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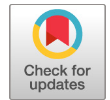

Phenotypic and Molecular Antimicrobial Susceptibility of *Helicobacter pylori*

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ABSTRACT Failure to eradicate *Helicobacter pylori* infection is often a result of antimicrobial resistance, which for clarithromycin is typically mediated by specific point mutations in the 23S rRNA gene. The purpose of this study was to define current patterns of antimicrobial susceptibility in *H. pylori* isolates derived primarily from the United States and to survey them for the presence of point mutations in the 23S rRNA gene and assess the ability of these mutations to predict phenotypic clarithromycin susceptibility. Antimicrobial susceptibility testing was performed using agar dilution on 413 *H. pylori* isolates submitted to Mayo Medical Laboratories for susceptibility testing. For a subset of these isolates, a 150-bp segment of the 23S rRNA gene was sequenced. A total of 1,970 MICs were reported over the 4-year study period. The rate of clarithromycin resistance was high (70.4%), and elevated MICs were frequently observed for metronidazole (82.4% of isolates had an MIC of >8 μg/ml) and ciprofloxacin (53.5% of isolates had an MIC of >1 μg/ml). A total of 111 archived *H. pylori* isolates underwent 23S rRNA gene sequencing; we found 95% concordance between genotypes and phenotypes ($P = 0.9802$). Resistance to clarithromycin was most commonly due to an A2143G mutation (82%), followed by A2142G (14%) and A2142C (4%) mutations. Clinical *H. pylori* isolates derived primarily from the United States demonstrated a high rate of clarithromycin resistance and elevated metronidazole and ciprofloxacin MICs. The relative distribution of point mutations at positions 2143 and 2142 in the 23S rRNA gene in clarithromycin-resistant *H. pylori* was similar to that reported from other parts of the world; these mutations predict phenotypic resistance to clarithromycin.

KEYWORDS 23S rRNA, *Helicobacter pylori*, antibiotic resistance, macrolide resistance

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The most commonly prescribed initial treatment regimen for *Helicobacter pylori* infection in the United States has been a three-drug regimen that includes clarithromycin, a proton pump inhibitor (PPI), and amoxicillin or metronidazole for 14 days. This regimen is now only recommended in regions where *H. pylori* clarithromycin resistance is known to be $<$15% and in patients with no history of prior macrolide exposure (1). Patients with an allergy to penicillin or who have previous macrolide exposure may be prescribed a nitroimidazole, tetracycline, bismuth, and a PPI for 10 to 14 days (1). Other treatment regimens include sequential therapy (5 to 7 days of amoxicillin and a PPI followed by 5 to 7 days of clarithromycin, a nitroimidazole, and a PPI), concomitant therapy (clarithromycin, a nitroimidazole, amoxicillin, and a PPI for 10 to 14 days), hybrid therapy (7 days of amoxicillin and a PPI followed by 7 days of clarithromycin, a nitroimidazole, amoxicillin, and a PPI), levofloxacin triple therapy (amoxicillin, levofloxacin, and a PPI for 10 to 14 days), and fluoroquinolone sequential therapy (5 to 7 days of amoxicillin and a PPI followed by 5 to 7 days of a fluoroquinolone, a nitroimidazole, and a PPI (1, 2). Because treatment failures are often a result of

antimicrobial resistance, patients with refractory *H. pylori* infection may require endoscopy of their upper gastrointestinal tract so that gastric mucosal biopsy specimens can be obtained for culture and susceptibility testing. For *H. pylori* susceptibility testing, the Clinical and Laboratory Standards Institute (CLSI) guidelines recommend agar dilution with Mueller-Hinton agar containing aged sheep blood and incubation at 35°C for 72 h in a microaerophilic (5% O_2, 10% CO_2, and 85% N_2) atmosphere, but these guidelines provide interpretive criteria only for clarithromycin (3).

Antimicrobial resistance in *H. pylori* has been increasing worldwide, including in the United States (2). Resistance to clarithromycin has been reported in several countries to be caused by specific point mutations in the 23S rRNA gene, particularly at positions 2143 and 2142 of domain V. Studies that have evaluated the presence of these mutations in clinical isolates from the United States in comparison to susceptibility determined using the gold standard agar dilution method are lacking (1). Our laboratory routinely performs testing on *H. pylori* isolates for susceptibility to clarithromycin, amoxicillin, metronidazole, tetracycline, and ciprofloxacin. The purpose of this study was to define current patterns of antimicrobial resistance in *H. pylori* isolates from the United States, assess for the presence of point mutations in the 23S rRNA gene, and determine the ability of point mutations in the 23S rRNA gene to predict phenotypic clarithromycin susceptibility.

RESULTS

A total of 1,970 MICs were reported over the 4-year period from which susceptibility results were retrieved (Table 1). Of these isolates, 70.4% ($n = 290$) were interpreted as resistant to clarithromycin, 1.9% ($n = 8$) as intermediate, and 27.7% ($n = 114$) as susceptible. A wide distribution of MICs (in μg/ml) against metronidazole was found, i.e., $\leq$8 (17.6%, $n = 58$), 16 (7.3%, $n = 24$), 32 (20.0%, $n = 66$), 64 (35.2%, $n = 116$), 128 (19.1%, $n = 63$), 256 (0.6%, $n = 2$), and $>$256 (0.3%, $n = 1$). We found 330 instances where both clarithromycin and metronidazole were tested on the same isolate. Of these isolates, 60.6% ($n = 200$) were resistant to clarithromycin and had a metronidazole MIC of $>$8 μg/ml. The most frequent MICs for the other antibiotics were $\leq$2 μg/ml (98.8%, $n = 405$) for amoxicillin, $\leq$0.06 μg/ml (38.1%, $n = 156$) and 1 μg/ml (60.1%, $n = 246$) for tetracycline, and $\leq$1 μg/ml (46.5%, $n = 190$) and $>$2 μg/ml (51.6%, $n = 211$) for ciprofloxacin.

A total of 118 archived *H. pylori* isolates were submitted for 23S rRNA gene sequencing. Seven isolates yielded insufficient *H. pylori* DNA for sequencing or did not have available phenotypic clarithromycin susceptibility results and were therefore excluded from further analysis. For the remaining 111 isolates, there was concordance between phenotypic susceptibility testing and genotypic results in 106 (95%) isolates ($P = 0.9802$). Of these isolates, 20% ($n = 21$) were susceptible and 80% ($n = 85$) were resistant to clarithromycin. An A2143G mutation was identified in 82% ($n = 70$) of the resistant isolates, an A2142G mutation in 14% ($n = 12$), and an A2142C mutation in 4% ($n = 3$). There was discordance between antimicrobial susceptibility testing and genotype results in 5% ($n = 5$) of the 111 isolates; 3 had a resistant phenotype but a wild-type sequence, and 2 had a susceptible phenotype but an A2143G mutation. The isolates corresponding to these discordant results were initially successfully cultured but failed to grow in subsequent subcultures performed for repeat or additional testing.

DISCUSSION

Clinical *H. pylori* isolates submitted to our reference laboratory (Mayo Medical Laboratories) for antimicrobial susceptibility testing between 2011 and 2016 demonstrated a high rate of clarithromycin resistance (70.4%) and a high rate of elevated metronidazole MICs (82.4% of isolates had an MIC of $>$8 μg/ml) compared to those reported from two previously published studies (4, 5). In addition, elevated MICs were observed for ciprofloxacin (53.5% of isolates had an MIC of $>$1 μg/ml). In contrast, most isolates had a low amoxicillin MIC (98.8% were $\leq$2 μg/ml) and a low tetracycline MIC

TABLE 1 Antimicrobial susceptibility test results for *Helicobacter pylori* isolates ($n = 413$)

Antibiotic	No. (%) of isolates with an MIC (μg/ml) of:																			Total no. of isolates
	≤0.06	≤0.25	0.5	>0.5	≤1	1	≤2	2	>2	4	≤8	8	>8	16	32	64	128	256	>256	
Amoxicillin							405 (98.8)			1 (0.2)		2 (0.5)	2 (0.5)							410
Ciprofloxacin					190 (46.5)			8 (2.0)	211 (51.6)											409
Clarithromycin[a]		114 (27.7) (S)	8 (1.9) (I)	290 (70.4) (R)																412
Metronidazole											58 (17.6)			24 (7.3)	66 (20.0)	116 (35.2)	63 (19.1)	2 (0.6)	1 (0.3)	330
Tetracycline	156 (38.1)					246 (60.1)		3 (0.7)	4 (1.0)											409

[a]Clinical and Laboratory Standards Institute interpretive criteria exist only for clarithromycin; S, susceptible; I, intermediate; R, resistant.

(98.3% were ≤1 μg/ml). A majority (60.6%) of the isolates were resistant to clarithromycin and had a metronidazole MIC of >8 μg/ml. These findings differ from those in previous reports. In an evaluation of 17 U.S.-based antimicrobial treatment trials between 1993 and 1999, clarithromycin and metronidazole susceptibility testing was performed on initial-visit *H. pylori* isolates obtained from gastric antrum, and occasionally corpus, biopsy specimens via Etest and/or agar dilution (4). MICs of >1 and >8 μg/ml were interpreted as resistant to clarithromycin and metronidazole, respectively. A total of 3,439 *H. pylori* isolates were tested by Etest, and 3,193 were tested by the agar dilution method. Over the 6-year study period, clarithromycin resistance was at its lowest level in 1993 (6.1%), gradually increased to its peak in 1997 (14.5%), and then declined in 1998 (11.1%) and 1999 (9.0%). The overall resistance rates were 10.6% (versus our study rate of 70.4%; $P < 0.0001$) for clarithromycin and 35% for metronidazole (versus our rate of 82.4% with an MIC of >8 μg/ml; $P < 0.0001$). More recently, Shiota et al. performed cultures on *H. pylori* samples from 656 randomly selected patients at the Houston Veterans Affairs Medical Center between 2009 and 2013 (5). *H. pylori* isolates were recovered from 135 gastric antral biopsy specimens. MICs were determined via Etest for clarithromycin, metronidazole, amoxicillin, tetracycline, and levofloxacin; EUCAST breakpoints were applied, and isolates with MICs of >0.5, >8, >0.12, >1, and >1 μg/ml, respectively, were interpreted as resistant (6). Of the patients with *H. pylori* infection, 110 were treatment naive; 54.5% of *H. pylori* isolates from these patients were susceptible to all tested antimicrobials, whereas 14.5% of the isolates were resistant to clarithromycin (versus our rate of 70.4%; $P < 0.0001$), 17.3% to metronidazole (versus our rate of 82.4% with an MIC of >8 μg/ml; $P < 0.0001$), 0% to amoxicillin, 0.8% to tetracycline (versus our rate of 1.71% with an MIC of >1 μg/ml; $P = 0.0385$), and 29.1% to levofloxacin (versus our rate of 53.6% with an MIC of >1 μg/ml for ciprofloxacin; $P < 0.0001$). Dual resistance to clarithromycin and metronidazole was identified in 1.8% of the isolates. When isolates were grouped into those from 2009 to 2010 ($n = 66$) and those from 2011 to 2013 ($n = 62$), there was an increase in clarithromycin resistance, from 9.1% in the earlier period to 24.2% in the later period ($P = 0.02$). There was no significant change in metronidazole resistance between the two time frames.

Of the 111 *H. pylori* isolates for which both phenotypic antimicrobial susceptibility and 23S rRNA gene sequencing data were available, there was a high degree (95.5%) of concordance between the genotypes and phenotypes. Of the 106 concordant isolates, 66.0% had an A2143G point mutation, 11.3% an A2142G point mutation, and 2.8% an A2142C point mutation. In a review by Megraud, mutation prevalences in countries excluding the United States had a distribution of mutations similar to ours (7). The combined prevalence rates, derived from 196 isolates, were 69.8% for the A2143G mutation (versus our rate of 66.0%; $P = 0.3989$), 11.7% for the A2142G mutation (versus our rate of 11.3%; $P = 0.9033$), and 2.6% for the A2142C mutation (versus our rate of 2.8%; $P = 0.8816$). More recently, Mitui et al. reported on *H. pylori* 23S rRNA sequencing performed on formalin-fixed paraffin-embedded gastric biopsy specimens obtained at the Children's Medical Center in Dallas, TX (8). Nineteen of 38 biopsy specimens yielded a 23S rRNA gene mutation (A2143G mutation in 89% [$n = 17$], A2142G mutation in 11% [$n = 2$]).

This study has several important limitations. First, the levels of resistance in our *H. pylori* isolates are likely inflated due to selection bias; many specimens submitted to our laboratory were likely from patients who had already failed an initial course of therapy. Therefore, the rates of resistance reported herein should be used only as a reference for expected susceptibility for *H. pylori* isolated from patients for whom gastric biopsy is indicated rather than as an estimate of the baseline prevalence of resistance in *H. pylori* in untreated populations. Unfortunately, not every antimicrobial agent was tested against every isolate. Also, although a majority of our laboratory specimens come from U.S. clients, we do receive specimens from international clients. It is possible that some of the isolates were from outside the United States, but the exact origin of each of the isolates studied is unknown. Based on our experience with *H. pylori* testing, however, we

expect that almost all, if not all, isolates were from the United States. Finally, sequencing was performed on only a 150-bp fragment of amplified DNA surrounding the 2143 and 2142 positions of the 23S rRNA gene. We cannot exclude mutations, such as A2223G and C2694A, that may have occurred elsewhere in the gene or in a different gene. Notably, the A2115G, G2141A, C2147G, T2190C, and C2195T mutations were not identified in any of our sequences, and novel mutations were not identified in the region sequenced. We did not seek other mechanisms of resistance, such as efflux pumps.

In summary, clinical *H. pylori* isolates submitted for susceptibility testing, expected to be primarily from the United States, had a high rate of clarithromycin resistance and elevated metronidazole and ciprofloxacin MICs while having relatively low amoxicillin and tetracycline MICs. The presence of 23S rRNA gene point mutations predicts phenotypic resistance to clarithromycin, which was most commonly conferred by the A2143G mutation, followed by the A2142G and A2142C mutations. The relative distribution of each mutation reported herein is similar to that reported from other parts of the world.

MATERIALS AND METHODS

Antimicrobial Susceptibility Testing. Susceptibility results from 413 clinical *H. pylori* isolates tested between November 2011 and January 2016 at Mayo Medical Laboratories in Rochester, MN, were retrospectively retrieved from the Mayo Clinic clinical microbiology electronic database. Susceptibility testing was performed using the agar dilution method following CLSI guidelines (2). MICs for clarithromycin, amoxicillin, metronidazole, tetracycline, and/or ciprofloxacin, either as a panel or individually, were determined per clinical request. Clarithromycin MICs were interpreted based on CLSI breakpoints ($\leq$0.25 μg/ml, susceptible; 0.5 μg/ml; intermediate; $\geq$1.0 μg/ml, resistant); the other four agents do not have established CLSI interpretive criteria (3).

23S rRNA Gene Sequencing. A subset of the isolates that underwent susceptibility testing had been archived in the freezer from July 2014 to January 2016. These isolates were thawed, cultured on Columbia agar (Hardy Diagnostics, Santa Maria, CA), and incubated under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) at 35°C for 3 to 5 days until sufficient growth occurred. A loopful of bacterial colonies was lysed following a previously described rapid DNA preparation method (9). Five microliters of lysed sample was added to a 96-well plate containing 45 μl of master mix. PCR master mix was created by combining 45 μl of 1.1× Platinum PCR SuperMix (Invitrogen, Carlsbad, CA) with 0.5 μl each of 0.5 μM forward (5′-GAGCTGTCTCAACCAGAGATTC-3′) and 0.5 μM reverse (5′-CTTCAAAGCCTCCCACCTATC-3′) primers (per sample volumes) targeting a segment of domain V within the 23S rRNA gene. PCR was performed using a Veriti 96-well thermal cycler (firmware version 1.3.0; Applied Biosystems, Foster City, CA) with an initial 2-min denaturation step at 95°C followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 1 min, and extension at 72°C for 2 min. A final extension of 2 min at 72°C was provided prior to conclusion of the cycling program. The resultant amplified DNA fragment was verified by running it in 2.2% agarose using the FlashGel system (Lonza, Basel, Switzerland) and confirming the presence of a 150-bp band.

Prior to Sanger sequencing, 5 μl of amplified DNA was combined with 2 μl of ExoSAP-IT (Affymetrix, Santa Clara, CA) and added to each well of a new 96-well PCR plate, and the plate was placed into the Veriti 96-well thermal cycler for an initial 15 min at 37°C, followed by 15 min at 80°C. One microliter of the stock (50 μM) forward and reverse primers diluted 1:10 was added to each well. The plate was then sent to the Mayo Clinic Genomics Core Facility for bidirectional sequencing using dye terminator technology via the ABI 3130xl genetic analyzer (Applied Biosystems). Sequence files were analyzed with CLC Genomics Workbench, version 9 (Qiagen, Valencia, CA), and the presence or absence of mutations at the 2143 and/or 2142 position was recorded. Testing was performed in a blinded fashion, and only once it was completed were results compared to those of phenotypic susceptibility testing.

Statistical Methods. The proportions of drug resistance found in this study were compared with proportions cited in the literature using a test of single proportion. A comparison of agar dilution versus sequencing results was performed using McNemar's test of paired proportions. Analysis was performed using SAS 9.4 (SAS, Inc., Cary, NC).

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