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Helicobacter pylori Mutations Detected by Next-Generation Sequencing in Formalin-Fixed, Paraffin-Embedded Gastric Biopsy Specimens Are Associated with Treatment Failure

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ABSTRACT *Helicobacter pylori* antibiotic resistance is widespread and increasing worldwide. Routine detection of *H. pylori* mutations that invoke antimicrobial resistance may be a useful approach to guide antimicrobial therapy and possibly avert treatment failure. In this study, formalin-fixed, paraffin-embedded (FFPE) gastric biopsy specimens from a cohort of individuals from northern Ohio in the United States were examined using a next-generation sequencing (NGS) assay to detect *H. pylori* mutations that are known to confer resistance to clarithromycin, levofloxacin, and tetracycline. From January 2016 to January 2017, 133 *H. pylori*-infected gastric biopsy specimens were identified histologically and subsequently analyzed by NGS to detect mutations in *gyrA*, 23S rRNA, and 16S rRNA genes. The method successfully detected *H. pylori* in 126 of 133 cases (95% sensitivity). Mutations conferring resistance were present in 92 cases (73%), including 63 cases with one mutation (50%) and 29 cases with mutations in multiple genes (23%). Treatment outcomes were available in 58 cases. Sixteen of the 58 cases failed therapy (28%). Therapy failure correlated with the number of mutated genes: no failure in cases with no mutations (0/15), 19% (5/27) failure in cases with one gene mutation, and 69% (11/16) failure in cases with more than one mutated gene. Common 23S rRNA mutations (A2142G or A2413G) were present in 88% (14/16) of failed cases as opposed to in only 10% (4/42) of eradicated cases ($P < 0.001$). This NGS assay can be used on remnant specimens collected during standard-of-care testing to detect mutations that correlate with increased risk of treatment failure. A prospective study is needed to determine if the risk of treatment failure can be decreased by using this assay to guide antibiotic therapy.

KEYWORDS DNA sequencing, *Helicobacter pylori*, antibiotic resistance, mutation

Helicobacter pylori infects close to one-half of the global population and is the main cause of peptic ulcer disease and a trigger for gastric cancer (1, 2). The current recommended empirical treatment for *H. pylori* eradication includes two or three antibiotics (typically clarithromycin and either amoxicillin or metronidazole) and one antisecretory drug for 14 days, with the eradication goal being higher than 80% (3–5). However, *H. pylori* acquires antibiotic resistance by mutation, which has dramatically increased over the past decades (6–8). The rate of clarithromycin resistance in the United States has increased from 10% to 24% in the 1990s to 24% to 70% in recent years (1, 3, 9). Increasing rates of resistance have also been reported in Europe and Asia (1, 2, 6, 10). This has caused a sharp global decline in the effectiveness of the recommended treatments that used to be >90% effective in the 1990s (5, 6, 11–13). Studies have shown that while a proton pump inhibitor (PPI)-clarithromycin-amoxicillin triple therapy has up to 88% eradication rate for clarithromycin-susceptible strains, the

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eradication rate drops to only 18% in clarithromycin-resistant strains (1, 14). Therefore, it is suggested to identify the *H. pylori* strains that are likely to fail empirical therapy and subsequently to choose a personalized therapeutic regimen with high likelihood of successful eradication. Several studies have reported that a treatment based on antimicrobial susceptibility testing is more effective than empirical treatment (15–17).

Susceptibility testing using bacterial culture followed by MIC phenotypic resistance testing is the gold standard technique to detect resistance. However, this practice is not widely used due to the fastidious nature of the organism and the long incubation time required. Enzyme immunoassay, fluorescence *in situ* hybridization (FISH), and several PCR-based methods have been described to detect *H. pylori* and the mutations related to clarithromycin resistance from biopsy specimens, gastric fluid, colonies, and even stool samples (12, 16). PCR methods based on the detection of point mutations offer high sensitivity and specificity and hence are alternatives to phenotypic testing, but PCR only allows for the detection of resistance mutations at a limited number of sites (16, 18). Currently, there is no simple and widely used method for predicting antimicrobial resistance, but there is opportunity to use molecular methods to develop a rapid and clinically useful approach to detect mutations associated with resistance. In this study, we investigated the ability of a novel next-generation sequencing (NGS) assay to detect resistance mutations in *H. pylori* by sequencing *H. pylori* DNA from formalin-fixed, paraffin-embedded (FFPE) gastric biopsy specimens. We also evaluated the correlation of these resistance mutations to clinical outcomes.

MATERIALS AND METHODS

Samples for NGS assay validation. The study was approved by the Institutional Research Board of the University Hospitals Cleveland Medical Center/Case Western Reserve University (UHCMC/CWRU). One hundred thirty-three gastric biopsy specimens positive for *H. pylori* (by morphology or immunohistochemistry) collected between January 2017 and January 2018 were selected by a retrospective search of the Department of Pathology archives database. Medical records were reviewed for each patient, including demographics, treatment of *H. pylori* infection, and treatment outcome. Only the first biopsy specimen obtained from a subject was analyzed in this study. Clinical eradication was defined as negative fecal antigen test, negative urea breath test, negative repeat biopsy, or clinical impression of cure (symptom relief) on follow-up assessments. Treatment failure was defined as a persistent positive fecal antigen test, urea breath test, repeat biopsy, or persistence of symptoms of dyspepsia in follow-up assessments.

DNA extraction and ion semiconductor-targeted NGS. FFPE tissue blocks were retrieved, and 7-μm curls were prepared. DNA was extracted from the curls using the QIAamp FFPE DNA Isolation kit (catalog number 5110; Qiagen, Valencia, CA) according to the manufacturer's instructions. The primers were designed to target the DNA regions that have been associated with antibiotic resistance. Clarithromycin resistance is due to single point mutations in the peptidyl transferase region of domain V of 23S rRNA (3). Tetracycline resistance is due to mutation in the binding site of tetracycline in 16S rRNA (14). Fluoroquinolone resistance is due to point mutations in the quinolone resistance determining region (QRDR) of *gyrA* at positions encoding amino acids 86, 87, 88, 91, or 97 (14, 19). We used 3 pairs of primers to amplify the three gene regions: *gyrA*, (forward) 5′-AAGGTTAGGCAGACGGCT-3′ and (reverse) 5′-TTAACCACCCCCATGGCGA-3′; 23S rRNA gene, (forward) 5′-GGTGGTATCTCAAGGATGGC-3′ and (reverse) 5′-GATCTAACCGCGGCAAGACG-3′(20); and 16S rRNA gene, (forward) 5′-TGGAGCATGTGG TTTAATTCGA-3′ and (reverse) 5′-TGCGGGACTTAACCCAACA-3′(21). An input of 30 ng of DNA was used for the multiplex PCR. The amplicons were then subjected to DNA end repair, deoxyribosyladenine (dA) tailing, and adapter ligation per the manufacture's protocol (Thermo Fisher). The IonChef and Hi-Q View 200 kits were used for template amplification and enrichment, and sequencing was performed on either an Ion Torrent PGM with v318 Chip at approximately 1% of chip space per sample or an S5 GeneStudio with 510 Chip at approximately 5% of chip space.

Informatics pipeline and variant interpretation. The low-quality bases from the end of the read (base $<$ Q20) and raw reads (read lengths $<$ 100 bp) were trimmed by Trimmomatic (version 0.38). The remaining clean reads were mapped with BWA (22) (version 0.7.5a-r405) against the database of 16S rRNA gene sequences compiled by Miao et al. (23). Reads that best mapped to *H. pylori* and had $>$99% similarity to *H. pylori* were extracted and aligned to the 16S rRNA gene of the reference genome (NC_000915). Single nucleotide variants (SNVs) and indel were called using SAMtools's (24) (version 1.7) Mpileup module and Bcftools (minimum base and mapping quality of $>$10 and minimum coverage of $>$100 reads); finally, a series of mutations were generated. For determining variations in 23S rRNA and *gyrA* genes, all reads were aligned to the corresponding gene from the reference genome (NC_000915) followed by the identification of variants as described above for 16S. There is limited published data describing the clinical significance of a majority of variants for *gyrA* and 23S, and so only variants with a clear link to antibiotic resistance were considered clinically significant mutations (3, 8, 25–35).

Statistical analysis. Clinical and demographic characteristics were compared using statistical tests (Fisher's exact tests, chi-square, Mann-Whitney rank sum test, and Student's *t* test) to assess differences

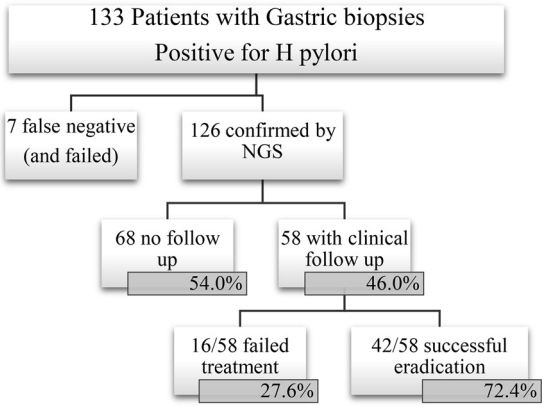

FIG 1 Consort flow diagram of the study.

between groups in categorical and nominal variables in the relationships between variations of multidrug resistance genes and the presence or absence of resistance mutations. A P value of ≤ 0.05 was considered statistically significant. Statistical analyses were performed using SigmaPlot 11.0.

RESULTS

The NGS assay detected *H. pylori* in 126 of 133 histologically positive cases (Fig. 1), yielding 94.7% sensitivity. Table 1 shows the demographic data of the 126 NGS positive cases. Bacteria other than *H. pylori* were also detected using the 16S sequencing assay, and these bacteria and the frequency of their detection are reported in Table S1 in the supplemental material.

Prevalence of gene variants and resistance mutations. The assay detected 11 different variants in 23S with at least one variant identified in 52 of 126 specimens, 28 different variants in *gyrA* in 75 specimens, and 4 different variants in 16S present in 12 specimens (see Tables S2 and S3). Of these variants, a subset has been described as invoking antibiotic resistance, and these included 6 mutations in 23S, 6 mutations in *gyrA*, and 4 mutations in 16S (Table 2). The prevalence of mutations known to cause antibiotic resistance was 48 (38.1%) in 23S, 66 (52.4%) in *gyrA*, and 12 (9.5%) in 16S (Table 3). The most common 23S mutations were A2143G, A2142G, and T2182C, and the most prevalent *gyrA* mutations were N87T and N87I (Table 2).

Treatment outcomes. Correlation with therapy outcomes was studied in a subset of 58 patients for which records of clinical follow-up were available. Clarithromycin (54 cases) and amoxicillin (47 cases) were the most commonly used antibiotics (Table 5). Of these 58 subjects, 16 (28%) failed treatment and 42 (72%) experienced successful eradication of *H. pylori* infection. Mutations were detected in all (16/16) in the failed therapy group, but only in 64% (27/42) in the successful eradication group ($P < 0.01$) (Table 4). There was no significant difference in the antibiotics prescribed when comparing the successfully eradicated and failed therapy groups, except for tetracy-

TABLE 1 Demographic information ($n = 126$)

Characteristics	Value(s)
Age (yr)	
Mean ± SD	49.8 ± 20.0
Range	7–89
Sex (*n*)	
Male	39
Female	87
Race (*n*)	
White	50
African-American	55
Other	21

TABLE 2 Prevalence of mutations

Gene	No. among all positive cases ($n = 126$)	Positive cases with follow-up data			
		Treatment failed ($n = 16$)		Eradication ($n = 42$)	
		No.	%	No.	%
23S rRNA					
A2143G	30	11	69	4	10
A2142G	10	4	25	1	2
T2182C	9	0	0	3	7
C2195T	8	1	6	3	7
G2141A	3	0	0	1	2
A2223G	1	1	6	0	0
No mutations	78	2	13	32	76
gyrA					
N87T	35	5	31	10	24
N87I	18	2	13	8	19
D91N	10	6	38	2	5
N87K	4	2	13	0	0
D91G	4	1	6	0	0
D91Y	3	2	13	0	0
No mutations	50	3	19	24	52
16S rRNA					
A928C	6	2	13	3	7
A926G	5	1	6	1	2
A928T	1	1	6	0	0
G927T	0	0	0	0	0
No mutations	114	12	75	38	90

cline, which was used more frequently in the failed group than in the eradicated group ($P = 0.03$) (Table 5).

Of the 16 failed cases, 15 received clarithromycin, and 13 of these harbored 23S mutations associated with macrolide resistance. Of 42 eradicated cases, 39 had received clarithromycin, and only 8 of these 39 cases harbored 23S mutations. Regarding 16S mutations, of 16 failed cases, 5 received tetracycline, and 2 of these 5 cases harbored 16S mutations associated with tetracycline resistance. Of 42 eradicated cases, 3 had received tetracycline, and none of these 3 cases harbored mutations associated with tetracycline resistance (Tables 4 and 5). In the case of *gyrA* mutations and levofloxacin resistance, only 2 of 58 patients had received levofloxacin, and so we could not test a correlation between the therapy failure and mutation presence. Therapy failure positively correlated with the number of mutated genes. No therapy failures were reported in 15 specimens without mutations. The failure rate was 19% (5/27) in patients with 1 gene mutation and 69% (11/16) in specimens with mutations in more than one gene (Fig. 2). Total mutation burden (cumulative number of mutations of three genes) was

TABLE 3 Clinically relevant mutation patterns and prevalence of *H. pylori* in the northeast Ohio cohort ($n = 126$)

Mutation pattern	No. (%) of cases
No mutation	34 (27.0)
Mutation in one gene	63 (50.0)
16S	5 (4.0)
23S	20 (15.9)
gyrA	38 (30.2)
Mutation in more than one gene	29 (23.0)
23S and 16S	1 (0.8)
gyrA and 23S	22 (17.5)
gyrA and 16S	1 (0.8)
gyrA and 23S and 16S	5 (4.0)

TABLE 4 Clinically relevant mutations and antibiotic therapy in cases with follow-up

Case	Treatment outcome[a]	Mutation(s) in:[b]				
		23S in cases treated with macrolide (clarithromycin)	*gyrA* in cases treated with fluoroquinolone (levofloxacin)	16S in cases treated with tetracycline (doxycycline)	Cases treated with amoxicillin (mutations not analyzed)[b]	Cases treated with metronidazole (mutations not analyzed)[b]
1	F	A2143G, C2195T	N87T			
2	F	A2142G	N87I, D91N	A928C		
3	F		N87T, D91N			
4	F	A2143G	N87T			
5	F	A2143G				
6	F	A2143G, A2142G	D91N			
7	F	A2143G, A2223G	D91N			
8	F	A2143G				
9	F		N87T, D91N			
10	F	A2142G	D91N			
11	F	A2143G	N87T, D91G			
12	F	A2143G	D91Y			
13	F	A2143G	N87K, D91Y	A926G		
14	F	A2143G	N87I	A928T		
15	F	A2143G				
16	F	A2142G	N87K	A928C		
17	S		N87T			
18	S					
19	S					
20	S	T2182C				
21	S					
22	S	C2195T				
23	S					
24	S			A926G		
25	S		N87T			
26	S					
27	S	A2143G				
28	S		N87T			
29	S					
30	S		N87I			
31	S	T2182C				
32	S					
33	S					
34	S		N87T, D91N			
35	S		N87T			
36	S		N87I			
37	S	A2143G	N87I			
38	S					
39	S		N87T			
40	S			A928C		
41	S		N87I, D91N			
42	S		N87I	A928C		
43	S	A2143G, A2142G, G2141A		A928C		
44	S					
45	S	C2195T				
46	S					
47	S					
48	S		N87T			
49	S		N87I			
50	S		N87T			
51	S	T2182C				
52	S	C2195T	N87T			
53	S	A2143G	N87I			
54	S					
55	S		N87I			
56	S					
57	S		N87T			
58	S					

[a]F, failed; S, successful eradication.
[b]Shading indicates that the corresponding antibiotic was received.

TABLE 5 Antibiotics used in cases with clinical follow-up

Antibiotic(s) or characteristic	No. of cases treated ($N = 58$)	No. (%) of cases	
		Treatment failure ($N = 16$)	Eradication ($N = 42$)
Amoxicillin	47	13 (81.2)	34 (80.9)
Clarithromycin	54	15 (93.7)	39 (92.9)
Tetracycline	8	5 (31.2)	3 (7.1)[a]
Levofloxacin	2	2 (12.5)	0 (0)
Metronidazole	12	6 (37.5)	6 (14.3)
Combination antibiotic therapy	56	16 (100)	40 (95.2)
Mutation detected that invokes resistance to at least one of the prescribed antibiotics	23	15 (93.75)	8 (19.0)[b]

[a]*P* value <0.05.
[b]*P* value <0.001.

greater in the failed treatment group than in the successful eradicated group (2.5 ± 1.0 versus 1.1 ± 1.0, respectively, $P < 0.001$).

23S rRNA mutations were significantly more common in treatment failure cases than in successful eradication cases (88% versus 24%, $P < 0.001$) (Table 4). Two 23S mutations of A2142G and A2143G were present in 88% of cases in the failed treatment group (14/16) as opposed to in only 10% of cases in the eradicated group (4/42) ($P < 0.001$) (Fig. 3).

DISCUSSION

Our data demonstrated that targeted NGS testing of *H. pylori* from FFPE is a feasible approach to detect mutations associated with antimicrobial resistance and that the presence of these mutations correlated with treatment failure. In our cohort, clarithromycin resistance mutations were present in 38.1% (48/126), levofloxacin resistance mutations were present in 52.4% (66/126), and tetracycline resistance mutations were present in 9.5% (12/126) of specimens with detectable amounts of *H. pylori* DNA (Table 3). Amoxicillin resistance is attributed to penicillin binding protein mutations, and resistance is uncommon and was not investigated in this study. Metronidazole resistance is commonly encountered and has been associated with mutations in *rdxA* and/or *frxA*, but data regarding the value of detecting these mutations in predicting antibiotic resistance is limited; therefore, in this study, we did not investigate the mutations associated with metronidazole resistance (2, 36). Previous studies have shown that tailored treatments based on resistance data improve eradication rates and increase cost efficiency compared to the use of standard empirical triple therapy, especially in

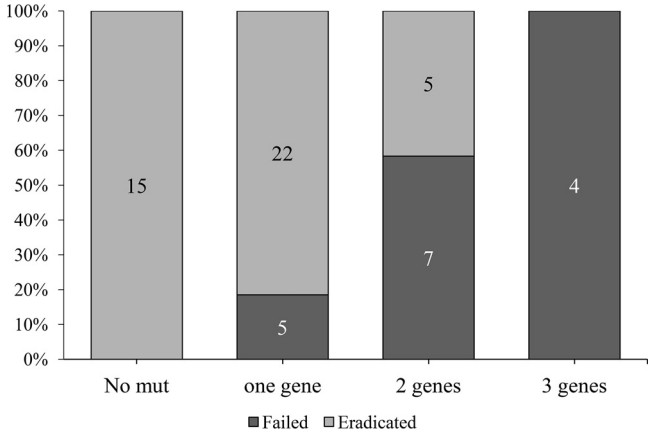

FIG 2 Failure rate based on the number of mutated genes. In our group of 58 patients with follow-up results, therapy failure increased with the number of gene mutations detected, while no therapy failure was reported in patients without mutations. Failure rate was 18.5% (5/27) in patients with 1 gene mutation and 68.8% (11/16) in patients with mutations in more than one gene.

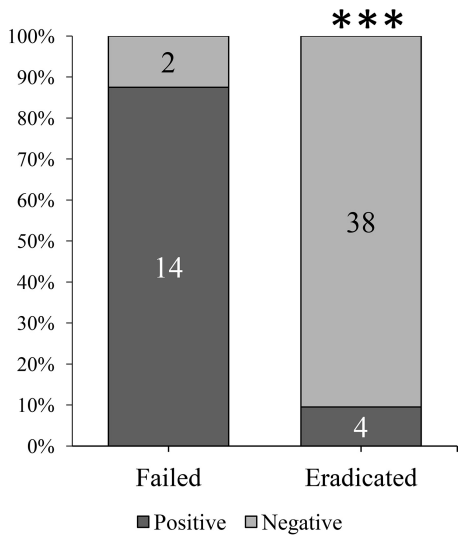

FIG 3 Failure rate based on A2143G and/or A2142G mutations. In our group of 58 patients with follow-up results, subjects with one of these mutations were significantly more likely to fail treatment than those with unmutated 23S rRNA ($P < 0.001$): 87.5% of patients who failed therapy, as opposed to only 9.5% in the eradicated group.

areas where clarithromycin resistance is higher than 15%, such as the United States (37, 38). In the future, this NGS assay could be used to tailor *H. pylori* therapy.

Clarithromycin was the most commonly used antibiotic in our cohort, and we identified a strong correlation between 23S mutation (which would invoke clarithromycin resistance) and treatment failure, even though these infections were treated with combination therapy in which one of the other antibiotics (e.g., amoxicillin) may have been active. Resistance to at least one of the prescribed antibiotics was detected in 93.7% of failed cases as opposed to in only 19% of eradicated cases (Table 5). This correlation highlights that resistance to even one antibiotic can significantly affect the clinical outcome of *H. pylori* eradication treatment.

When considering the results of 23S rRNA mutations alone, the presence of A2142G or A2143G alone correlated with treatment failure, and these mutations were previously reported to be associated with clarithromycin resistance (11, 32, 39). Of the 16 cases that failed therapy, 69% (11/16) had A2143G and 25% (4/16) had A2142G; cases with specimens containing one of these mutations were significantly more likely to fail treatment than those with unmutated 23S rRNA ($P < 0.00$) (Fig. 3).

Among the 6 common mutations in *gyrA* that invoke fluoroquinolone resistance, N87 and D91 are reported to be the most commonly encountered (14, 19, 40), and these mutations were the most frequent *gyrA* mutations in our cohort (Table 4). However, we did not observe a significant correlation between *gyrA* mutations and treatment success. Many subjects with *gyrA* mutations achieved successful eradication, and this may be because only three individuals in our study (3/58) received fluoroquinolone therapy. For similar reasons, 16S mutations also did not demonstrate a significant correlation with treatment success. In contrast, nearly all subjects in our study had received clarithromycin therapy (Table 5), and treatment failure correlated strongly with 23S mutations invoking macrolide resistance. However, despite no individual correlation between *gyrA* or 16S mutation and treatment success, we observed a direct correlation between the mutation burden across 16S, 23S, and *gyrA* genes and the likelihood of empirical treatment failure, regardless of the treatment regimen used (Table 5; see also Fig. S1 in the supplemental material). This finding suggests that *H. pylori* total mutation burden may be an independent risk factor of treatment failure, although additional studies are needed to confirm this possibility.

Our assay detected 11 different mutations in 23S and 28 mutations in *gyrA*, from which 6 mutations in 23S and 6 in *gyrA* have shown clinical correlation to antibiotic

resistance. The rest of these mutations were regarded as variants of unknown significance. *In vitro* susceptibility testing of cultured isolates containing these variants of unknown significance may provide insight into whether these variants invoke antibiotic resistance. It is also worth noting that our assay was not designed to differentiate different strains of *H. pylori* or to identify mixed infections caused by more than one *H. pylori* strain.

Previous studies have shown a correlation between the presence of *H. pylori* mutations and *in vitro* antibiotic resistance (3), and the present study demonstrates that mutation screening directly from biopsy specimens can predict treatment failure. The NGS assay described in this study can be performed with FFPE specimens, which are routinely obtained as part of standard of care. This NGS assay has prognostic value, and it could potentially be used for personalized therapy to avoid the use of antibiotics that are likely to be ineffective. Our study corroborates others' findings that specific mutations in 23S rRNA (A2142G or A2413G) correlate with increased likelihood of treatment failure when clarithromycin is used. This study is the first to suggest that total mutation burden across 16S rRNA, 23S rRNA, and *gyrA* genes correlates with increased likelihood of treatment failure; further investigation should be performed to test the validity of this finding.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.01834-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

ACKNOWLEDGMENT

We declare no conflicts of interest.

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