Novel *Helicobacter pylori* Sequencing Test Identifies High Rate of Clarithromycin Resistance

*Midori Mitui, †Ashish Patel, *N. Kristine Leos, *Christopher D. Doern, and *Jason Y. Park

See ''Resistance Testing for *Helicobacter pylori* Infection: Is It Finally Ready for Prime Time?'' by Gold on page 3.

ABSTRACT

Objectives: Eradication therapy selection for *Helicobacter pylori* gastritis requires knowledge of the local resistance rate to clarithromycin. There is minimal population-based or regional data in the United States on pediatric clarithromycin resistance. Although commercial methods such as fluorescence in situ hybridization and DNA probe assays are available in Europe for the evaluation of *H pylori* 23S rRNA mutations associated with resistance, clinical testing for 23S rRNA in the United States is not widely available. This study examined a single pediatric institution's clarithromycin resistance rate by a DNA polymerase chain reaction/sequencing assay applied to archived gastric biopsy specimens.

Methods: From the period 2010 to 2012, 38 *H pylori*–infected gastric biopsies were examined from archived formalin-fixed paraffin-embedded (FFPE) material. The *23S rRNA* gene of *H pylori* was polymerase chain reaction amplified and sequenced for the identification of point mutations that are associated with clarithromycin therapeutic resistance.

Results: By *23S rRNA* gene sequencing, 50% (n = 19) of the specimens contained *H pylori* with mutations significant for clarithromycin resistance.

Conclusions: This study is consistent with other pediatric reports suggesting significant *H pylori* clarithromycin resistance in the United States. Furthermore, the method used in this study can be used by hospital-based clinical laboratories to assess local clarithromycin resistance from archived biopsy material.

Key Words: antibiotic resistance, clarithromycin, formalin-fixed paraffin-embedded material, *Helicobacter pylori*

(*JPGN* 2014;59: 6–9)

Helicobacter pylori infection is the major cause of gastric ulcer disease and gastritis and a risk factor in the development of gastric cancer. The estimated *H pylori* infection prevalence is 80% to 90% in developing countries and 35% to 40% in the United States (1). Clarithromycin-based triple therapy is often used as the first-line therapy, but resistance to clarithromycin has been increasing worldwide, leading to eradication failure. European and American pediatric gastroenterology societies (the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition and the North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition) (2) consider a high prevalence of resistance to be >20%, and pediatric gastroenterology guidelines recommend antibiotic susceptibility testing in areas of high resistance rate. Resistance to clarithromycin is caused by the presence of point mutations in domain V of the *23S rRNA* gene of *H pylori*. The analysis of the *23S rRNA* gene has been demonstrated to have a 91.5% correlation with minimum inhibitory concentration (MIC) by E test for the detection of resistance (3).

Studies have reported a clarithromycin resistance rate of 10% to 13% in adult populations (1,4), but only a few studies have reported clarithromycin resistance in pediatric populations. Pediatric studies in the United States have described a resistance rate of 38% to 41% (5,6). In a recent study of Japanese pediatric patients, the resistance rate was reported to be 36% (7), and a recent European multicenter study reported a resistance rate of 32% in children (8).

In this report, the clarithromycin resistance rate in a tertiary care pediatric hospital was investigated using a novel DNA polymerase chain reaction (PCR)/sequencing assay targeted to domain V of the *23S rRNA* gene of *H pylori*.

METHODS

Patient Population and Materials

The study was performed according to a protocol approved by the University of Texas Southwestern Medical Center institutional review board. Patient data, archived slides, and formalin-fixed paraffin-embedded (FFPE) blocks were obtained from Children's Medical Center (Dallas, TX). The electronic medical records were reviewed for all cases. A search of the laboratory information system from 2010 to 2012 was performed for gastric biopsies with reports either positive or negative for *H pylori* gastritis. Specimens that were negative for *H pylori* were examined to demonstrate the specificity of the PCR/sequencing assay. All specimens had been previously analyzed by hematoxylin and eosin stain, and immunohistochemistry (IHC) specific for *H pylori*.

DNA Extraction From FFPE Samples

Human and bacterial DNA were extracted from 20-μm FFPE sections. Either 2 or 5 sections were obtained depending on the amount of tissue present in the block. The QIAamp DNA FFPE Tissue Kit (QIAGEN, Valencia, CA) was used and the DNA was eluted in 40 μL. The number of paraffin sections used was inversely proportional to the amount of tissue present in the block.

Primer Design

Primers were designed in highly conserved regions of the *23S rRNA* gene domain V of *H pylori* targeting the mutation sites. To

Received December 31, 2013; accepted March 20, 2014.

From the *Department of Pathology, and the †Division of Pediatric Gastroenterology, Children's Medical Center, University of Texas Southwestern Medical Center, Dallas.

Address correspondence and reprint requests to Midori Mitui, PhD, Children's Medical Center,1935 Medical District Dr, Dallas, TX 75235 (e-mail: midori.mitui@childrens.com).

This article has been developed as a Journal CME Activity by NASPGHAN. Visit *http://www.naspghan.org/wmspage.cfm?parm1=742* to view instructions, documentation, and the complete necessary steps to receive CME credit for reading this article.

The authors report no conflicts of interest.

DOI: 10.1097/MPG.0000000000000380

identify conserved regions, an alignment of different *H pylori* sequences that were available on GenBank was performed using MegAlign software (DNAstar, Madison, WI). The sequences of the primers are forward 5′-AGGTAGCGAAATTCCTTGTCGGT-3′ and reverse 5′-AGCTAACAGAAACATCAAGGGTGGT-3′.

Specificity of the Primers

The primers were tested against different bacterial and fungal isolates to evaluate potential cross-reactivity. The bacterial *16S rRNA* gene and the fungal ITS sequence were amplified as positive controls.

PCR Amplification, Sequencing, and Mutation Analysis

The extracted DNA was PCR-amplified using the HotStarTaq Plus Master Mix (QIAGEN) and sequenced on the 3130xl Genetic Analyzer (Life Technologies, Foster City, CA). Human β-actin gene was used as an internal control to check for the quality of the extracted DNA sample. The sequencing results were compared with the reference sequence GenBank: U27270.1 using Mutation Surveyor (SoftGenetics, State College, PA).

RESULTS

Case Selection

During the 3-year period 2010 through 2012, a total of 4089 patients underwent gastric biopsies at our pediatric institution. *H pylori* was identified in 4.5% (n = 184) of these gastric biopsies by histology and/or IHC. Thirty-eight *H pylori* positive FFPE specimens were examined. Positive cases were selected as consecutive groups with the following distribution by year: 2010, 5 cases; 2011, 5 cases; and 2012, 28 cases. In addition, 15 consecutive gastric biopsy cases that were negative for *H pylori* were examined as analytical controls.

TABLE 1. Characteristics of patients with *H pylori* gastritis

Patient	Mutation status	Age	Sex	Ethnicity	Prior antibiotic	*H pylori* treatment	Cured	Follow-up, y
1	A2143G	10	Female	White	None	CAP	Yes	1.1
2	A2143G	17	Female	White	Doxycycline	CAP	Yes	1.2
3	A2143G	14	Female	White	None	CAP	Yes†	3.0
4	A2143G	16	Male	Hispanic	None	CAP	Yes†	0.7
5	A2143G	16	Male	Asian	None	CAP	No follow-up	1.1
6	A2143G	13	Female	White	CAP*	CAP	No follow-up	2.5
7	A2142G	16	Female	White	None	CAP	No follow-up	1.9
8	A2143G	12	Male	Asian	None	CAP	No	0.8
9	A2143G	16	Male	White	Doxycycline	CAP	No	0.9
10	A2142G	15	Female	Hispanic	CAP*	CAP	No	0.9
11	A2143G	13	Male	Hispanic	None	CAP	No	1.2
12	A2143G	17	Female	Hispanic	Ciprofloxacin	CAP	No	0.6
13	A2143G	10	Male	White	None	CAP	No	0.6
14	A2143G	8	Male	Asian	Ampicillin-sulbactam	CAP, SUC	No	2.6
15	A2143G	18	Female	Hispanic	None	CAP	No	2.4
16	A2143G	15	Female	African American	None	MET, AMOX, PPI	Yes	1.2
17	A2143G	11	Male	Asian	None	MET, AMOX, PPI	Yes	1.1
18	A2143G	16	Female	Hispanic	None	MET, TET, PPI	Yes	0.6
19	A2143G	6	Male	Asian	None	MET, AMOX, PPI	No	0.8
20	Wild-type	16	Male	Hispanic	None	CAP	Yes†	2.1
21	Wild-type	3	Male	Hispanic	None	CAP	Yes†	1.8
22	Wild-type	11	Female	African American	MET	CAP	Yes	1.3
23	Wild-type	15	Male	Hispanic	Minocycline	Clarithromycin, MET	Yes	0.7
24	Wild-type	7	Male	Hispanic	None	CAP	Yes	0.5
25	Wild-type	18	Female	Hispanic	None	CAP	No follow-up	2.9
26	Wild-type	5	Female	Hispanic	CAP*	CAP	No follow-up	2.7
27	Wild-type	15	Male	African American	None	CAP	No follow-up	1.6
28	Wild-type	19	Male	Hispanic	None	CAP	No	0.8
29	Wild-type	8	Male	White	Azithromycin	CAP	No	0.7
30	Wild-type	14	Female	Hispanic	None	CAP	No	0.6
31	Wild-type	14	Male	African American	None	MET, TET, PPI	Yes	1.1
32	Wild-type	18	Female	Hispanic	None	MET, AMOX, PPI	Yes	0.9
33	Wild-type	16	Female	Hispanic	CAP*	MET, TET, PPI	Yes	0.8
34	Wild-type	11	Male	White	None	MET, AMOX, PPI	Yes	0.8
35	Wild-type	14	Male	Hispanic	None	MET, TET, PPI	No follow-up	0.6
36	Wild-type	17	Female	Hispanic	None	MET, TET, PPI	No follow-up	0.7
37	Wild-type	16	Female	Hispanic	None	MET, AMOX, PPI	No	1.2
38	Wild-type	17	Male	Hispanic	None	MET, TET, PPI	No	0.7

* Previously treated for *H pylori*; AMOX = amoxicillin; CAP = clarithromycin, ampicillin, proton pump inhibitor; MET = metronidazole; PPI = proton pump inhibitor; SUC = sucralfate; TET = tetracycline.
† Breath test or stool antigen confirmed.

Specificity of the Primers

No cross-reactivity of the primers was detected with the following bacterial organisms: *Staphylococcus aureus*, *S epidermidis*, *Pseudomonas aeruginosa*, *Bacteroides fragilis*, *Salmonella* spp, *Lactobacillus* spp, *Clostridium* spp, *Campylobacter jejuni*, group A *Streptococcus*, *S mitis*, *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and the following fungal isolates: *Fusarium* spp, *Trichosporon* spp, and *Candida* spp.

Two gastric biopsy cases with a diagnosis of *H heilmannii* were examined by the *H pylori* 23S rRNA assay. The PCR amplification revealed cross-reactivity of the primers; however, by sequencing the PCR product, no mutations associated with clarithromycin resistance were identified, and the identity was determined to be *Candidatus Helicobacter felis* (data not shown).

Limit of Detection, Sensitivity, and Specificity

The limit of detection of the PCR reaction was 50 fg based on serial dilutions of purified DNA from the American Type Culture Collection *H pylori* strain 26695. The sensitivity and specificity of the PCR/sequencing assay were 100% as compared with IHC when the samples contained a bacterial load of $\geq$48 *H pylori* organisms on a representative single 5-μm–stained tissue section.

Detection of Nucleotide Substitutions

Seven different nucleotide substitutions were identified in *H pylori*–positive samples: C2131T, A2142G, A2143G, T2182C, A2187G, A2222G, and A2223G. Of these, only 2 substitutions, A2142G and A2143G, have been consistently correlated with clarithromycin resistance in previously published studies. We identified the A2142G or A2143G mutation in 19 of the 38 *H pylori*–positive cases (50%) (Table 1).

Patients' Characteristics

The patients' ages ranged from 3 to 19 years old (Table 1). There were 18 girls and 20 boys. Of these 38 patients with *H pylori* infection, 86.8% (n = 33) had endoscopic findings of gastritis (eg, nodular gastric mucosa, erythema, erosions). All of the patients with *H pylori* infection were symptomatic with gastrointestinal symptoms (eg, abdominal pain, nausea, vomiting, diarrhea).

Of the 19 cases that had a 23S mutation, 17 were A2143G and 2 were A2142G (Fig. 1 and Table 1). Six of the 19 patients with 23S mutant *H pylori* had prior antibiotic exposure. Two of the 6 patients with prior antibiotic exposure had been previously treated in the outpatient setting for *H pylori* gastritis with a regimen of clarithromycin, amoxicillin, and prevacid. Fifteen of the 19 mutant cases were treated with a clarithromycin combination therapy; the other 4 cases were treated with regimens including metronidazole. Of the 15 mutant 23S cases treated with clarithromycin, 3 had no follow-up information. The remaining 12 mutant 23S cases were cured by clarithromycin, amoxicillin, and Prevacid in only 4 cases (33.3% cure using clarithromycin regimen). Of the 8 cases that failed clarithromycin-based therapy, 5 were treated with a second antibiotic combination regimen based on either clarithromycin (n = 2) or metronidazole (n = 3). Both patients on a second regimen based on clarithromycin failed therapy again. Two of 3 patients on a second regimen based on metronidazole were cured.

Five of the 19 patients with infection by wild-type *H pylori* had prior antibiotic exposure (Table 1). Two of the 5 patients with prior antibiotic exposure had been previously treated for *H pylori* gastritis with a regimen of clarithromycin, amoxicillin, and prevacid. Eleven of the 19 patients were treated with a clarithromycin combination therapy; the other 8 patients were treated with regimens including metronidazole. Three of the patients with wild-type *H pylori* were treated with clarithromycin and had no follow-up information. Of the remaining 8 patients with wild-type *H pylori* infection, 5 had cure by a clarithromycin regimen (62.5%). The remaining 3 patients with wild-type *H pylori* infection failed clarithromycin-based therapy and have not been started on a second antibiotic regimen.

DISCUSSION

The assay described in our study detects *H pylori* mutations that are associated with clarithromycin resistance. Fifty percent of the *H pylori*–infected patients had the A2142G or A2143G mutation. This rate of clarithromycin resistance is much higher than the rates seen in the last national surveys of the United States

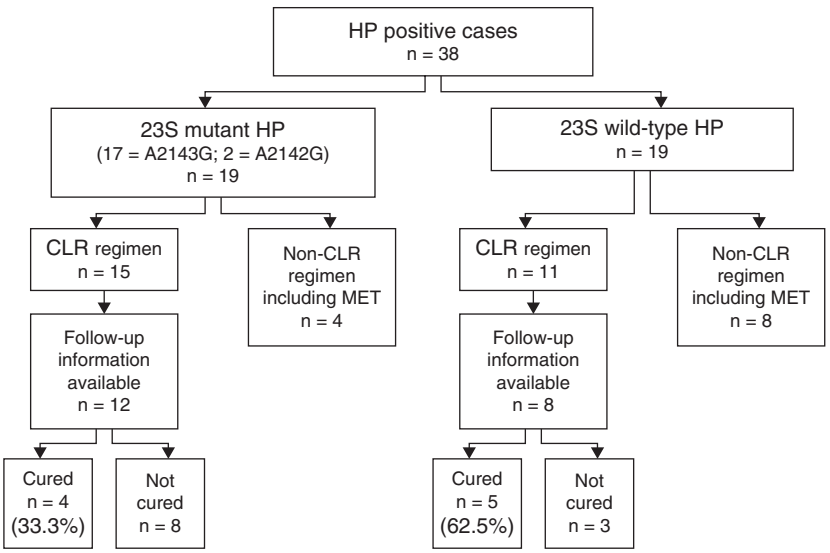

FIGURE 1. Patient characteristics corresponding to 38 *H pylori*–positive cases. CLR, clarithromycin; FFPE, formalin-fixed paraffin-embedded; HP, *H pylori*; MET, metronidazole.

(1,4), which reported no geographic region with a resistance rate of greater than 15%.

The overall concordance between clarithromycin resistance by E test and the presence of *23S rRNA* gene mutations is 81% to 91% (3,9). Considering phenotypic resistance testing as the criterion standard, a report demonstrated that the sensitivity and specificity of point mutations at positions 2142 and 2143 were 75% and 98.3%, respectively (10). Some studies have noted that the mutation A2143G is associated with high MIC values (3,11), whereas others have found high MIC values associated with the A2142G mutation (12–14).

The eradication rate by clarithromycin combination therapy (clarithromycin, amoxicillin, and Prevacid) in our patient population infected with wild-type *H pylori* was 62.5%. This rate is lower than the previously published rates of approximately 83% (15,16). Possible explanations include poor patient compliance, amoxicillin resistance, or clarithromycin resistance not detected by *23S rRNA* gene mutation analysis.

The PCR/sequencing method used in this study is designed to test FFPE samples. This method allows for the identification of mutant *H pylori* strains before initiation of clarithromycin therapy. This is especially relevant in areas of high clarithromycin resistance rates or in patients refractory to *H pylori* therapy. Furthermore, the flexibility of testing archived FFPE samples will enable hospital-based clinical laboratories to rapidly determine their local clarithromycin resistance rate and will aid in the management of antibiotic-resistant *H pylori* gastritis.

REFERENCES

1. Duck WM, Sobel J, Pruckler JM, et al. Antimicrobial resistance incidence and risk factors among *Helicobacter pylori*-infected persons, United States. *Emerg Infect Dis* 2004;10:1088–94.
2. Koletzko S, Jones NL, Goodman KJ, et al. Evidence-based guidelines from ESPGHAN and NASPGHAN for *Helicobacter pylori* infection in children. *J Pediatr Gastroenterol Nutr* 2011;53:230–43.
3. Versalovic J, Osato MS, Spakovsky K, et al. Point mutations in the 23S rRNA gene of *Helicobacter pylori* associated with different levels of clarithromycin resistance. *J Antimicrob Chemother* 1997;40:283–6.
4. Meyer JM, Silliman NP, Wang W, et al. Risk factors for *Helicobacter pylori* resistance in the United States: the surveillance of *H. pylori* antimicrobial resistance partnership (SHARP) study, 1993–1999. *Ann Intern Med* 2002;136:13–24.
5. Elitsur Y, Lawrence Z, Russmann H, et al. Primary clarithromycin resistance to *Helicobacter pylori* and therapy failure in children: the experience in West Virginia. *J Pediatr Gastroenterol Nutr* 2006;42:327–8.
6. Tolia V, Brown W, El-Baba M, et al. *Helicobacter pylori* culture and antimicrobial susceptibility from pediatric patients in Michigan. *Pediatr Infect Dis J* 2000;19:1167–71.
7. Kato S, Fujimura S. Primary antimicrobial resistance of *Helicobacter pylori* in children during the past 9 years. *Pediatr Int* 2010;52:187–90.
8. Megraud F, Coenen S, Versporten A, et al. *Helicobacter pylori* resistance to antibiotics in Europe and its relationship to antibiotic consumption. *Gut* 2013;62:34–42.
9. Agudo S, Perez-Perez G, Alarcon T, et al. High prevalence of clarithromycin-resistant *Helicobacter pylori* strains and risk factors associated with resistance in Madrid, Spain. *J Clin Microbiol* 2010;48:3703–7.
10. Moder KA, Layer F, Konig W, et al. Rapid screening of clarithromycin resistance in *Helicobacter pylori* by pyrosequencing. *J Med Microbiol* 2007;56 (Pt 10):1370–6.
11. Stone GG, Shortridge D, Versalovic J, et al. A PCR-oligonucleotide ligation assay to determine the prevalence of 23S rRNA gene mutations in clarithromycin-resistant *Helicobacter pylori*. *Antimicrob Agents Chemother* 1997;41:712–4.
12. Taylor DE, Ge Z, Purych D, et al. Cloning and sequence analysis of two copies of a 23S rRNA gene from *Helicobacter pylori* and association of clarithromycin resistance with 23S rRNA mutations. *Antimicrob Agents Chemother* 1997;41:2621–8.
13. van Doorn LJ, Glupczynski Y, Kusters JG, et al. Accurate prediction of macrolide resistance in *Helicobacter pylori* by a PCR line probe assay for detection of mutations in the 23S rRNA gene: multicenter validation study. *Antimicrob Agents Chemother* 2001;45:1500–4.
14. Owen RJ. Molecular testing for antibiotic resistance in *Helicobacter pylori*. *Gut* 2002;50:285–9.
15. Hojsak I, Kos T, Dumancic J, et al. Antibiotic resistance of *Helicobacter pylori* in pediatric patients—10 years' experience. *Eur J Pediatr* 2012; 171:1325–30.
16. Kalach N, Benhamou PH, Campeotto F, et al. Clarithromycin resistance and eradication of *Helicobacter pylori* in children. *Antimicrob Agents Chemother* 2001;45:2134–5.