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Clinical, Epidemiologic, and Genomic Studies (SWOG S1119) of *Helicobacter Pylori* in Lima, Peru: Role of Contaminated Water

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ABSTRACT

Background: *Helicobacter pylori* is a Group 1 carcinogen because of its causal relationship to gastric carcinoma. We examined the role of drinking water as a potential mechanism of transmission in Peru, where gastric carcinoma is the most common cancer and cause of cancer death. **Materials and Methods:** Patients from high and low gastric cancer risk districts within Lima were enrolled to the study. Patients with histologically proven *H. pylori* infection were registered to the treatment and in-home water sampling portions of the trial. Gastric biopsies, filtered water, and biofilm specimens were analyzed for *H. pylori* by culture and quantitative polymerase chain reaction. Eradication was assessed by urea breath test at 6-8 weeks and one year from registration. **Results:** A total of 109 patients (89 from high-risk and 20 from low-risk districts) tested positive for *H. pylori* and were registered in the trial. Eradication rate by urea breath test at 6-8 weeks was 66%. Twenty-three patients who tested positive at 6-8 weeks responded favorably to second-line treatment (74.1%). Thus, the overall response rate at one year was 85%. *H. pylori* from 13 patients who failed to respond at 6-8 weeks demonstrated *in vitro* antibiotic resistance to amoxicillin, levofloxacin, and metronidazole. There was evidence of reinfection in five patients. Attempts to culture *H. pylori* from drinking water failed. *H. pylori* was detected by quantitative polymerase chain reaction in 97% of gastric mucosa, 50% of drinking water, and 30% of water biofilm specimens. **Conclusions:** We detected *H. pylori* by polymerase chain reaction in Lima's drinking water, suggesting water as a source of infection (ClincialTrials.gov number NCT015128). (J CANCEROL. 2016;2:52-63)

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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* 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 1 x 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 µm 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 Laboratories, 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 indeterminate.

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 H_2O to bring the reaction

volume to 20 µl. The standard curve comprised 10^1 to 10^6 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[25]. 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[23]. 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 between 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 the protocol is available on the SWOG website[26].

Table 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

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

Table 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 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

high-risk districts (p = <0.0001) (Tables 1 and 2). Patient characteristics and district of residence are shown in tables 2 and 3, respectively. Most patients were female, had a normal or overweight body mass index (BMI) class, and were over the age of 40.

Table 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%)

*Included patients receiving second-line therapy.

Table 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

*One value was > 0.125. MIC: minimum inhibitory concentration.

Response to treatment at 6-8 weeks and at one-year is shown in table 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 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 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

Table 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

CL: chlorine; qPCR: quantitative polymerase chain reaction.

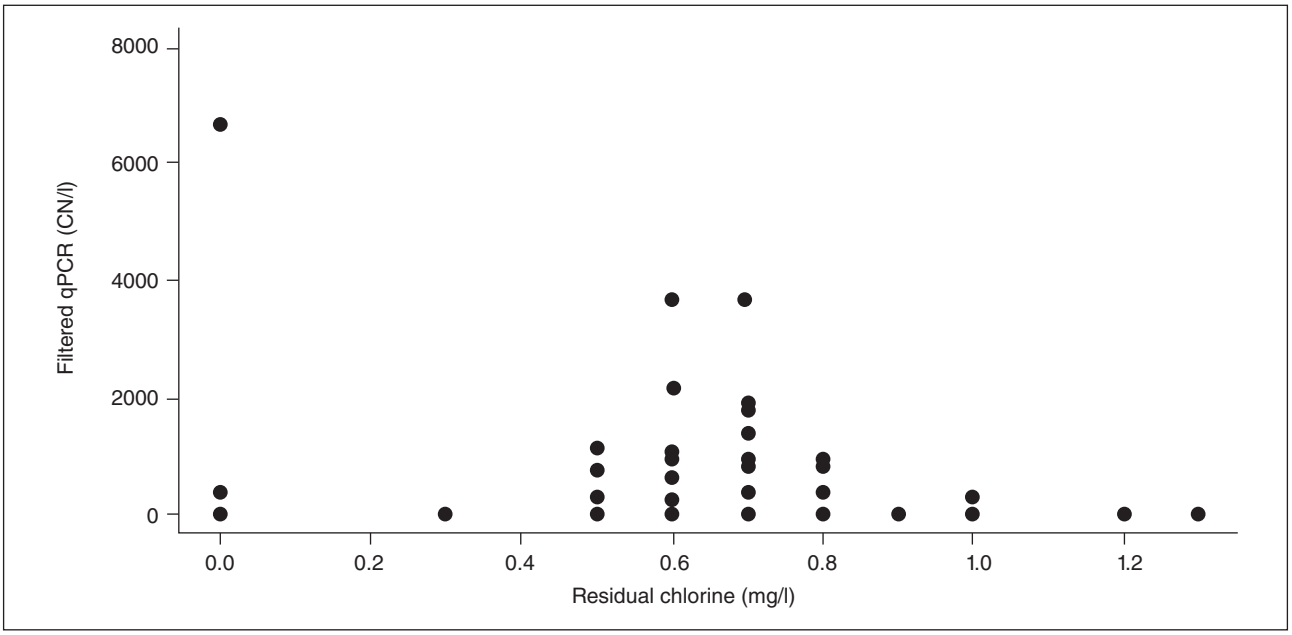

Figure 1. Correlation between quantitative polymerase chain reaction in filtered drinking water and residual chlorine. qPCR: quantitative polymerase chain reaction.

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. 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 (micromhos per centimeter; range, 40-939); me-

dian 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[25,27,28]. 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[28-30] and *H. pylori* has also been reliably detected in recreational and drinking water using molecular biology techniques[27,31]. Finally, the viable but non-culturable form of *H. pylori* has been shown to be infectious in mice via gavage[32] and we previously showed that waterborne *H. pylori* is infectious in mice as well[33]. 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[27,34,35].

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[39].

The levels of residual chlorine in water in about half of the water samples were below the median of 0.7 mg/l. However, all but four residual chlorine measurements on this study meet the standard set forth by the Peruvian Ministry of Health that the level of residual chlorine in any point of the water distribution system should not be less than 0.5 mg/l[40].

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[32]. 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 different 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[41].

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[42]. 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[43].

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[44]. 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[45]. The findings of this study support that strategy.

DISCLOSURE OF INTEREST

Laurence Baker receives personal fees from Morphotek and Marathon Pharmaceuticals, which are outside the submitted work. All other authors declare that they have no conflicts of interest.

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