pylori* in the prior day's sample.

4 | DISCUSSION

To our knowledge, this is the longest time-series sampling study of drinking water contamination with *H. pylori* in Lima, Peru. Based on our sampling, it appears that drinking water in Lima is contaminated with *H. pylori* 20.3% of the time (49 of 241 positive samples), in both the drinking water collected in Lince and the wells used to supplement the treated drinking water supply. Based on the null associations

TABLE 1 Water characteristics from June 2015 to May 2016 sampling campaign

	N	Range	Median	Mean	Notes
H. pylori genome copies/L	241	0-2.56E3	0	60.68	49 of 241 samples positive, 12 above the detectable limit
pH	238	5.16-8.43	6.68	6.54	96 samples were 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 μS/cm	232	326-616	512	504.8	
Free chlorine residual (FAC) in mg/L	209	0.01-0.78	0.37	0.3658	17 of 209 samples below WHO recommendation of 0.2-0.5 mg/L of FAC

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

TABLE 2 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 for the presence/absence of *H. pylori* with smoothing (β, SE)	Model 1b: Logistic Regression for the presence/absence of *H. pylori*—auto-correlation adjustment (β, SE)	Model 2a: Log-transformed Simple Linear Regression for quantities of *H. pylori* (β, SE)	Model 2b: Log-transformed Simple linear Regression for quantities of *H. pylori* (β, SE) with influential outlier removed
Intercept	9.73 (5.42)	5.41 (5.12)	0.29 (2.25)	1.54 (2.31)
Prior day the 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)

*$P < 0.1$, **$P < 0.05$.

found between the 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 previous laboratory study, the optimal pH for *H. pylori* survival in water was found to be 5.8-6.9, but that does not account for other relevant factors, such as co-exposure with chlorine.[44] Multiple studies have shown that *H. pylori* can survive longer at lower temperatures in both well water and river water.[16,45] However, as all cells went into the VBNC state, the authors could not comment on persistence or reproduction at these temperatures. Although our results are in line with other literature, more research is needed to tease out whether *H. pylori* dies more quickly at higher temperature or simply moves into a VBNC state more rapidly. The lack of an explanatory mechanism for how pH might be positively associated with the quantity of *H. pylori* makes it uncertain whether these relationships are meaningful. Given this, it seems likely that other, unaccounted for biotic and abiotic factors might be important in relation to the 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 system, and the stochastic shedding of cells from biofilms in the pipes.

Despite this, our current study shows that there is 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,[5,26] Sweden,[46] Pakistan,[47] Iraq,[19] Iran,[22,48] Costa Rica,[23] and Spain[37] have shown contamination of drinking water with *H. pylori* using PCR, culture, and microscopy techniques such as fluorescent in situ hybridization. In contrast, studies in Bangladesh[40] and Japan[49] failed to detect *H. pylori* in treated drinking water, although the study in Japan and a further study in Scandinavia detected *H. pylori* via PCR in untreated well water used as drinking water.[10,49] The results from our study are consistent with those that reported contamination, although 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, respectively.[26,37]

4.1 | Limitations

As we used a DNA-based method of detection, we could not determine between viable and nonviable *H. pylori* cells in drinking water, so we are unable to infer whether the DNA amplified was from culturable, viable but nonculturable, or nonviable *H. pylori* cells. Thus, examining our results in a risk assessment format would be problematic, as we cannot determine the relative proportions of each form, and the relative infectiousness of the VBNC compared to the culturable form is not well characterized. However, Sen et al[50] 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. While this may suggest that the DNA detected in our and other studies in chlorinated drinking water may have come from VBNC or viable, culturable *H. pylori* cells, it is in no way conclusive.

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 widespread of contamination that this did not appear to be linked to a specific district.[26] Given that there are leaks in the distribution system and a large amount of unaccounted for water,[51] 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.

4.2 | 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.[10,26,37] By collecting water from a single location over a year, we better characterized the annual body burden of *H. pylori* from drinking water in the Lince district in Lima, which can be used to more accurately assess risk of infection from this exposure route. However, this relationship may be modified by downstream water treatment techniques, such as boiling and bleach disinfection. Indeed, according to a survey by the World Bank's Water and Sanitation Program, 89% of people treat tap water (primarily through boiling) before they drink it.[52]

The biggest limitation of our and other quantitative surveys of *H. pylori* contamination of drinking water is that they could not distinguish between VBNC, viable culturable, and nonviable *H. pylori* cells. Thus, although several samples in our and other studies[26,37] had quantities of *H. pylori* found to be infectious in either mice, humans, or monkeys,[34,53,54] it is unclear whether the sampled water poses the same infectious risk found in dosing trials as these trials used the viable, culturable state of *H. pylori*. In the only quantitative microbial risk assessment performed thus far for *H. pylori* in drinking water, Ryan et al[55] recommended that the maximum contaminant level goal for *H. pylori* be set at <1 organism/L in drinking water based on the downstream risk of infection and gastric cancer. That study used quantities of *H. pylori* found in surface and recreational water,[35,36] which, when accounting for the efficiency of municipal water treatment in the USA, were substantially lower than those found in our study. Further, the contamination of treated water from La Atarjea[5,26] and the consistent contamination of well water used to supplement the treated drinking water (Boehnke KF, Brewster RK, Xi C, unpublished data) highlight 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.

5 | CONCLUSIONS

Over a 1-year sampling period, we detected *H. pylori* in 20.3% of drinking water samples from Lima, Peru, using qPCR, which suggests that there is continued contamination of the water supply in the Lince district. We found no significant relationship between sampling date and likelihood of *H. pylori* contamination, but found that increased temperature was associated with a lower likelihood of the presence of *H. pylori* and that increased pH was associated with a higher quantity of *H. pylori*. Further studies are required to examine whether this is true in other districts in Lima. Future studies should aim to identify potential sources for contamination and better characterize the risk of *H. pylori* in drinking water, as well as examine the effectiveness of downstream drinking water treatments, such as boiling. 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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DISCLOSURES OF INTERESTS

All authors declare no conflicts of interest.

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