




Helicobacter pylori Antimicrobial Resistance and Gene Variants in High- and Low-Gastric-Cancer-Risk Populations

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ABSTRACT Colombia, South America has one of the world's highest burdens of *Helicobacter pylori* infection and gastric cancer. While multidrug antibiotic regimens can effectively eradicate *H. pylori*, treatment efficacy is being jeopardized by the emergence of antibiotic-resistant *H. pylori* strains. Moreover, the spectrum of and genetic mechanisms for antibiotic resistance in Colombia is underreported. In this study, 28 *H. pylori* strains isolated from gastric biopsy specimens from a high-gastric-cancer-risk (HGCR) population living in the Andes Mountains in Túquerres, Colombia and 31 strains from a low-gastric-cancer-risk (LGCR) population residing on the Pacific coast in Tumaco, Colombia were subjected to antibiotic susceptibility testing for amoxicillin, clarithromycin, levofloxacin, metronidazole, rifampin, and tetracycline. Resistance-associated genes were amplified by PCR for all isolates, and 29 isolates were whole-genome sequenced (WGS). No strains were resistant to amoxicillin, clarithromycin, or rifampin. One strain was resistant to tetracycline and had an A926G mutation in its 16S rRNA gene. Levofloxacin resistance was observed in 12/59 isolates and was significantly associated with N871/K and/or D91G/Y mutations in *gyrA*. Most isolates were resistant to metronidazole; this resistance was significantly higher in the LGCR (31/31) group compared to the HGCR (24/28) group. Truncations in *rdxA* and *frxA* were present in nearly all metronidazole-resistant strains. There was no association between phylogenetic relationship and resistance profiles based on WGS analysis. Our results indicate *H. pylori* isolates from Colombians exhibit multidrug antibiotic resistance. Continued surveillance of *H. pylori* antibiotic resistance in Colombia is warranted in order to establish appropriate eradication treatment regimens for this population.

KEYWORDS Colombia, South America, *Helicobacter pylori*, PCR, adaptive mutations, antibiotic resistance, antimicrobial susceptibility testing, levofloxacin, metronidazole, whole-genome sequencing

Helicobacter pylori, a Gram-negative, spiral-shaped, microaerophilic flagellated bacterium, is considered a pathogen that colonizes the human stomach and is the causative agent of acute and chronic gastritis, peptic ulcer disease (10 to 20%), gastric adenocarcinoma (1 to 2%), and gastric lymphoma (<1%) (1–4). In addition, it has more recently been suggested that *H. pylori* may also be associated with extraintestinal diseases such as immune thrombocytopenic purpura, refractory iron deficiency anemia, and vitamin B12 deficiency (5, 6). Therefore, eradication of *H. pylori* infection for treatment or prevention of these disorders is commonly prescribed; however, emerging

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antimicrobial resistance is increasing worldwide and is the main factor for the failure of *H. pylori* eradication (7).

The prevalence of *H. pylori* infections approaches 50% worldwide (5), and can be as high as 80 to 90% in developing countries (1, 8). For example, in emerging nations such as Vietnam, India, and Saudi Arabia, or Canadian aboriginal populations, >80% of the populations are infected (9), whereas the infection rate prevalence in industrialized nations is as low as 30% (8, 10). Also noted are differences in the prevalence of infection both within different geographical locations within a country and between countries (8), as well as differences in the antimicrobial-resistance rates (1, 11). One of the highest gastric cancer rates in the world occurs in Colombia, South America, where >80% of the population is estimated to be infected with *H. pylori* (12–18). Interestingly, the incidence rates of gastric cancer in Colombia differ markedly between high-risk individuals residing in the Andes Mountains in Túquerres (~150/100,000) compared to those living on the coast in Tumaco, where there is a low risk of gastric cancer (6/100,000) (19, 20).

As a result of the high prevalence rate of *H. pylori* and its association with serious gastric disorders, different anti-*H. pylori* treatment regimens have emerged, in some cases depending upon the resistance to clarithromycin (CLR) and metronidazole (MTZ). For example, in one study, >85% of the *H. pylori* strains in Colombia were resistant to MTZ (21). While first-line therapy, typically a proton pump inhibitor (PPI) plus two of the three antibiotics CLR, MTZ, or amoxicillin (AMX), first proposed in the Maastricht Consensus Report (22), has generally been associated with successful cure rates, the rise of multidrug-resistant (MDR) strains of *H. pylori* has led to treatment failures. MDR has resulted from multiple antibiotics previously prescribed or, in some cases, indiscriminate use of “over-the-counter” antibiotics. Mutations in the 23S rRNA (A2142G/C, A2143G), 16S rRNA (AGA926-928TTC), and *gyrA* (QRDR) genes are responsible for CLR, tetracycline (TET), and fluoroquinolone resistance, respectively, as well as mutations in genes *fixA/rdxA*, *rpoB* (500 to 545), and *pbp1A*, conferring resistance to MTZ, rifampin (RIF), and AMX, respectively. Knowledge of these mutations provides guidance to management and treatment options. The aim of this study was to ascertain the prevalence and mechanism of *H. pylori* antibiotic resistance of six commonly used antibiotics in Colombian populations with high and low risk of gastric cancer, with each population having a $\geq 90\%$ prevalence of *H. pylori* infection.

MATERIALS AND METHODS

Study participants, histopathology, and *Helicobacter pylori* culture. Individuals from two locations in Colombia with contrasting risks of gastric cancer were invited to participate. The two locations were Tuquerres, in a high-gastric-cancer-risk region (HGCR), and Tumaco, in a low-gastric-cancer-risk region (LGCR). Inclusion criteria were age between 40 and 60 years and dyspeptic symptoms meriting upper gastrointestinal tract endoscopy. Exclusion criteria were use of proton pump inhibitors, H₂-receptor antagonists, or antimicrobials in the month before the endoscopy, history of chronic conditions, or a prior gastrectomy. Informed consent was obtained from all participants. The ethics committees of the participating hospitals and the Universidad del Valle in Colombia, and the Institutional Review Board of Vanderbilt University approved all study protocols. Gastric mucosa biopsy samples were obtained from all participants between 2006 and 2010, from the gastric antrum, incisura angularis, and corpus for histopathology. One additional antral biopsy specimen from each participant was frozen in glycerol/thioglycolate for *H. pylori* culture. Histopathological diagnoses were determined by two pathologists (authors P.C. and M.B.P.), according to internationally accepted criteria (23, 24). *H. pylori* strains in this study included 37 from individuals from a previous study (25), from which *H. pylori* isolates were available. Additionally, 22 strains were selected from a total of 271 enrolled participants following a similar protocol. Individuals from both regions were matched by age and sex, selecting those with the least advanced histological lesions (NAG or MAG). However, a few subjects with more advanced lesions were included as there were not enough cases with less advanced lesions to match, mainly in the HGCR area. In total, 59 representative strains of *H. pylori* were isolated from human gastric antrum biopsy samples; 31 strains were recovered from the samples taken from patients in the LGCR, and 28 strains were recovered from biopsy specimens from patients in the HGCR. Biopsies were homogenized in a sterile tissue grinder containing Brucella broth (BBL, Becton, Dickinson and Co., Sparks, MD) with 20% glycerol. Aliquots (50 μ l) were plated onto Glaxo blood agar plates containing vancomycin, bacitracin, naladixic acid, and amphotericin B (26). Plates were incubated microaerobically at 37°C with a gas mixture of 80:10:10 (N₂, CO₂, H₂) for up to 14 days. *H. pylori* was identified by colony morphology and Gram stain, and confirmed by PCR and 16S rRNA sequencing.

TABLE 1 PCR primers used to amplify select portions of *H. pylori* genes

Antibiotic	Gene	Primer name	Nucleotide sequence	Product size (bp)	Reference
Metronidazole (MTZ)	<i>rdx</i>	forward primer rdx1	5'-GCC ACT CCT TGA ACT TTA ATT TAG G-3'	749	(63)
		reverse primer rdx4	5'-CGT TAG GGA TTT TAT TGT ATG CTA C-3'		
	<i>frx</i>	forward primer frx1	5'-CGA ATT GGA TAT GGC AGC CG-3'	913	(63)
		reverse primer frx4	5'-TAT GTG CAT ATC CCC TGT AGG -3'		
Clarithromycin (CLR)	23S	forward primer 23SF2	5'-CGG TGC TCG AAG GTT AAG AG-3'	913	This study
		reverse primer 23S R2	5'-TTC AGC GGT TAT CAC ATC CA -3'		
Levofloxacin (LEV)	<i>gyrA</i>	forward primer gyrA1	5'-TTT AGC TTA TTC AAT GAG CGT-3'	428	(64)
		reverse primer gyrA2	5'-GCA GAC GGC TTG GTA GAA TA -3'		
Rifampin (RIF)	<i>rpoB</i>	forward primer1369f	5'-AGG GAC CAC TTG GGC AAT CGT AGG-3'	498	(65)
		reverse primer1867r	5'-TAG CGG TCA AAT AAA TCG TCT CAC -3'		
Tetracycline (TET)	16S	forward primer-9F	5'-GAG TTT GAT YCT GGC TCA G-3'	1532	This study
		reverse primer-1541R	5'-AAG GAG GTG WTC CAR CC-3'		
Amoxicillin (AMX)	<i>pbp1</i>	forward primer pbpF1	5'-TGC GAA CAC CCT TTT AAA T-3'	2385	(66)
		reverse primer pbpR1	5'-GCG ACA ATA AGA GTG GCA-3'		

Determination of antimicrobial susceptibility. All antimicrobials were purchased from Sigma Chemical Company, St. Louis, MO. Antimicrobial susceptibility to AMX, CLR, levofloxacin (LEV), MTZ, TET, and RIF were tested by agar dilution using a Steers replicator, as recommended by the CLSI guidelines (27). Briefly, each strain was suspended in sterile saline at a density equivalent to a McFarland no.2 to 3 turbidity standard. Using the Steers replicator, approximately 5 μ l of each suspension was delivered onto Müeller-Hinton agar plates (MHB; Remel, Lenexa, KS) supplemented with 5% aged defibrinated sheep blood containing 2-fold serial dilutions of each antibiotic. An antibiotic-free plate was inoculated before and after each series of antibiotic plates to confirm viability of the inoculum and observe possible contamination. The plates were incubated under microaerobic conditions at 37°C for 3 days. Isolates were classified as resistant based on the following minimal inhibitory concentration (MIC) breakpoints: >8 μ g/ml for MTZ (28); ≥ 2 μ g/ml for RIF (28); ≥ 1 μ g/ml for LEV; ≥ 2 μ g/ml for AMX (28); and ≥ 2 μ g/ml for TET (28). CLR breakpoints were based on CLSI criteria (≤ 0.25 μ g/ml, susceptible; 0.5 μ g/ml, intermediate; and ≥ 1.0 μ g/ml, resistant) (29, 30). The MIC is defined as the lowest concentration of antibiotic at which there is a marked decrease in growth relative to the control. *H. pylori* ATCC 43504 was used as the quality control reference strain. Susceptibility assays were run in duplicate, a minimum of three separate times.

Mutation analysis of resistance genes. DNA was extracted from *H. pylori* using a High Pure PCR template preparation kit (Roche Molecular Biochemicals, Indianapolis, IN), according to the manufacturer's instructions. 16S rRNA and 23S rRNA gene fragments conferring resistance to TET and CLR, respectively, were amplified. The *rpoB*, *gyrA*, and *rdxA/frxA* genes were also amplified for RIF, LEV, and MTZ, respectively. The PCR primer pairs and the nucleotide sequences used in this study are listed in Table 1. An expanded high-fidelity PCR system (Roche Molecular Biochemicals) was used for PCR amplification. The following conditions were used for amplification: 35 cycles of denaturation at 94°C for 1 min, annealing at 55 to 58°C for 1.5 min, and elongation at 72°C for 2 min, followed by an elongation step of 7 min at 72°C. The PCR products were Sanger sequenced (Quintara Biosciences, Allston, MA). The sequence data were analyzed with DNASTAR Lasergene software.

Whole-genome sequencing and analysis. DNA was extracted from 29 isolates (9 HGCR, 20 LGCR) of the 59 strains using the High Pure PCR product purification kit (Roche Molecular Biochemicals). SMRTbell template libraries were prepared according to the instructions from Pacific Biosciences (PacBio; Menlo Park, CA), following the procedure and checklist for 10 kb template preparation. SMRT sequencing was performed on the PacBio Sequel system. The *de novo* assembly of the 29 genomes was performed following the instructions of the hierarchical genome assembly process (HGAP), version 4.0. A complete closed contig was obtained for each bacterial genome. The circular genomes were reoriented to a common start site 12 bp 3' of the *nusB* (HP0001) gene on the minus strand. Laboratory analyses were performed at the Frederick National Laboratory for Cancer Research, U.S. National Cancer Institute. Genomes were annotated using RAST, hosted by PATRIC (31). Using OrthoFinder, concatenated core gene sequences were determined followed by MAFFT for multi-sequence alignment and FastTree to infer approximate maximum-likelihood phylogenetic trees (32). Chromatiblock was used to produce and visualize whole-genome syntenic alignments (33). The gene sequences for *frxA*, *rdxA*, *fdxB*, *gyrA*, and *gyrB* were extracted from assembled contigs using BMap and translated sequences were aligned using Clustal Omega to build gene trees with the neighbor-joining method. Genomes were analyzed against the Comprehensive Antibiotic Resistance Database to identify additional antibiotic resistance genes (34). MLST 2.0 was used for *in silico* multilocus sequencing type (MLST) prediction (35). To build a phylogeographic tree, 380 globally distributed *H. pylori* isolates, belonging to seven ancestral haplogroups, were acquired from PubMLST (36, 37). Concatenated MLSTs were aligned using MAFFT followed by FastTree for the tree inference.

Statistical analysis. Fisher's exact tests and kappa coefficients were calculated using GraphPad QuickCalcs (<https://www.graphpad.com/quickcalcs/>). For Fisher's exact test, a *P* value of ≤ 0.05 was considered statistically significant. For kappa coefficients, a value of < 0.4 was considered low agreement, a

TABLE 2 Antimicrobial susceptibilities of *H. pylori* isolates

Antimicrobial	MIC range ($\mu\text{g/ml}$)	MIC ₅₀ ($\mu\text{g/ml}$) ^a	MIC ₉₀ ($\mu\text{g/ml}$) ^b	Resistant		Susceptible	
				No.	%	No.	%
Metronidazole	0.125–128	32	64	55/59	93.2	4/59	6.8
Clarithromycin	≤0.015–0.25	≤0.015	0.03	0/59	0	59/59	100
Levofloxacin	0.03–32	0.25	16	12/59	20.3	47/59	79.6
Rifampin	≤0.015–1	0.25	0.5	0/59	0	59/59	100
Tetracycline	≤0.015–2	0.125	0.5	1/59	1.7	58/59	98.3
Amoxicillin	≤0.015–0.25	≤0.015	0.06	0/59	0	59/59	100

^aMIC₅₀ = the MIC which inhibits 50% of the isolates.

^bMIC₉₀ = the MIC which inhibits 90% of the isolates.

value of 0.4 to 0.6 was considered moderate agreement, a value of 0.61 to 0.8 was considered substantial agreement, and a value of 0.81 to 1.0 was considered nearly perfect or perfect agreement.

RESULTS

Isolation of *H. pylori* from Colombian populations. A total of 59 strains of *H. pylori* were isolated from human gastric biopsy samples; 31 strains were recovered from patients in the LGCR area, and 28 strains were isolated from patients in the HGCR region. The patients in the LGCR and HGCR groups had similar ages, gender distributions, and gastric pathologies (Table S1 in the supplemental material).

Phenotypic antimicrobial resistance of *H. pylori* isolates. All 59 isolates were tested for antibiotic susceptibility to CLR, AMX, MTZ, TET, LEV, and RIF. MTZ resistance was observed in 93.2% (55 of 59) of the *H. pylori* isolates. MICs for MTZ ranged from 0.125 to 128 $\mu\text{g/ml}$, with only 4 isolates demonstrating MICs below 8 $\mu\text{g/ml}$ (Table 2). Resistance to LEV was observed in 20.3% (12 of 59) of the *H. pylori* isolates (MIC range, 0.03 to 32 $\mu\text{g/ml}$). Only one isolate exhibited resistance to TET. All isolates were susceptible to CLR, AMX, and RIF. The MIC₅₀ and MIC₉₀ for each antibiotic are shown in Table 2. All isolates (31/31) from LGCR patients were resistant to MTZ, compared to 24 of 28 isolates from the HGCR patients (Fisher's exact test, *P* value = 0.045) (Table 3). There was no statistical difference in the number of isolates resistant to TET or LEV between the risk groups (Table 3).

Genotypic determination of antimicrobial resistance. Antibiotic resistance-related genes for CLR, AMX, MTZ, TET, LEV, and RIF from all 59 isolates were amplified by PCR, followed by Sanger sequencing to identify mutations previously associated with resistance phenotypes. Additionally, whole-genome sequencing (WGS) was performed on 29 isolates (20 LGCR and 9 HGCR) (Table S2). WGS identified the same resistance mutations as targeted sequencing (i.e., PCR followed by Sanger sequencing) for all genes evaluated in this study.

Consistent with susceptibility to CLR, AMX, and RIF, no previously reported drug resistance-associated mutations were present in the 23S rRNA (38, 39), *rpoB* (40, 41), or *pbp1A* (42–44) genes in any of the isolates. The single isolate resistant to TET had a mutation in the 16S rRNA gene at position A926G (Table 4), which has been associated with low-level (1 to 4 $\mu\text{g/ml}$) resistance (45, 46).

TABLE 3 Summary of resistant *H. pylori* strains in gastric cancer populations in Colombia

Parameter	MTZ (MIC > 8 $\mu\text{g/ml}$) ^b		LEV (MIC > 1 $\mu\text{g/ml}$) ^b		TET (MIC > 1 $\mu\text{g/ml}$) ^b	
	LGCR	HGCR	LGCR	HGCR	LGCR	HGCR
Range ($\mu\text{g/ml}$)	16–128	0.125–128	0.06–32	0.03–16	≤0.015–1	≤0.015–2
No. resistant strains	31/31	24/28	9/31	3/28	0/31	1/28
% Resistance	100	86	29	11	0	4
<i>P</i> value ^a	0.045*		0.0621		0.4746	

^aAsterisk (*) indicates *P* value < 0.05 for Fisher's exact test between the low- and high-risk groups.

^bLEV, levofloxacin; TET, tetracycline; MTZ, metronidazole.

TABLE 4 *H. pylori* isolate MIC values and drug resistance-associated mutations for levofloxacin, tetracycline, and metronidazole^a

Isolate ID ^b	Cancer risk group	LEV	<i>gyrA</i> (LEV)	TET	16s rRNA (TET)	MTZ	<i>rdxA</i> (MTZ)	<i>frxA</i> (MTZ)
MT5110	LGCR	8-32	N87I	S		16-64	>217 ELONGATED	
MT5118*	LGCR	8	N87K	S		32-64		
MT5125*	LGCR	32	N87I	S		32-64	M1V	73 STOP
MT5127	LGCR	4-8	D91Y	S		64-128		38 STOP
MT5156*	LGCR	S	N87T	S		64-128	10 STOP	73 STOP
MT5165*	LGCR	S		S		16-32	86 STOP	108 STOP
MT5175*	LGCR	S		S		16-32	145 STOP	
MT5178	LGCR	S		S		32-64		73 STOP
MT5179	LGCR	32	N87I	S		64-128	122 STOP	73 STOP
MT5101*	LGCR	S		S		64	M1V	38 STOP
MT5105*	LGCR	8-32	N87I	S		32-64		73 STOP
MT5106*	LGCR	S		S		64		38 STOP
MT5107	LGCR	S		S		32-64	49 STOP	38 STOP
MT5111*	LGCR	S	N87T	S		64-128	82 STOP	73 STOP
MT5113	LGCR	S	N87T	S		64	49 STOP	
MT5114*	LGCR	16	N87I	S		64	73 STOP	73 STOP
MT5116*	LGCR	S		S		64	145 STOP	38 STOP
MT5117	LGCR	S	N87T	S		64-128		73 STOP
MT5119*	LGCR	S		S		32-64		73 STOP
MT5120*	LGCR	S		S		64-128		167 STOP
MT5124*	LGCR	16-32	N87I	S		128		73 STOP
MT5126	LGCR	S		S		32-64	64 STOP	129 STOP
MT5131*	LGCR	S	N87T	S		64	26 STOP	73 STOP
MT5135*	LGCR	S	N87T	S		32-64		73 STOP
MT5136*	LGCR	S	N87T	S		64	26 STOP	73 STOP
MT5139	LGCR	4	N87T, D91Y	S		16-64	32 STOP	
MT5155	LGCR	S		S		32		67 STOP
MT5176*	LGCR	S		S		16-64		
PZ5004*	LGCR	S		S		32-64	101 STOP	73 STOP
PZ5024	LGCR	S	N87T	S		32-64	211 ELONGATED	73 STOP
PZ5026*	LGCR	S	N87T	S		64-128	129 STOP	38 STOP
MT2139	HGCR	S		S		64		148 STOP
MT2141*	HGCR	S		S		64	209 STOP	38 STOP
MT2158*	HGCR	S	N87T	S		64-128	49 STOP	73 STOP
MT2164*	HGCR	S		S		32-64		110 STOP
MT2143	HGCR	S		S		>32	49 STOP	38 STOP
MT2171*	HGCR	S		S		S		
MT2174*	HGCR	S		S		64-128		
MT2102	HGCR	S		S		S		38 STOP
MT2106	HGCR	S	N87T	S		32-64		148 STOP
MT2108	HGCR	S		S		S		38 STOP
MT2112	HGCR	S		S		16-32	10 STOP	
MT2114	HGCR	S		S		16-32	215 ELONGATED	
MT2115	HGCR	S		S		8		38 STOP
MT2118	HGCR	S		S		32	58 STOP	73 STOP
MT2120*	HGCR	S		S		64	157 STOP	38 STOP
MT2122*	HGCR	S		S		32-64		38 STOP
MT2124	HGCR	16-32	N87I	S		16-64	109 STOP	73 STOP
MT2127	HGCR	S		S		64-128		38 STOP
MT2129*	HGCR	S		S		64	51 STOP	38 STOP
MT2130	HGCR	S		S		16-32		38 STOP
MT2131	HGCR	S		S		8-16		90 STOP
MT2133	HGCR	2-4	D91G	S		32-64		38 STOP
MT2136	HGCR	S	N87T	S		64-128		38 STOP
MT2156	HGCR	S		S		64		38 STOP
MT2160*	HGCR	4-8	D91Y	S		64	29 STOP	38 STOP
PZ5056	HGCR	S		2	A926G	32-64	157 STOP	19 STOP
PZ5080	HGCR	S		S		S		73 STOP
PZ5086	HGCR	S		S		64-128		206 STOP
26695 (ATCC 700392)	Reference strain							
ATCC 43504	Reference strain						72 STOP	38 STOP

^aPhenotypically resistant MIC values ($\mu\text{g/ml}$) and drug resistance-associated mutations are highlighted in gray shading. Truncated and elongated *rdxA* and *frxA* genes are indicated by their length in amino acid residues and "STOP" or "ELONGATED," respectively. LEV, levofloxacin; TET, tetracycline; MTZ, metronidazole.

^bAsterisk (*) indicate isolates evaluated by PCR and whole-genome sequencing (WGS).

The 428-bp region of the *H. pylori* quinolone resistance-determining region (QRDR) of the *gyrA* gene was evaluated from the 12 LEV-resistant strains. All resistant strains had a nonsynonymous mutation occurring at amino acid positions N87 and/or D91 (Table 4), which has been previously reported to confer resistance to fluoroquinolones (47, 48). High-level resistance coincided with N87I or N87K mutations, while low-level resistance was present in isolates with D91G or D91Y mutations. Twelve isolates with N87T mutations were susceptible to LEV. One isolate coharboring N87T and D91Y mutations had low-level LEV resistance. Several reports have described N87T mutations in the *gyrA* gene of LEV-susceptible *H. pylori* strains (49–53). The N87T mutation has been associated with hypersensitivity to quinolone agents (53). No mutations associated with quinolones were found in *gyrB* gene sequences. Mutations in the *gyrA* gene were significantly associated with LEV resistance (Fisher's exact test, $P < 0.00001$). The kappa coefficient equaled 0.543 (95% confidence interval [CI]: 0.337 to 0.748), suggesting moderate agreement between phenotypic and genotypic assays to predict LEV resistance in these isolates. When N87T mutations were ignored, the *gyrA* genes remained significantly associated with LEV resistance (Fisher's exact test, $P < 0.00001$), and the kappa coefficient had a perfect agreement of 1.000 (95% CI: 1.00 to 1.00).

In agreement with the high prevalence of MTZ resistance, variant sequences for the *rdxA* and *frxA* genes were frequently present in these isolates (Table 4). Of the 59 isolates, 25 had truncated *rdxA* genes, yielding predicted products of ≤ 157 residues versus 210 residues for wild-type *rdxA* found in the *H. pylori* type strain ATCC 26695. Two isolates had point mutations changing the start codon from M to V. One isolate lacked a stop codon and had a predicted *rdxA* product of > 217 residues. In addition to truncation events, mutations at positions R16H/C/P, H25R, H53R/A, D59N, L62V, A68T/V/S/N, G98S, G163V/D, V204I, and A206T in the *rdxA* gene product were present in nearly all isolates (Table S3). These substitutions have been previously associated with MTZ resistance (54–57). Forty-nine isolates had truncated *frxA* gene sequences yielding predicted products of ≤ 167 compared to 217 residues of the wild-type *frxA* in the *H. pylori* type strain ATCC 26695. Nonsynonymous point mutations were also present in the *frxA* genes of nearly all isolates (Table S4). All resistant strains had at least one truncated *rdxA* or *frxA* gene, except for four isolates (MT5118, MT2174, MT5176, and MT2114). However, mutations in at least one residue previously associated with drug resistance (54, 55) were present in these four strains (Table S3, Table S4). Three of the four susceptible strains had truncated *frxA* sequences but intact *rdxA* genes, suggesting inactivation of *rdxA* may be more important in the mechanism of MTZ resistance in some *H. pylori* isolates. The fourth susceptible strain had intact genes for both *rdxA* and *frxA*. There was no statistical association between *rdxA* or *frxA* gene mutations and resistance phenotype by Fisher's exact tests or kappa coefficients, likely because the tests were underpowered by having only four susceptible strains versus 55 resistant strains for comparison. Dendrograms built from the *rdxA*, *frxA*, or the concatenation of both gene sequences did not reveal a clear phylogenetic relationship between gene sequence, risk group, or MIC levels (Fig. S1).

Complete genomes of 29 isolates (9 HGCR and 20 LGCR) were sequenced using PacBio to further evaluate mechanisms of antibiotic resistance. Additional antibiotic resistance genes for CLR, AMX, MTZ, TET, LEV, and RIF were not detected. Whole genome alignments showed extensive variation exists in the chromosomal structures among isolates, including rearrangement in the location of the syntenic blocks encoding of *frxA*, *rdxA*, and *gyrA* genes (Fig. 1). These rearrangements suggest chromosomal instability that may have influenced the emergence of antibiotic resistance in these genes.

Whole-genome phylogenetic analysis of *H. pylori* isolates. The complete genomes of 29 representative isolates (9 HGCR, 20 LGCR) were also assessed to determine phylogenetic origins. Interestingly, antibiotic resistance profiles for MTZ or LEV did not correlate with whole-genome phylogeny (i.e., the strains with similar resistance levels did not cluster together). Based on a whole-genome phylogenetic tree constructed from multisequence alignment of concatenated core gene sequences, isolates mainly clustered according to risk group (Fig. 2). The *H. pylori* type strain ATCC 43504 grouped in

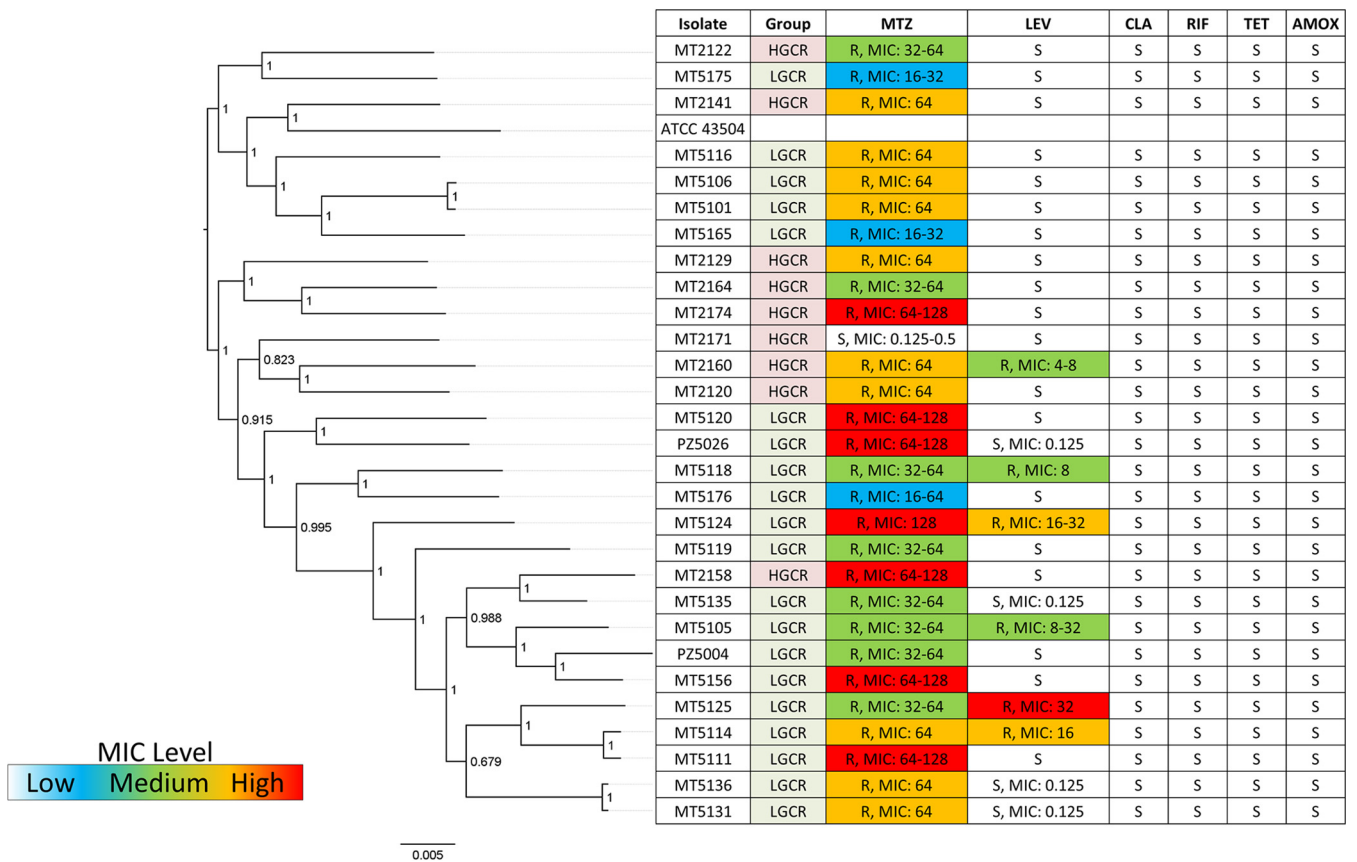


FIG 2 Whole-genome phylogenetic tree constructed from multisequence alignment of concatenated core gene sequences of 29 Colombian *H. pylori* isolates and the reference strain ATCC 43504. Local support values are indicated on the branches.

the HGCR clade. According to multilocus sequence typing, isolates from the LGCR cohort were in general more similar to hspWAfrica, hspSAfrica, and hpEurope strains, while those from HGCR patients associated most closely with hpEurope (Fig. 3), which is similar to the findings by Sabet et al. (58).

DISCUSSION

In this study, we have determined antibiotic resistance in 59 *H. pylori* strains cultured from stomach biopsy specimens of Colombian patients from high- and low-risk-gastric-cancer populations. As the prevalence of *H. pylori* in these populations exceeds 90%, effective use of antibiotics to eradicate infection and mitigate the development of subsequent gastric diseases, including cancer, is critically important. In addition to quantifying phenotypic levels of antibiotic resistance to six antibiotics commonly used in *H. pylori* eradication modalities (AMX, CLR, LEV, MTZ, RIF, and TET), mutational events in antibiotic resistance-related genes were also identified via PCR and product sequencing, as well as whole-genome sequencing (WGS).

In our study, we used PacBio for WGS of select *H. pylori* isolates to complement our characterization of antimicrobial resistance. PacBio sequencing technology yields long sequencing reads up to ~25 kb that enables assembly of complete, continuous bacterial chromosomes and plasmids (59). Conversely, Illumina and other short-read sequencing platforms typically can produce only up to ~500-bp read lengths and yield incompletely assembled genomes that are fragmented into noncontinuous sequences called contigs (59). While PacBio and short-read sequencing technologies can both provide highly accurate sequence coverage for detection of base pair mutations, contigs produced from short sequencing reads can sometimes result in missing or fragmented genes. Such confounding artifacts are avoided with PacBio, giving this technology a significant

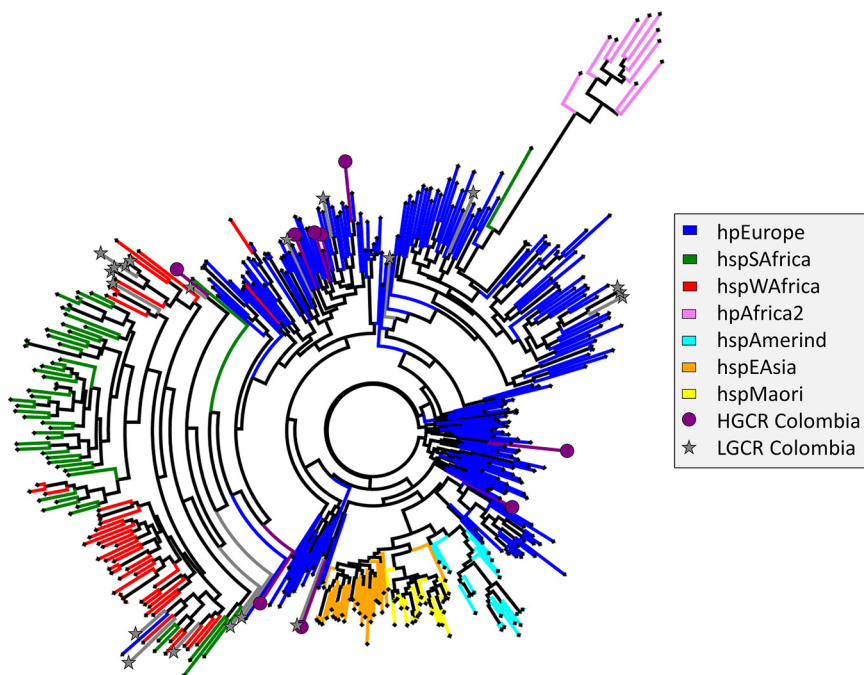


FIG 3 Phylogeographic tree constructed from multisequence alignment of concatenated multilocus sequencing types of 29 Colombian *H. pylori* isolates with whole-genome sequences. Also included were 380 reference strains with known distinct ancestral haplogroups. Branches are colored according to ancestral haplogroup.

advantage for gene detection and sequence analysis. Unlike PCR and Sanger sequencing, WGS analysis is also a time- and cost-effective approach for detecting and evaluating antibiotic-resistant gene profiles. Both PacBio and short-read sequencing technologies have been used to study the evolution, as well as pathogenic mechanisms, of *H. pylori* (60, 61). While Illumina and Sanger sequencing have also been used to identify mutations in antibiotic-resistance genes in *H. pylori* (62), our study is the first to our knowledge to use PacBio to study the mechanisms of antimicrobial resistance in this organism. Thus, in addition to detecting specific mutations associated with antibiotic resistance, we were able to analyze how whole-genome phylogenetic relationships, other potential resistance genes, and chromosomal rearrangements may have impacted the antibiotic resistance phenotypes of the HGCR and LGCR Colombian *H. pylori* isolates.

No drug resistance to CLR, AMX, and RIF, nor drug resistance-associated mutations in the 23S rRNA, *rpoB*, or *pbp1A* genes, was observed in any of the 59 isolates. This is in contrast to a systematic review of *H. pylori* antibiotic resistance in Colombia that reported the prevalence of CLR and AMX resistance to be 16% (7 to 28%) and 6% (2 to 12%), respectively (63). In Latin American countries (Argentina, Brazil, Chile, Colombia, Costa Rica, Cuba, Ecuador, Mexico, Paraguay, Peru, Uruguay, and Venezuela) the prevalence of CLR and AMX resistance is 12% (9 to 16%) and 4% (2 to 8%), respectively (63).

However, in a recent study conducted in patients from Tumaco and Túquerres, Colombia whose *H. pylori* infection status was 88.7% and 85.4%, respectively, the prevalence of *H. pylori* resistance to CLR and AMX in patients with dyspepsia symptoms was significantly higher in the LGCR group in Tumaco (20.5% and 22.8%, respectively) than in the high-risk group in Túquerres (3.4% and 5.4%, respectively) (P value <0.05) (17). Of the 74 *H. pylori* isolates reported in this paper, 57 were cultured from low-risk patients. Of these 57, 28 isolates were resistant to CLR, and 29 were susceptible. Of the 17 *H. pylori* strains isolated from the high-risk patients, 12 were susceptible to CLR and 5 were resistant. A subset of these isolates was analyzed for mutations noted in the PCR products of the 23S rRNA gene V domain. At least one mutation was noted in the region V domain in 31 (55.3%) of the *H. pylori* isolates,

with 17 (33.3%) resistant and 14 (25%) susceptible to CLR. Interestingly, 9 (16.1%) of the resistant *H. pylori* isolates did not have mutations in the 23S rRNA region amplified. The authors, by analyzing kappa coefficients, surmised there was no relationship between the presence of mutations and *in vitro* resistance to CLR (17). Similarly, the authors found there was no statistical relationship to the lack of mutation in the domain V of the 23S rRNA in a particular strain and its *in vitro* susceptibility to CLR. Nevertheless, the authors concluded that therapeutic failure of eradication treatment (omeprazole 20 mg, CLR 500 mg, and AMX 1,000 mg for 14 days) was associated with punctual mutations of the 23S rRNA gene in CLR-resistant *H. pylori* (17). While none of the 59 *H. pylori* isolates from our study had point mutations in the 23S rRNA gene associated with CLR resistance, several of these isolates had identical 23S rRNA mutations found in the CLR-susceptible strains isolated by Matta et al. (17).

TET, LEV, and MTZ drug-resistant phenotypes and genotypes were detected in the 59 *H. pylori* strains isolated from the low- and high-risk Colombian populations in our study. Only a single isolate exhibited TET resistance, which was confirmed by a mutation in its 16S rRNA gene at position A926, a putative drug-binding site that has been previously associated with drug resistance in *H. pylori* (64). A recent systematic review and meta-analysis reported that TET resistance is present in ≤ 10 to 14% of *H. pylori* isolates worldwide (65). In this meta-analysis, no studies evaluated TET resistance in Colombia; however, one study conducted in Lima, Peru found that $\sim 4\%$ of *H. pylori* isolates exhibited TET resistance (65). A systematic review of Latin American countries, including Colombia, noted the prevalence of TET resistance was 6% (2 to 14%) (63).

In our study, the prevalence of resistance for LEV was 20.3% (12/59 isolates). While 9/30 (30%) *H. pylori* isolates from the low-risk gastric cancer versus 3/29 (11%) in the high-risk group were resistant to LEV, there was no statistical association between risk group and drug resistance. However, there was a significant association between drug resistance phenotype and the presence of mutations at N87 and/or D91 within the quinolone resistance-determining region (QRDR) of the *gyrA* gene among all 59 isolates. In agreement with our study, mutations at N87 and D91 have been previously reported to confer high- and low-level fluoroquinolone resistance, respectively (47, 48). Worldwide, more than 15% of *H. pylori* isolates exhibit LEV resistance (65). Prevalence of LEV resistance ranges from 9 to 36% in South American countries (only data from Argentina, Brazil, and Peru were included in the meta-analysis) (65). In all Latin American countries, the reported prevalence of LEV or ciprofloxacin resistance was estimated to be 15% (6 to 28%) (63).

Nearly all isolates in this study exhibited MTZ resistance. These resistant isolates often contained truncated *rdxA* and/or *frxA* genes encoding multiple nonsynonymous point mutations implicated with MTZ resistance in previous studies (54–56). Experimentally, it has been shown that MTZ resistance in isolates coharboring both truncated *rdxA* and *frxA* genes compared to strains with a single truncated gene alone had higher levels of resistance (66). Of the 59 strains in this study, 20 were resistant to MTZ and contained truncated *rdxA* and *frxA* genes. In Peru and Brazil, the prevalence of MTZ-resistant *H. pylori* ranged from 40 to 62% (65). A study of Colombians from Pasto, Nariño published in 2001 noted that about 50% of the population had *H. pylori* strains resistant to MTZ (67). In our study, nearly all *H. pylori* isolates from Tumaco and Túquerres Colombian populations were resistant to MTZ, suggesting uncontrolled, over-the-counter use of this drug may in large part be responsible for MTZ resistance. The prevalence of MTZ resistance in Tumaco and Túquerres, Colombia found in our study was substantially higher than the 53% (46 to 60%) estimated across Latin America (63).

H. pylori strains evaluated by WGS indicated the isolates from Tumaco and Túquerres originated from different phylogeographic locations. Consistent with the study by Sablet et al. (58), the LGCR isolates were of either European or African origin, while the HGCR isolates were more related to the European phylogeographic origin. Likewise, phylogenetic analysis of core gene sequences indicated that *H. pylori* isolated from the LGCR and HGCR patients were in general distinct from each other. Interestingly, antibiotic phenotypes did not appear to correlate with phylogenetic origin. However, *rdxA* mutations

previously found to be enriched in *H. pylori* isolates of the European or African phylogeographic origin (57) were frequently present in the strains from our study (Table S4). While large chromosomal rearrangements were apparent among the genomes, including in the regions harboring *gyrAB*, *frxA*, and *rdxA*, antibiotic resistance likely emerged due to the selective pressures of drug use by patients during attempts to eradicate *H. pylori* or during antibiotic treatment for other infections.

Interestingly, MTZ resistance was the most frequently observed resistance phenotype and was present at a similar rate, although statistically different, between the LGCR and HCGR populations (31/31 versus 24/28 strains). Resistance to other antibiotics evaluated in our study was not detected or not significantly different between these groups. As mentioned previously, over-the-counter access and use of antibiotics may have driven the selection of antibiotic resistance in *H. pylori* in these Colombian populations, irrespective of their risk for gastric cancer. This surprising finding reinforces the important clinical ramifications of determining community level information regarding *H. pylori* resistance rates, specifically regarding the selection of personalized first-line eradication therapies versus nonspecific empirical regimens. The primary determinant with respect to selection of triple therapies versus quadruple therapies is the local resistance rate of *H. pylori* to CLR. Further, rates of resistance to other antibiotics may influence the selection of bismuth-based quadruple therapy (PPI, bismuth, MTZ, TET) versus non-bismuth-based quadruple therapy (PPI, AMX, CLR, MTZ). Knowledge regarding rates of resistance to antibiotics commonly used in eradication regimens may therefore represent an inflection point in optimizing the care of *H. pylori*-infected persons.

In conclusion, our study found *H. pylori* strains isolated from Colombians with low and high risk for gastric cancer exhibited multidrug antibiotic resistance. Furthermore, we found antibiotic resistance phenotypes were associated with mutations in genes previously implicated in *H. pylori* drug resistance. Thorough detection and characterization of antibiotic resistance in *H. pylori* strains from Colombia is necessary in order to provide efficacious treatment regimens that will eradicate infection in high-risk patients. Thus, the findings from this study indicate that continued monitoring of antibiotic resistance using susceptibility assays and genotyping is warranted in Colombia due to the high occurrence of *H. pylori* infection and gastric cancer in this country.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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