Antibiotic resistance among *Helicobacter pylori* clinical isolates in Lima, Peru

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Objectives: Gastric carcinoma is the most common cancer and cause of cancer mortality in Peru. *Helicobacter pylori,* a bacterium that colonizes the human stomach, is a Group 1 carcinogen due to its causal relationship to gastric carcinoma. While eradication of *H. pylori* can help prevent gastric cancer, characterizing regional antibiotic resistance patterns is necessary to determine targeted treatment for each region. Thus, we examined primary antibiotic resistance in clinical isolates of *H. pylori* in Lima, Peru.

Materials and methods: *H. pylori* strains were isolated from gastric biopsies of patients with histologically proven *H. pylori* infection. Primary antibiotic resistance among isolates was examined using E-test strips. Isolates were examined for the presence of the cagA pathogenicity island and the vacA m1/m2 alleles via polymerase chain reaction.

Results: Seventy-six isolates were recovered from gastric biopsies. Clinical isolates showed evidence of antibiotic resistance to 1 (27.6%, n=21/76), 2 (28.9%, n=22/76), or ≥3 antibiotics (40.8%). Of 76 isolates, eight (10.5%) were resistant to amoxicillin and clarithromycin, which are part of the standard triple therapy for *H. pylori* infection. No trends were seen between the presence of cagA, vacA m1, or vacA m2 and antibiotic resistance.

Conclusion: The rate of antibiotic resistance among *H. pylori* isolates in Lima, Peru, is higher than expected and presents cause for concern. To develop more targeted eradication therapies for *H. pylori* in Peru, more research is needed to better characterize antibiotic resistance among a larger number of clinical isolates prospectively.

Keywords: *H. pylori,* antibiotic resistance, Peru, amoxicillin

Introduction

*Helicobacter pylori* is a stomach bacterium that colonizes ~50% of people globally.1 *H. pylori* infection 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 with <20% of asymptomatic Caucasians in the USA.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 to reduce recurrence of *H. pylori* infection.

Unfortunately, there is no broad consensus about an optimal antibiotic therapy for the 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) with various other therapies.8-11
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 seven sites of six Latin American countries that showed that the 14-day triple therapy was superior to the 5-day concomitant quadruple therapy, and no different than the 10-day sequential quadruple therapy.11,12 These inconsistencies reflect localized differences in antibiotic use practices, such as the use of clarithromycin for upper respiratory infections.13

The differences in efficacy of antibiotic therapy are supported by primary antibiotic resistance data. For example, \textit{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.14 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.15 Thus, we searched the literature for reports of primary antibiotic resistance to \textit{H. pylori} in Peru. Three studies were identified, which reported 36.9% resistance to levofloxacin,16 an average of 66% resistance to metronidazole,17,18 50% resistance to clarithromycin,17 and 0% resistance to tetracycline.17 Unfortunately, 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 \textit{H. pylori} infection is an important step toward prevention of gastric carcinoma,5,19 we assessed primary \textit{H. pylori} antibiotic resistance among 76 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 second- and third-line therapies.11,20 Our data showed significant primary antibiotic resistance to first- and second-line antibiotics among \textit{H. pylori} isolates from a clinical setting in Lima, Peru.21

\textbf{Materials and methods}

\textbf{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, MI, USA. The cohort of patients from whom \textit{H. pylori} isolates were obtained has been previously described.22 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 were obtained before enrollment in the trial. Study participants were aged 20–70 years and had symptoms of dyspepsia for at least 6 months. Patients with gastric cancer or peptic ulcer disease were excluded from this study. Stomach biopsies were obtained via endoscopy under sedation from 109 adult symptomatic patients. The diagnosis of \textit{H. pylori} infection was made histologically. Six biopsies per patient were obtained: four for histologic studies and two for culture, which were stored in 1.5 mL of 1× phosphate-buffered saline (PBS) with 20% glycerol at −80°C until processing. Following endoscopy, infected patients were treated with esomeprazole, amoxicillin, and clarithromycin twice a day for 14 days. Indigent patients received treatment free of charge. Patients were followed up 1 year after treatment to check for \textit{H. pylori} infection status via the urea breath test.21

\textbf{H. pylori isolation}

Gastric biopsy samples were thawed on ice and then homogenized using OMNI probes at maximum speed (Omni International, Kennesaw, GA, USA). A volume of 50 µL of the homogenized sample was plated onto both 5% Sheep’s Blood Tryptic Soy Agar plates (Remel, Columbus, OH, USA) and on \textit{H. pylori} selective media (Columbia blood agar base with 10% horse blood, 10 mg/L vancomycin, 5 mg/L trimethoprim, 5 mg/L cefsulodin, 5 mg/L amphotericin B, 300 mg/L urea, and 3500 U polymyxin B/L).22 Plates were incubated at 37°C in microaerobic conditions for 3–7 days. Presumptive \textit{H. pylori} isolates were subcultured, then confirmed by morphology and checked for urease activity using a urease indicator broth (0.33 M urea, 0.2% phenol red, 0.02% NaN₃, 0.01 M NaPO₄ buffer [pH 6.5]). Glycerol stocks of each isolate were prepared in Brucella Broth (Remel) with 15% glycerol.

\textbf{Detecting vacA and cagA with PCR}

\textit{H. pylori} isolated DNA was tested for the presence of \textit{H. pylori} \textit{cagA} and \textit{vacA} genes by polymerase chain reaction (PCR) using previously described primers and the Takara
PCR kit (Clontech, Mountain View, CA, USA). For \textit{cag}A, primers F1 (5'–GATAACGAGCGGCTTCTTTGAG-3') and B1 (5'-CTGCAAAAGATTGTTGCGAG-3') were used to amplify a 349 base pair product. Primers VAG-F (5’-CAATCTGTCCAATCAAGCGAG) and VAG-R (5’-GCGTCAAAATAATTCCAAGG) were used to amplify the m1/m2 subunits of the \textit{vacA} gene, yielding a 570 or 645 base pair product. 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 (Durham, NC, USA) instructions, \textit{H. pylori} isolates were tested for susceptibility to amoxicillin, clarithromycin, levofloxacin, metronidazole, rifampicin, and tetracycline using E-test®. Isolates were subcultured and then grown on Mueller Hinton agar supplemented with 5% sheep’s blood (Remel, Columbus, Ohio, USA). Colonies were collected and suspended in 1× PBS and visually compared with a 3.0 McFarland turbidity standard. Cell suspensions were then spread on Mueller Hinton agar with 5% sheep’s blood (MH) 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 37°C in microaerobic conditions. Results were interpreted per the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards, which are “based on epidemiological cut-off values, which distinguish wild-type isolates from those with reduced susceptibility”.\(^{25}\)

**Quality control**

ATCC strain 43504 (\textit{H. pylori}) and ATCC 25922 (\textit{Escherichia coli}) were used as quality control strains. See Table 1 for expected quality control 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 subcultured onto an MH plate 24 hours before testing. Colonies were suspended in 1× PBS to a visual density of a 0.5-McFarland standard and were 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**

\textit{H. pylori} plates were read after 72 hours of incubation in microaerobic conditions. \textit{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”.\(^{25}\)

**Statistical analysis**

Antibiotic resistance MICs were examined using descriptive statistics. Student’s \(t\)-tests were used to examine whether isolates with the m1 versus m2 subunit of the \textit{vacA} gene were resistant to different numbers of antibiotics.

**Results**

Seventy-six \textit{H. pylori} strains were isolated from the gastric biopsies and were tested for primary antibiotic resistance (Table 2). Metronidazole was the antibiotic to which isolates were most commonly resistant (61.8%), while isolates showed least resistance to tetracycline (3.9%). About one-third of isolates were resistant to either clarithromycin or amoxicillin, which are typically used for the standard triple therapy, and 10.5% were resistant to both (Table 3). Further, 40.1% of strains were resistant to >3 of the tested antibiotics.

**Polymerase chain reaction**

By PCR, all 76 strains were positive for the \textit{cag}A pathogenicity island, 57 (75%) were positive for \textit{vacA} m1 and 19 (25%) were positive for \textit{vacA} m2. No differences were seen between the presence of \textit{vacA} m1/m2 and the mean number of antibiotics to which isolates were resistant.
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Table 2 Primary antibiotic resistance of clinical isolates from Lima, Peru

<table>
<thead>
<tr>
<th>Resistance cutoff (mg/L)</th>
<th>Amoxicillin</th>
<th>Clarithromycin</th>
<th>Tetracycline</th>
<th>Levofloxacin</th>
<th>Metronidazole</th>
<th>Rifampicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolates susceptible (%)</td>
<td>≥0.125</td>
<td>≥0.5</td>
<td>&gt;1</td>
<td>&gt;1</td>
<td>&gt;8</td>
<td>&gt;1</td>
</tr>
<tr>
<td>Isolates resistant (%)</td>
<td>51/76 (67.1)</td>
<td>49/76 (64.5)</td>
<td>73/76 (96.1)</td>
<td>35/76 (46.1)</td>
<td>29/76 (38.2)</td>
<td>41/76 (53.9)</td>
</tr>
<tr>
<td>MIC30 (mg/L)</td>
<td>0.0555</td>
<td>0.094</td>
<td>3</td>
<td>0.048</td>
<td>265</td>
<td>0.875</td>
</tr>
<tr>
<td>MIC90 (mg/L)</td>
<td>256</td>
<td>256</td>
<td>32</td>
<td>0.125</td>
<td>256</td>
<td>32</td>
</tr>
<tr>
<td>MIC range (mg/L)</td>
<td>0–256</td>
<td>0–256</td>
<td>0.25–32</td>
<td>0.032–32</td>
<td>0.25–256</td>
<td>0–256</td>
</tr>
</tbody>
</table>

Notes: Results of MIC testing using E-test strips from bioMérieux. Susceptibility and resistance were determined using EUCAST standards. *Five isolates had a 0.5-µg/mL MIC for clarithromycin, which falls between the susceptible and resistant cutoffs for clarithromycin. For the purposes of this analysis, they were considered resistant.

Abbreviation: MIC, minimum inhibitory concentration.

Table 3 Primary resistance to >1 antibiotic among clinical isolates

<table>
<thead>
<tr>
<th>No. resistance</th>
<th>1</th>
<th>2</th>
<th>3+</th>
<th>Resistant to clarithromycin and amoxicillin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of isolates, n/N (%)</td>
<td>2/76 (2.6)</td>
<td>21/76 (27.6)</td>
<td>22/76 (28.9)</td>
<td>31/76 (40.8)</td>
</tr>
</tbody>
</table>

Notes: Nearly all isolates were resistant to at least one antibiotic, and 70% were resistant to two or more. Eight of 76 isolates were resistant to both clarithromycin and amoxicillin, which are both used in the triple therapy.

Discussion

To our knowledge, this is the first study characterizing H. pylori primary antibiotic resistance to amoxicillin and rifampicin in Peru. When comparing our study results to published studies, we found that the MIC cutoffs 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.15,26 We conducted a brief meta-analysis compiling all primary antibiotic resistance data in Peru from ours and other reports from the literature (Table 4).

This study demonstrates a high incidence of primary H. pylori antibiotic resistance in Lima, Peru, to antibiotics used in the standard triple therapy. Inference from our results is limited due to our small sample size and that our patient population is likely not generalizable to Peru. However, 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. 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 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.12,15–17,19,26–28 This observation, coupled with reports of H. pylori reinfection after successful antibiotic treatment,12,17 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.2,17 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.

Table 4 Reported primary antibiotic resistance among Helicobacter pylori isolates from Peru using EUCAST guidelines

<table>
<thead>
<tr>
<th></th>
<th>Amoxicillin, %</th>
<th>Clarithromycin, %*</th>
<th>Tetracycline, %</th>
<th>Levofloxacin, %</th>
<th>Metronidazole, %</th>
<th>Rifampicin, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vasquez et al14</td>
<td>N/A</td>
<td>38.9 (7/18)</td>
<td>0 (0/5)</td>
<td>N/A</td>
<td>27.8 (5/18)</td>
<td>N/A</td>
</tr>
<tr>
<td>Berg et al17</td>
<td>N/A</td>
<td>4.2 (1/24)</td>
<td>N/A</td>
<td>N/A</td>
<td>63.6 (49/77)</td>
<td>N/A</td>
</tr>
<tr>
<td>Mochizuki Tamayo et al15</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>36.8 (35/95)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Totals, including present study</td>
<td>32.9 (25/76)</td>
<td>29.7 (34/118)</td>
<td>3.7 (3/81)</td>
<td>44.4 (76/171)</td>
<td>59.1 (101/171)</td>
<td>46.1 (35/76)</td>
</tr>
</tbody>
</table>

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

Abbreviations: MIC, minimum inhibitory concentration; N/A, not applicable.
Conclusion
We show high rates of primary antibiotic resistance to H. pylori clinical isolates in Lima, Peru. More studies are needed to confirm this finding to optimize the clinical treatment of H. pylori infection in Peru.

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Author contributions
All authors participated in the design and conduct of the study. MV, AB, and INR directed the clinical trial. CX, KFB, KCT, and SO conducted the laboratory studies. RS performed the statistical analyses. All authors reviewed, critically revised, and approved the final manuscript.

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