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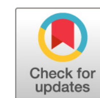

Clinical Evaluation of a Real-Time PCR Assay for Simultaneous Detection of *Helicobacter pylori* and Genotypic Markers of Clarithromycin Resistance Directly from Stool

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ABSTRACT *Helicobacter pylori* infection is mainly diagnosed noninvasively, with susceptibility testing traditionally requiring endoscopy. Treatment is empirical, with clarithromycin-based triple therapy recommended where resistance rates are below 15%. Rising rates of clarithromycin resistance, resulting in high clarithromycin-based therapy failure rates, are seen worldwide, but U.S. data are limited. We developed a real-time PCR assay for simultaneous detection of *H. pylori* and genotypic markers of clarithromycin resistance directly from stool specimens. The assay was validated by testing 524 stool samples using an *H. pylori* stool antigen test as the reference method for detection accuracy and Sanger sequencing to confirm genotypic susceptibility results. A separate set of 223 antigen-positive stool samples was tested and retrospective medical record review conducted to define clinical utility. PCR resulted in 88.6% and 92.8% sensitivity in the validation and clinical study sets, respectively. Sequencing confirmed correct detection of clarithromycin resistance-associated mutations in all positive validation samples. The PCR-predicted clarithromycin resistance rate was 39% in the clinical data set overall and 31% in treatment-naive patients; the clarithromycin-based triple therapy eradication rate in treatment-naive patients was 62%. The clarithromycin-based triple therapy success was lower when resistance was predicted by PCR (41%) than when no resistance was predicted (70%; $P = 0.03$). PCR results were positive in 98% of antigen-positive stools from patients tested for eradication. The described PCR assay can accurately and noninvasively diagnose *H. pylori*, provide genotypic susceptibility, and test for eradication. Our findings support the need for susceptibility-guided therapy in our region if a clarithromycin-based regimen is considered.

KEYWORDS *H. pylori*

Helicobacter pylori has been identified as a cause of chronic gastritis and peptic ulcer disease since 1984 and was recognized in 1994 as a human carcinogen by the World Health Organization International Agency for Research on Cancer (1, 2). Today, *H. pylori* infection is estimated to affect approximately 4.4 billion individuals globally, with an estimated pooled prevalence of 36% in the United States, and it is the most common infectious cause of cancer worldwide (3, 4). Infection is thought to be acquired during childhood and can persist for decades in the acidic gastric environment. A series of bacterial and host factors determine long-term effects of infection, including the risk of peptic ulcer disease, gastric adenocarcinoma, and mucosa-associated lymphoid

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tissue (MALT) lymphoma. Eradication of *H. pylori* by appropriate antibiotic therapy is recommended for all infected individuals and has been shown to decrease the risk of developing peptic ulcer disease and gastric cancer and to treat early-stage MALT lymphoma (5–8). Today, therapy is mostly empirical; guidelines from the American College of Gastroenterology (ACG) recommend clarithromycin-based triple therapy as a first-line treatment option in regions where clarithromycin resistance is known to be below 15% and in patients with no history of macrolide exposure (8). Data on regional clarithromycin resistance rates to guide selection of empirical therapy are limited, with some studies showing that resistance has been increasing in many countries over the past decade. In the United States, the current prevalence of clarithromycin resistance has been estimated to be close to 13%, up to 18% in some adult populations, and even higher in children (9–11). Clarithromycin resistance is most commonly caused by point mutations in the 23S rRNA (rRNA) gene, including A2143G, A2142G, and A2142C, which result in decreased macrolide binding to the 23S rRNA ribosomal subunit; clarithromycin resistance is considered the main cause of clarithromycin therapy failure (8, 9). Clarithromycin-based therapy eradication rates worldwide and in the United States are estimated to be close to or below 80% and are considered inadequate, which has raised a discussion around the need for susceptibility-guided rather than empirical therapy (8, 10, 12, 13).

H. pylori infection is typically diagnosed by highly sensitive and specific nonendoscopic methods such as the urea breath test or the stool antigen test, unless endoscopy-based testing is indicated. The same methods are recommended to test for proof of eradication after treatment (8). When therapy fails, a biopsy is required for culture and phenotypic susceptibility testing to guide further therapy, but culture is slow, not always successful, and not widely available (8, 14). Molecular assays for the detection of *H. pylori* and genotypic antibiotic resistance markers have been described, with many requiring endoscopically collected tissue and a few using noninvasively collected samples (15). To address the need for rapid, accurate, and noninvasive diagnosis and susceptibility testing, we describe a PCR assay for simultaneous detection of *H. pylori* and clarithromycin resistance prediction directly from stool samples. In addition to evaluating the assay's analytical and clinical performance characteristics, we examined its clinical utility, defining rates of clarithromycin resistance in our patient population along with therapy outcomes in relation to genotypically predicted resistance.

MATERIALS AND METHODS

Nucleic acid extraction. A pea-sized to marble-sized (~50- to 100-mg) portion of mixed fresh stool was transferred into a 5-ml vial containing 50% Stool Transport and Recovery (S.T.A.R.) buffer with 100 copies of lambda phage DNA (internal control). Fecal specimens in culture and sensitivity (C&S) stool medium vials (Cardinal Health, Dublin, OH) were sampled by swirling a sterile cotton swab in medium and transferring the swab into a 50% S.T.A.R. vial (STAR-IC). Vials were vortexed and particulate matter allowed to settle. STAR-IC vials were placed onto a Hamilton Microlab Star liquid handling device with a customized deck to perform DNA extraction using Mayo MicroLab Maxwell high-throughput (HT) fecal DNA purification kit chemistry (Promega, Madison, WI). Briefly, the liquid handler transfers a portion of STAR-IC sample and combines it with cetyltrimethylammonium bromide (CTAB) for preprocessing to enhance inhibitor removal (16). An aliquot of the preprocessed sample was carried through an automated solid-phase extraction method, yielding 100 μl of eluted material.

PCR assay design. A 108-bp region of the 23S rRNA gene in *H. pylori* NCTC 11637 (GenBank accession number LS483488.1) was selected as the target. An optimized primer-probe set (Table 1) was combined with LightCycler Master DNA HybProbe (Roche Life Science, Indianapolis, IN) mastermix containing 3 mM (total) $MgCl_2$ and 0.025 U of LightCycler uracil-DNA glycosylase (UNG; Roche Life Science). Complete master mix (25 μl) was combined with 25 μl of DNA extract on the Hamilton Star device in a LightCycler 480 white 96-well plate (Roche Life Science). The LightCycler 480 II instrument assay conditions were as follows: (i) UNG digestion and denaturation, 10 min at 40°C followed by 10 min at 95°C; (ii) PCR thermocycling (50 cycles, quantification analysis), 95°C for 10 s, 65°C for 15 s, and 72°C for 15 s for five cycles; 95°C for 10 s, 1°C/cycle annealing target temperature touchdown, and 72°C for 15s for five cycles, followed by 95°C for 10 s, 60°C for 15 s, and 72°C for 15 s for 40 cycles; (iii) melting curve analysis, denature at 95°C for 30 s, cool to 59°C and hold for 10 s, then cool to 40°C for 15 s, then increase temperature at a ramp rate of 0.19°C/s to 85°C with continuous florescent monitoring; (iv) cooldown, 40°C for 30 s. Real-time amplification detection was monitored with a double-quenched TexasRed-labeled TaqMan probe. Following amplification, a fluorescein-labeled SimpleProbe designed against an 18-mer region known to contain the three common single-nucleotide point mutations (SNPs) conferring clarithromycin resistance (A2143G, A2142G, and A2142C) was annealed, and a melting temperature (T_m)

TABLE 1 Primer and probe sequences

Sequence name	Sequence (5′–3′)[a]
Hpylori F2	GAGCTGTCTCAACCAGAGATT
Hpylori R3ex	GCAGTGCTAAGTTGTAGTAAAGGTC
Hpylori TM3	TEX-TGGAGGTGAAAXTTCCTCCTACCCG-phosphate
Hpylori SP2	GCAXIAGACGGAAAGACCCC-phosphate
Lambda F	ATGCCACGTAAGCGAAACA
Lambda R	GCATAAACGAAGCAGTCGAGT
Lambda TM	LC670-ACCTTACCGAAATCGGTACGGATACCGC-BBQ

[a]TEX, TexasRed fluorophore; LC670, LightCycler red 670 fluorophore; BBQ, BlackBerry quencher; X, internal BHQ2 (BlackHole quencher); XI, internal FAM (fluorescein) label.

peak was generated and used to distinguish wild-type and the clarithromycin resistance-associated SNPs (see Fig. S1 in the supplemental material). A signal generated solely by the SimpleProbe was not considered positive. The assay internal control was monitored with a LightCycler red 670 fluorophore (LC670)-labeled TaqMan probe.

Assay verification. (i) Cross-reactivity. The primer and probe sequences were searched in the NCBI BLAST database to determine any potential *in silico* cross-reactivity. Additionally, a specificity panel of quantified genomic human DNA and DNA from 73 microbes was assayed with mastermix to detect cross-reactivity *in vitro* (see Table S1 in the supplemental material).

(ii) Analytical sensitivity and limit of detection. Analytical sensitivity and linearity were assessed for each sequence type (wild type, A2143G, A2142G, and A2142C) with quantified plasmid diluted in extraction kit elution buffer. Six replicates of 9 10-fold serial dilutions, prepared in antigen- and PCR-negative pooled fecal samples, were assayed. Limit of detection (LoD) studies were carried out using 10-fold serial dilutions of quantified genomic DNA from *H. pylori* clinical isolates with wild-type, A2143G, A2142G, and A2142C sequence types spiked into clinical samples at defined concentrations. Each sample was extracted in triplicate and extracts were assayed in duplicate for a total of six replicates per dilution. Dilutions were carried through to detection extinction. The LoD was defined as the last dilution at which all six replicates were detected. These experiments were performed for freshly frozen feces and feces in C&S stool transport vials. These data were also used to provide a probit estimate of LoD.

(iii) Accuracy sample set. *(a) Fecal sample testing.* Accuracy was measured using a blinded set of residual fecal specimens from Mayo Clinic and Mayo Clinic Laboratories patients ($n = 535$) submitted for routine *H. pylori* stool antigen (HPSA) testing with the Premier Platinum HpSA Plus assay (Meridian Bioscience, Cincinnati, OH). Positive samples underwent bidirectional Sanger sequencing (GenScript, Piscataway, NJ) to assess the ability of the PCR assay to accurately detect the targeted SNPs associated with clarithromycin resistance. Raw sequence files were trimmed and assembled with CLC Genomics Workbench software version 19 (Qiagen, Germantown, MD). Contiguous sequences were aligned to the wild-type reference sequence (GenBank accession number LS483488.1) to distinguish genotypes.

(b) Isolate testing to verify prediction of clarithromycin susceptibility. In total, 111 archived DNA samples from *H. pylori* isolates that underwent agar dilution susceptibility testing, conventional PCR, and Sanger sequencing for a previous study (17) were tested with the new PCR assay.

(iv) Statistics. Statistical measurements, including probit estimate of LoD, sensitivity, specificity, positive/negative predictive values, simple kappa coefficient, and respective 95% confidence intervals (95% CI), were performed with SAS version 9.4 (Cary, NC).

Clinical utility study. In total, 223 freshly frozen fecal samples collected in 2015 to 2018 from 197 unique Mayo Clinic patients with a positive HPSA test result using the Premier Platinum HpSA Plus assay were archived at −80°C for this part of the study. Seventy-seven of the samples were placed into C&S medium prior to archiving. Samples underwent 3 freeze-thaw cycles prior to testing with the *H. pylori* PCR assay. Samples were collected from Mayo Clinic patients from clinical facilities located mainly in Minnesota and Wisconsin.

A retrospective review of the electronic medical records was conducted for each subject. Demographic information, history of documented prior macrolide use, and longitudinal data pertaining to diagnosis, treatment, follow-up testing, and treatment outcome of *H. pylori* infection were documented. Treatment outcome was defined as treatment success or failure when a test of eradication by any method was documented. A positive result was interpreted as treatment failure, and a negative result was interpreted as success. Cases were classified in one of two groups, the "initial diagnosis test" group and the "test of eradication" group. The initial diagnosis test group comprised mostly treatment-naive cases tested for diagnosis of *H. pylori*. Some cases of patients with a history of successfully treated *H. pylori* infection, as documented by testing history, or remote, treated infection presenting with a new positive diagnostic test presumed to be a new episode, were included in this group. The test of eradication group comprised positive HPSA samples from patients being tested for follow-up proof of eradication after receiving antibiotic therapy for *H. pylori*.

Percent agreement between HPSA and PCR results was determined for the cases in total and within each group. A subset of cases within the initial diagnosis group with documented test of eradication was defined. Within this subset, treatment outcome (failure versus success) of cases treated with clarithromycin-based triple therapy and other therapeutic combinations was recorded and analyzed in the

context of molecular resistance prediction. Within the test of eradication group, results of *H. pylori* detection and clarithromycin resistance marker detection by PCR were compared in cases treated with clarithromycin-based triple therapy and with other combinations. When evaluating therapy outcome related to clarithromycin resistance prediction by PCR, clarithromycin-based triple therapy was strictly defined as the combination of a proton pump inhibitor (PPI), clarithromycin, and amoxicillin. Other first-line and salvage antibiotic combinations, some containing clarithromycin, were classified as "other combination therapy."

(i) Statistics. Statistical analysis was performed using Fisher's exact test.

(ii) Ethics statement. Collection of the samples and the associated clinical history review were approved by the Mayo Clinic Institutional Review Board (IRB no. 16-002892).

Vendor notification. Samples collected for this study were tested with the HPSA assay between 2015 and 2018. In June 2017, Meridian Bioscience notified customers of a minor manufacturing change to the Premier Platinum HpSA Plus assay that could potentially result in higher optical density readings for weak or moderate positive tests. It is unclear if the manufacturing change had any effect on positivity rates and test performance.

RESULTS

PCR assay verification. (i) Cross-reactivity. The primers demonstrated some homology to zoonotic/rarely encountered *Helicobacter* species. The region selected for the probe sequences is highly conserved across many *Epsilonproteobacteria*. *In vitro* testing of available *Helicobacter* species and other members of the *Epsilonproteobacteria* demonstrated some cross-reactivity with non-*pylori Helicobacter* species, albeit at reduced sensitivity (see Table S1 in the supplemental material). Nonspecific and amplification-free hybridization of the SimpleProbe was not noted in the presence of high concentrations of homologous template DNA (Table S1).

(ii) Analytical sensitivity and LoD. Analytical sensitivity of the PCR assay was less than a single copy of the target for all genotypes targeted. The linear range of the assay ranged from 10^6 to 10^{-1} target copies/μl. The LoD in freshly frozen feces was 24 genomes/μl (wild type), 35 genomes/μl (A2143G), 40 genomes/μl (A2142G), and 35 genomes/μl (A2142C). For feces in C&S medium, LoD values were 24 genomes/μl (wild type), 35 genomes/μl (A2143G), 40 genomes/μl (A2142G), and 3.5 genomes/μl (A2142C).

(iii) Accuracy. *(a) Fecal samples.* Eleven samples were excluded from the accuracy set due to inhibition (rate of 2%), bringing the final accuracy sample count to 524, of which there were 101 true-positive, 398 true-negative, 13 false-negative, and 12 false-positive sample results. Inhibition was defined as a lack of detection of the λ-phage internal control in an *H. pylori* target-negative sample. The overall sensitivity and specificity were 89% (95% CI, 81 to 94%) and 97% (95% CI, 95 to 99%), respectively. Positive and negative predictive values were 89% (95% CI, 82 to 94) and 97% (95% CI, 95 to 98%), respectively. The simple kappa coefficient value was 0.86 (95% CI, 0.81 to 0.91), classifying the assay's performance as strong to almost perfect (18, 19).

Among the 113 PCR-positive samples, 77 (68%) were characterized by melt curve analysis as having a wild-type genotype or clarithromycin-susceptible prediction and 36 (32%) as possessing one of the three aforementioned SNPs or clarithromycin resistance prediction. Bidirectional Sanger sequencing analysis demonstrated 100% agreement. Of note, four of the samples with SNP T_m values showed dual T_m, peaks, suggesting a possible mixed population of clarithromycin-susceptible and -resistant genotypes, which has been previously observed (20, 21). Sanger sequencing trace files in 2/4 samples showed dual base signal peaks for A and G at positions 2142 and 2143. Only the G mutant was detected at position 2143 in the remaining two files.

(b) Isolate collection. The assay matched phenotypic susceptibility testing in 106/111 isolates (96% categorical agreement), with a major error rate of 9% (2/23) and very major error rate of 3% (3/88) compared to phenotypic susceptibility testing by agar dilution. Real-time PCR results perfectly matched those of a conventional PCR assay/Sanger sequencing for all isolates, including the 5 phenotypically mismatched isolates.

Clinical utility study. A total of 223 fecal samples belonging to 197 unique Mayo Clinic patients previously positive by HPSA testing were tested by the PCR assay. Patient ages ranged from 3 to 91 years, with most being over 18 years of age; 139 were female and 84 were male (see Table S2 in the supplemental material). Clarithromycin-based triple therapy was the most frequently used initial therapy combination after *H.*

TABLE 2 Clinical study *Helicobacter pylori* stool antigen and PCR assay agreement

Assay results	No. (%) of cases:		
	Total ($n = 223$)	Initial diagnosis group ($n = 169$)	Test of eradication group ($n = 54$)
HPSA$^+$/PCR$^+$	207 (92.8)	154 (91.1)	53 (98.1)
HPSA$^+$/PCR$^-$	16 (7.2)	15 (8.9)	1 (1.9)

pylori diagnosis (67%), followed by bismuth quadruple therapy (18%) and other combinations (10%). Other combinations included clarithromycin in 14 cases. There was no documented antibiotic therapy in 11 cases.

In the clinical study data set, the PCR assay detected *H. pylori* in 207 of 223 (93%) of samples previously positive by the HPSA test. A total of 16 cases (7%) that were previously positive by the HPSA test were negative by PCR. Of these, 15 belonged to the initial diagnosis test group, and one to the test of eradication group (Table 2). Clarithromycin resistance-associated SNPs were detected in 81 of 207 (39%) positive samples. Within the initial diagnosis test group, mostly treatment-naive cases, clarithromycin resistance was detected in 47 of 154 (31%) PCR-positive cases.

In total, 169 cases were assigned to the initial diagnosis test group, and 54 cases to the test of eradication group. Within the initial diagnosis test group, 92 of 169 (54%) cases had a documented test of eradication following therapy that allowed evaluation of treatment outcome. HPSA test and urea breath test were commonly used to test eradication. Of these 92 cases, 29 (32%) had a positive follow-up posttherapy test, indicating therapy failure, and 63 (69%) had a negative follow-up test, indicating therapy success. The initial therapy success rate was 13/27 (48%) when resistance was predicted by PCR versus 43/58 (74%) when no resistance was predicted ($P = 0.02$). Of this subset of 92 cases, 69 were initially treated with clarithromycin-based triple therapy, and 23 were treated with other combination therapies. Other combination therapies included 20 cases treated with bismuth quadruple therapy, one clarithromycin-containing regimen in a combination other than defined triple therapy, and two additional regimens not clearly defined. Of the 69 initially treated with clarithromycin, 26 failed initial therapy, and 43 had a successful outcome, resulting in a clarithromycin-based triple therapy success or eradication rate of 62%. The PCR assay detected clarithromycin resistance in 13/26 (50%) of failed therapy cases initially treated with clarithromycin-based triple therapy. The clarithromycin-based triple therapy success rate in this group was 9/22 (41%) when resistance was predicted by the PCR assay versus 30/43 (70%) when no resistance was predicted ($P = 0.03$) (Table 3). The *H. pylori* eradication rate was 9/22 (41%) when clarithromycin resistance was detected for cases treated with clarithromycin-based triple therapy versus 4/5 (80%) when clarithromycin resistance was detected for cases treated with other combination therapy ($P = 0.16$). The eradication rate when clarithromycin resistance was not detected was 30/43 (70%) for cases treated with clarithromycin-based triple therapy versus 13/15 (87%) for cases treated with other combination therapy ($P = 0.31$).

In the test of eradication group, 35 of 54 cases had been previously treated with clarithromycin-based triple therapy prior to testing positive for *H. pylori* by the HPSA test. The PCR assay detected clarithromycin resistance in 24 of 35 (69%) and did not detect resistance in 11 of 35 (31%) cases that failed clarithromycin-based triple therapy. The PCR assay detected clarithromycin resistance in 10 of 19 cases previously treated with other combination therapies (Table 4). Within this subgroup, other combination therapies included 5 cases treated with bismuth quadruple therapy, 9 clarithromycin-containing regimens in a combination other than defined triple therapy, and five regimens containing other combinations.

DISCUSSION

H. pylori infection is highly prevalent worldwide, and eradication is necessary to prevent associated complications. High rates of clarithromycin resistance contribute to

TABLE 3 Clinical study initial diagnosis test group treatment outcome and *Helicobacter pylori* PCR assay results

Clarithromycin resistance results	No. of cases:					
	Total ($n = 92$)		Received clarithromycin-based triple therapy ($n = 69$)		Received other combination therapy ($n = 23$)	
	Failure ($n = 29$)	Success ($n = 63$)	Failure ($n = 26$)	Success ($n = 43$)	Failure ($n = 3$)	Success ($n = 20$)
PCR$^+$/resistance detected	14	13	13	9	1	4
PCR$^+$/resistance not detected	15	43	13	30	2	13
PCR negative	0	7	0	4	0	3

observed low rates of eradication in current times. Prior studies have demonstrated a significant and strong association between clarithromycin resistance and clarithromycin-based treatment failure, with resistance reducing therapy success by over 50% (10, 13, 22–24). Empirical therapy with a clarithromycin-based regimen is currently recommended when the local resistance rate is documented to be below 15%, but regional data on resistance rates are limited (8). PCR-based assays have been described in the literature for detection of *H. pylori* and clarithromycin resistance markers directly from tissue samples, bypassing the need for culture but still requiring invasive collection methods (25–28). PCR-based assays that can detect *H. pylori* and genotypic clarithromycin resistance markers directly from stool specimens have been described and evaluated, mostly in European countries, with various performance characteristics in adults and children (29–39). Next-generation sequencing has been applied for the same purpose from formalin-fixed paraffin-embedded tissue and isolates, but not, to our knowledge, from stool (40, 41).

The described real-time PCR assay targets a 108-bp region of the 23S rRNA gene in *H. pylori* and can detect within the amplified product three common SNPs associated with clarithromycin resistance that can be distinguished from the wild type by T_m analysis. A PCR assay that can be used directly from stool samples is ideal for patients who do not meet criteria for endoscopy, but it can be challenging due to small amounts of target DNA in the sample, the presence of inhibiting substances, and the potential presence of other *Helicobacter* species with sequence homology in the sample and requires specialized methods for extraction and careful design to optimize performance (13, 33). The described PCR assay uses a customized extraction kit that employs methods to enhance inhibitor removal and maximize DNA extraction from stool samples.

In our verification studies, the assay showed good analytical sensitivity (less than a single copy of the target) for detection of *H. pylori*. Although there was *in vitro* cross-reactivity with a few non-*pylori Helicobacter* species, most are unlikely to be encountered in human specimens. *Helicobacter heilmannii*, which can be clinically significant, showed cross-reactivity, but was detected with reduced sensitivity. The assay demonstrated a 95% result agreement with antigen testing upon testing of 524 stool specimens. Compared to the antigen test, the PCR assay resulted in a sensitivity of 89% (101/114) and a specificity of 97% (398/410) for detection of *H. pylori*. All 113 PCR-positive stool samples characterized by the PCR assay as wild type (68%) or predicted clarithromycin resistant (32%) were in agreement with results obtained by sequencing. These 113

TABLE 4 Clinical study test of eradication group treatment regimens and *Helicobacter pylori* PCR assay results

Clarithromycin resistance results	No. of cases:		
	Total ($n = 54$)	Previously treated with clarithromycin-based triple therapy ($n = 35$)	Previously treated with other combination therapy ($n = 19$)
PCR$^+$/resistance detected	34	24	10
PCR$^+$/resistance not detected	19	11	8
PCR negative	1	0	1

samples include 12 cases that were positive by PCR and negative by HPSA test, suggesting that these cases were, in fact, true positives missed by antigen testing. The sensitivity of the HPSA assay as evaluated in prior studies is reported to range from 90 to 97% (42–44). The 13 cases that were negative by PCR and positive by HPSA test were not tested by any additional method.

A 96% categorical agreement was observed with our assay's predicted clarithromycin resistance status (all confirmed by sequencing) compared to culture-based phenotypic susceptibility testing in 111 tested *H. pylori* isolates. These results support the ability of genotypic resistance markers to predict phenotype and are consistent with our prior study, which found 95% concordance between 23S rRNA sequencing and agar dilution in determining resistance status to clarithromycin using *H. pylori* isolates (17).

In addition to our validation set, we evaluated a separate stool sample set from Mayo Clinic patients, including stools that had been previously found to be *H. pylori* positive by the HPSA test. Electronic medical record review of these cases revealed that clarithromycin-based triple therapy is the most commonly used initial therapy in our population. The PCR assay showed an overall 93% (207/223) positive agreement with the HPSA assay for detection of *H. pylori*. Cases positive by HPSA testing and negative by PCR could represent a limitation of the specificity of the HPSA assay or, if truly missed by PCR, could be related to stool samples undergoing multiple freeze-thaw cycles and not all being stored in a stool preservative.

The overall clarithromycin resistance rate predicted by PCR in our *H. pylori*-positive cohort was 39% (81/207), and within the initial diagnostic test group, the clarithromycin resistance rate was 31% (47/154). In our validation data set, which included samples from our institution and institutions utilizing our reference laboratory practice, a 32% resistance rate was detected. Taken together, our assay verification data and our clinical study data reveal a clarithromycin resistance rate of 37% detected directly from *H. pylori* positive stool specimens, higher than that estimated in current literature for the United States and very close to rates reported by Beckman et al. in a similar study (29). A previous study performed in our institution by sequencing of isolates recovered from cultures of internal and reference laboratory tissue samples found a 70% clarithromycin resistance rate (17). These samples can be assumed to represent mostly cases of therapeutic failure requiring endoscopy for guided therapy. Thus, the higher clarithromycin resistance rate in this group is not unexpected.

In our initial diagnosis test group of mostly treatment-naive patients, a 62% (43/69) eradication rate with clarithromycin-based triple therapy was observed, consistent with prior studies showing eradication rates below 80%. The PCR assay detected clarithromycin resistance in 50% of cases that went on to fail clarithromycin-based triple therapy as evidenced by our longitudinal data. The therapy success rate with clarithromycin-based triple therapy was significantly lower when resistance was predicted (41%) than when no resistance was predicted (70%) ($P = 0.03$), demonstrating a correlation of genotypic resistance prediction and therapy failure. This is consistent with the study by Beckman et al., in which a 62% success rate was found in patients with clarithromycin resistance mutations, and a 93% success rate was found in wild-type cases (29). Resistance was detected by the PCR assay in 50% (13/26) of failed therapy cases and in 21% (9/43) of successful eradication cases treated with clarithromycin-based triple therapy. These findings are in line with a study by Nezami et al. where 23S rRNA mutations detected by next generation sequencing were present in 88% of failed therapy cases and in only 10% of successful eradication cases (40).

In the test of eradication group, our assay detected clarithromycin resistance in 69% of the cases with failed clarithromycin-based triple therapy, suggesting that some failures might have been preventable with baseline susceptibility testing to guide a non-clarithromycin-based treatment course. Our findings support prior assertions that clarithromycin-based regimens are no longer suitable for unconditional empirical therapy (45). Both the ACG guidelines and the European Maastricht V/Florence consensus report on the management of *H. pylori* infection recommend clarithromycin susceptibility

testing by phenotypic or molecular methods over empirical therapy when clarithromycin resistance is documented to be greater than 15% (8, 14). Our study demonstrates resistance rates higher than this cutoff, and our clinical data suggest that susceptibility-guided therapy would help address the low therapy success rates observed with empirical therapy alone. Other studies have demonstrated that susceptibility-guided therapy, including by genotypic methods, is superior to empirical first-line clarithromycin treatment to eradicate *H. pylori* (46–48). We note that a large number of patients were lost to follow-up after initial testing (only 54% of our initial cases had posttherapy outcome data); the possibility of providing susceptibility-guided therapy can help improve the likelihood of initial therapy success in these patients.

The described PCR assay can inform and guide treatment choices at the time of diagnosis by providing baseline clarithromycin resistance status in a rapid, accurate manner from noninvasively collected stool specimens. Based on our findings, this practice might help reduce therapeutic failure and reduce the subsequent need for invasive diagnostic procedures and additional therapy courses, which in turn can result in decreased compliance with treatment and follow-up. In addition, the high positive agreement observed between the PCR and HPSA assays in the test of eradication group (98%) indicates that our assay can be used as a test of cure in a similar way as HPSA testing. Although the clinical data set comprised only cases known to be positive by HPSA testing, the larger validation data set included HPSA-positive and -negative cases with 95% agreement between assays, further supporting utility as a test of eradication. Because of the ease of collection of stool samples, the possibility of at-home patient-collected specimens to test for *H. pylori* can be considered, which would help with patient compliance with follow-up testing to confirm eradication and prevent long-term complications. Potential false-negative urea breath and stool antigen tests as a result of proton pump inhibitor (PPI) use are described; we could not assess whether PPIs would have a similar effect on the PCR performance, as most of the cases evaluated here did not have documented PPI use at the time of testing.

It is important to note that our assay is able to detect SNPs associated with resistance to clarithromycin only, and it does not provide susceptibility information on other antimicrobial agents that can alternatively and/or additionally be used to treat *H. pylori*. It also only detects three clarithromycin-associated SNPs. The assay can be performed on *H. pylori* isolates, which can be helpful if susceptibility information is needed before phenotypic susceptibility can be obtained or if phenotypic susceptibility testing is not successful. A limitation of our study, as with many *H. pylori* diagnostic studies, is an imperfect reference standard. Here, we used stool antigen; in a perfect study, a composite reference standard that includes stomach biopsy sample, culture, and possibly breath tests in addition to stool antigen testing might be a consideration.

This is one of few U.S. studies to assess regional clarithromycin resistance rates, to evaluate therapy outcome based on genotypic clarithromycin resistance prediction, and to evaluate the performance of a PCR assay as a test of cure. Our study highlights the need for susceptibility-guided therapy and, in cases where empirical therapy is considered, demonstrates that clarithromycin-based regimens are not appropriate in our region. The described assay is, to our knowledge, the first to be developed and clinically implemented for molecular detection and susceptibility testing of *H. pylori* from stool specimens in the United States. At our institution, based on these findings, the testing algorithm for *H. pylori* lists the described PCR assay as the preferred assay when endoscopy is not required. In addition to its utility as a diagnostic tool, the assay can be used to gather information on regional clarithromycin resistance rates that can then be used to inform future patient management.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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