Am. J. Trop. Med. Hyg., 96(5), 2017, pp. 1050–1059
doi:10.4269/ajtmh.16-0729

Clarithromycin-Based Triple Therapy Is Still Useful as an Initial Treatment for *Helicobacter pylori* Infection in the Dominican Republic

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Abstract. *Helicobacter pylori* antibiotic susceptibility in the Dominican Republic has not been monitored. We assessed *H. pylori* antibiotic susceptibility in the Dominican Republic, and analyzed *H. pylori* mutations associated with antibiotic resistance. We recruited 158 dyspeptic patients in Santo Domingo and used agar dilution to test susceptibility to five antibiotics. Polymerase chain reaction–based sequencing was used to assess *gyrA*, *gyrB*, *rdxA*, *frxA*, and 23S rRNA mutations; next-generation sequencing was used to identify other metronidazole resistance–associated genes. Among 64 *H. pylori* strains isolated, we identified two (3.1%), one (1.6%), and no strains with clarithromycin, amoxicillin, and tetracycline resistance, respectively. Moreover, high frequency of metronidazole resistance (53/64, 82.8%) was observed, whereas levofloxacin resistance is emerging (23/64, 35.9%). We identified many *rdxA* and *frxA* mutations in metronidazole-resistant strains, but no synergistic effect was apparent. We revealed novel mutations in *dppA*, *dppB*, *fdxA*, and *fdxB*, irrespective of *rdxA* and *frxA* mutations. Novel mutations at Ser-14 of *trx1* and Arg-221 of *dapF* were associated with different levels of metronidazole resistance. Most levofloxacin-resistant strains had a substitution at Asn-87 of *gyrA*, including the strain with the highest levofloxacin resistance, whereas only three substitutions were found at Ser-479 of *gyrB* with no synergistic effect. Besides the 23S rRNA A2142G mutation, we observed another mutation at T1958G in both clarithromycin-resistant strains. We confirmed high metronidazole and levofloxacin resistance associated with genetic mutations in the Dominican Republic. However, prevalence of clarithromycin resistance was low, suggesting that standard clarithromycin-based triple therapy remains useful as initial treatment of *H. pylori* infection.

INTRODUCTION

The emergence of drug resistance to antibiotics used to treat *Helicobacter pylori*, the Gram-negative bacterium responsible for severe gastroduodenal diseases, is a serious problem. According to current guidelines, triple therapy composed of a proton pump inhibitor and two antibiotics, amoxicillin (AMX) and clarithromycin (CAM) or metronidazole (MNZ), remains the standard first-line regimen for treatment of *H. pylori* infection,[1,2] although caution is advised with its use.[2] In recent years, efficacy of this regimen has been seriously challenged, and a cure rate below 70% has been reported in many countries.[3] Therefore, optimizing the first-line regimen based on local antibiotic resistance patterns is critical to prevent repeated courses of treatment and the spread of secondary antibiotic resistance.[4]

Helicobacter pylori genes with mutations implicated in drug resistance have been identified and can be detected by molecular methods. An understanding of *H. pylori* antibiotic resistance mechanisms is important in considerations of more rational antibiotic combinations. CAM resistance has been shown to be associated with any one of five recognized point mutations in *H. pylori* 23S rRNA. These mutations comprise an A to G substitution at nucleotide positions 2142 or 2143, A to C at 2142, A to T at 2144, T to C at 2717, and C to A at 2694.[5,6] The mechanisms of MNZ resistance are complex but largely associated with inactivation of *rdxA* (hp0954 in the *H. pylori* 26695 genome) and *frxA* (*hp*0642), which, together, have a synergistic effect that results in a high level of resistance.[7,8] Other identified mutations, including *rpsU* (*hp*0562),[9] *dppA* (*hp*0298), *dppB* (*hp*0299), *rps4* (*hp*1294), *ackA* (*hp*0903), *rnc* (*hp*0662), *dapF* (*hp*0566),[10] *recA* (*hp*0153),[11] *fdxA* (*hp*0277),[12] *fdxB* (*hp*0284),[13] and *trx1* (*hp*0824),[14,15] have also been associated with MNZ resistance. On the other hand, mutations in gyrase subunit A (*gyrA*) (*hp*0641), and *gyrB* (*hp*0501), have been associated with fluoroquinolone resistance.[16]

The prevalence of antibiotic resistance in Latin America varies by geographic region. The lowest resistance to CAM was reported to be 2% in Paraguay, and the highest was reported to be 50% in Peru.[17] A various prevalence of antibiotic resistance has also been reported in Latin American countries, 13–95%, 0–39%, and 0–86% resistance, respectively, to MNZ, AMX, and tetracycline (TCN).[17] The Dominican Republic is a nation that occupies the eastern part of the second largest island, Hispaniola, in the Caribbean Sea, with a total population of 10.41 million in 2014. The age-standardized rate of gastric cancer in the Dominican Republic is reported to be 7.3 per 100,000 per year (http://globocan.iarc.fr/). Although a recent study regarding the prevalence of *H. pylori* infection was reported (58.9%),[18] *H. pylori* antibiotic susceptibility in the Dominican Republic strains has not be monitored. According to the European Maastricht Consensus,[2,19] CAM-containing triple therapies administered without prior susceptibility testing should be abandoned in a region when the local CAM resistance frequency is greater than 15–20%. In populations with < 40% MNZ resistance, MNZ-based triple therapy is preferable.[19,20]

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Therefore, it is critical to examine current drug resistance frequencies to select the appropriate first-line regimen for *H. pylori* treatment in the Dominican Republic. In this study, we aimed to determine the antibiotic susceptibility of *H. pylori* and to identify mutations associated with antibiotic resistance in this pathogen in the Dominican Republic.

MATERIALS AND METHODS

Patients and *H. pylori*. This study included 158 consecutive patients (55 males and 103 females; age range, 17–91 years; mean age, 47.1 ± 16.2 years) who underwent endoscopy examination at the Digestive Disease Center, Dr. Luis E. Aybar Health and Hygiene City, Santo Domingo, Dominican Republic. Peptic ulcer diseases, including gastric and duodenal ulcers, were diagnosed by endoscopic observation, whereas chronic gastritis and gastric cancer were determined by histologic examination. Written informed consent was obtained from all participants, and the study protocol was approved by the ethics committees of Dr. Luis E. Aybar Health and Hygiene City; Institute of Microbiology and Parasitology, Autonomous University of Santo Domingo, Santo Domingo, Dominican Republic; and the Oita University Faculty of Medicine, Japan.

For *H. pylori* culture, antral biopsy specimens were homogenized and inoculated onto antibiotic selection plates, and then subcultured on Mueller Hinton II Agar medium (Becton Dickinson, Sparks, MD) supplemented with 7% horse blood without antibiotics. The plates were incubated up to 10 days at 37°C under microaerophilic conditions (10% O_2, 5% CO_2, and 85% N_2). *Helicobacter pylori* isolates were identified based on colony morphology; Gram staining results; and oxidase, catalase, and urease reactions. Isolated strains were stored at –80°C in *Brucella* broth (Becton Dickinson, Sparks, MD) containing 10% dimethyl sulfoxide and 10% horse serum.

Antibiotic susceptibility testing. The serial 2-fold agar dilution method was used to determine the minimum inhibitory concentrations (MICs) of AMX (Sigma Chemical Co., St. Louis, MO), CAM (Abbott Laboratories, Abbott Park, IL), MNZ (Sigma), TCN (Sigma), and levofloxacin (LVX) (Sigma). Briefly, bacteria were subcultured on Mueller Hinton II Agar medium (Becton Dickinson) supplemented with 10% defibrinated horse blood. The bacterial suspension, adjusted to a McFarland opacity standard of 3.0, was inoculated onto the plates. After 72 hours of incubation, the MIC of each antibiotic was determined. *Helicobacter pylori* ATCC 43504 was used for quality control testing. The resistance breakpoints were determined as described by the European Committee on Antimicrobial Susceptibility Testing (http://www.eucast.org/). Strains were considered resistant with MICs > 0.125 mg/L for AMX, > 0.25 mg/L for CAM, > 8 mg/L MNZ, and > 1 mg/L for TCN and LVX.

Molecular analysis of resistant strains. DNA was extracted from cultured *H. pylori* using a commercially available kit (Qiagen, Hilden, Germany) for amplification of *rdxA* and *frxA* from MNZ-resistant strains, *gyrA* and *gyrB* from LVX-resistant strains, and 23S rRNA peptidyl transferase from CAM-resistant strains using the primers as described previously.[21–23] As a control, we sequenced five randomly selected strains that were sensitive to MNZ and LVX and one sensitive to CAM from the Dominican Republic isolates. The sequences were then compared with the published sequence of *H. pylori* 26695 (GenBank accession number AE000511.1 GI: 6626253) using MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/) and confirmed by visual inspection.

To find genetic mutations other than the typical *rdxA* and *frxA* mutations, we also obtained full-length *rdxA*, *frxA*, *rpsU*, *dppA*, *dppB*, *rps4*, *ackA*, *rnc*, *dapF*, *recA*, *fdxA*, *fdxB*, and *trx1* sequences by next-generation sequencing (NGS) (MiSeq next-generation sequencer; Illumina, Inc., San Diego, CA). MiSeq output was integrated into contig sequences using CLC Genomics Workbench 7.0.4. (CLC Bio-Qiagen, Aarhus, Denmark) Genomics Workbench was also used for gene predictions and translation to protein sequences.

Statistical analysis. Discrete variables were tested using the χ^2 test, whereas continuous variables were tested using Mann–Whitney *U* and *t* tests. *P* values < 0.05 were considered statistically significant. SPSS statistical software package version 18.0 (SPSS, Inc., Chicago, IL) was used for all statistical analyses.

Ethical standards. We declare that all procedures performed for this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

RESULTS

Prevalence of antibiotic resistance. A total of 64 *H. pylori* strains were isolated from 19 male (age range, 21 to 69 years; mean age, 43.9 ± 12.4 years) and 45 female patients (age range, 17 to 82 years; mean age 43.8 ± 14.9 years). Of these patients, 47 had chronic gastritis, 16 had peptic ulcer diseases, and one had gastric cancer. The prevalence of CAM resistance was only 3.1% (2/64), in contrast to the higher prevalence reported in many other regions.[24] A low prevalence of AMX resistance (1/64, 1.6%) was observed, and no TCN resistance was detected (Table 1). However, similar to data reported from Africa,

TABLE 1
Antibiotic susceptibility of 64 *Helicobacter pylori* strains isolated from the Dominican Republic

	Number (%) of resistant isolates from patients					
Antibiotic	All patients (*N* = 64)	Gastritis (*N* = 47)	PUD (*N* = 16)	GC (*N* = 1)	Male (*N* = 19)	Female (*N* = 45)
AMX	1 (1.6)	0 (0.0)	1 (6.3)	0 (0.0)	0 (0.0)	1 (2.2)
CAM	2 (3.1)	2 (4.3)	0 (0.0)	0 (0.0)	0 (0.0)	2 (4.4)
MNZ	53 (82.8)	40 (85.1)	12 (75.0)	1 (100.0)	16 (84.2)	37 (82.2)
TCN	0 (0.0)	0 (0.0)	0 (0)	0 (0.0)	0 (0.0)	0 (0.0)
LVX	23 (35.9)	19 (40.4)	4 (25.0)	0 (0.0)	4 (21.1)	19 (42.2)

AMX = amoxicillin; CAM = clarithromycin; GC = gastric cancer; LVX = levofloxacin; MNZ = metronidazole; PUD = peptic ulcer disease; TCN = tetracycline.

Asia, and Europe,[24] the frequency of MNZ resistance was high (53/64, 82.8%). This frequency was also much higher than the neighboring countries of Costa Rica (42.0%) and Venezuela (59.0%).[17] Moreover, the prevalence of LVX resistance was also high (23/64, 35.9%). The distribution of patients' ages and antimicrobial resistance of isolates is shown in Table 2. Antibiotic resistance did not differ by age (P = 0.46, 0.51, 0.07, and 0.20 for AMX, CAM, MNZ, and LVX, respectively), sex (P = 0.51, 0.35, 0.85, and 0.11 for AMX, CAM, MNZ, and LVX, respectively), or disease group (P = 0.22, 0.69, 0.59, and 0.41 for AMX, CAM, MNZ, and LVX, respectively).

Overall, no strain was resistant to all antibiotics tested, and only two strains were resistant to three antibiotics (AMX, MNZ, and LVX or CAM, MNZ, and LVX) (Table 3). Of the study strains, 31.3% (20/64) showed dual-drug resistance to MNZ and LVX. No differences were observed in clinical outcomes between single-drug- and multidrug-resistant *H. pylori* infections (P = 0.13). There was no association between antibiotic resistance and clinical outcomes ($P > 0.05$). The MIC values for each antibiotic are shown in Figure 1. *Helicobacter pylori* showed a high level of resistance (with MIC values of 128 mg/L or greater) to MNZ in 23.2% (6/64) of isolates and to LVX (with MIC values of 32 mg/L or greater) in 7.8% (5/64) of isolates.

Detection of *H. pylori* gene mutations associated with antimicrobial resistance. The five MNZ-resistant strains did not show specific bands for *rdxA* by polymerase chain reaction, and for four strains, insufficient sequence data resulted. Therefore, a total of 44 MNZ-resistant and five sensitive (control) strains were analyzed in this study. A DNA sequence analysis of *rdxA* from MNZ-sensitive strains revealed intact reading frames (lacking nonsense mutations). A pairwise sequence alignment indicated that the MNZ-sensitive strains shared 94.6–97.1% identity with the reference strain, *H. pylori* 26695. On ignoring mutations that were present in both sensitive and resistant strains, all resistant strains but one contained mutations in *rdxA*. However, the one strain (DM4) that did not have an *rdxA* mutation had an *frxA* mutation (Table 4). Among resistant strains, 68.2% (30/44), 38.6% (17/44), and 31.8% (14/44) harbored missense mutations, premature stop codons, and translational frameshifts, respectively. Several *rdxA* alterations were categorized as class 1 mutations, which are expected to reduce affinity of the apoprotein for the flavin mononucleotide factor[25]; for example, an amino acid substitution at R16 was found in nine strains, at R200 in seven strains, and at S18 in DM118. Class II mutations, which are expected to destabilize dimer formation, were found at S43 in strains DM10 and DM118; Q50 in strains DM50 and DM143; and G145 in strains DM51 and DM87. Class III mutations were detected at C19 in strain DM103. No class IV mutations were found. Although a previous study[21] revealed the importance of amino acid substitutions A80H, A118T, A118S, Q197K, and V204I in resistance, we found these substitutions in both sensitive and resistant strains. In contrast, we confirmed the importance of a threonine replacement at position 51.[26] Other substitutions at E35, V85, G122, K190, and K198 might also be important in strains from the Dominican Republic.

We analyzed *frxA* in 40 resistant strains, because poor sequencing results were obtained for four strains (Table 4). Similar to mutational patterns in *rdxA*, *frxA* in MNZ-resistant strains also contained missense mutations and translational frameshifts (36/40, 90.0% and 5/40, 12.5%, respectively). In contrast with results of a previous study,[27] Q27E (57.5%, 23/40), N111H (57.5%, 23/40), N129T (55.0%, 22/40), S130D (57.5%, 23/40), E176K (45.0%, 18/40), and N182D (32.5%, 13/40) were predominant among the *frxA* mutations. Overall, although these two genes showed similarities in their patterns of mutation, no synergistic effect on MIC values was observed; for example, one MNZ-resistant strain with an MIC of 32 mg/L but showed no mutation in *frxA*, and this could not explain the different levels of MNZ resistance. Therefore, we randomly selected two sensitive strains, with MIC values of 32, 64, and 128 mg/L each and performed NGS (Table 5). Using the *H. pylori* strain 26695 and the MNZ-sensitive control strain, irrespective of their *rdxA* and *frxA* mutations, we revealed novel mutations associated with MNZ resistance in the full-length sequences of *dppA* (E473G), *dppB* (I322V), *fdxA* (N32S and S79I), and *fdxB* (V64I, M65L, and P245A). We did not obtain full-length *rdxA*, *frxA*, or *ackA* sequences from the NGS data. Moreover, we proposed that novel amino acid substitutions at Ser-14 of *trx1* and Arg-221 of *dapF* were associated with different levels of MNZ resistance.

Among the 23 LVX-resistant strains, 18 had amino acid substitutions associated with *gyrA* mutations (Table 6). Eleven of LVX-resistant strains (47.8%) showed an amino acid substitution at Asp-91, and 13 strains showed an amino acid substitution at Asn-87 (56.5%), including four strains with the highest LVX MIC values (32 mg/L). In addition, three strains exhibited amino acid substitutions associated with mutations in *gyrB*, including a substitution of interest at Ser-479. Although two strains with high levels of resistance showed mutations in both genes, one strain (DM62) that contained two substitutions of interest (Asp-91 [*gyrA*] and Ser-479 [*gyrB*]) had an LVX MIC value of 2 mg/L, suggesting there was no correlation between the degree

TABLE 2
Distribution of antibiotic resistance in patients of the Dominican Republic by age

Antibiotic	Number (%) per age group (years)					
	17–29	30–39	40–49	50–59	60–91	Total
Total	10	14	20	11	9	64
AMX	0 (0.0)	1 (7.1)	0 (0.0)	0 (0.0)	0 (0.0)	1
CAM	0 (0.0)	1 (7.1)	0 (0.0)	1 (9.1)	0 (0.0)	2
MNZ	8 (80.0)	14 (100.0)	17 (85.0)	7 (63.6)	7 (77.8)	53
TCN	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0
LVX	2 (20.0)	5 (35.7)	4 (20.0)	6 (54.5)	6 (66.7)	24

AMX = amoxicillin; CAM = clarithromycin; LVX = levofloxacin; MNZ = metronidazole; TCN = tetracycline.

TABLE 3
Multidrug resistance patterns of *Helicobacter pylori* in the Dominican Republic

Resistance patterns	*N*
Dual drugs	
CAM + MNZ	1 (1.6)
MNZ + LVX	20 (31.3)
Triple drugs	
AMX + MNZ + LVX	1 (1.6)
CAM + MNZ + LVX	1 (1.6)

AMX = amoxicillin; CAM = clarithromycin; LVX = levofloxacin; MNZ = metronidazole.

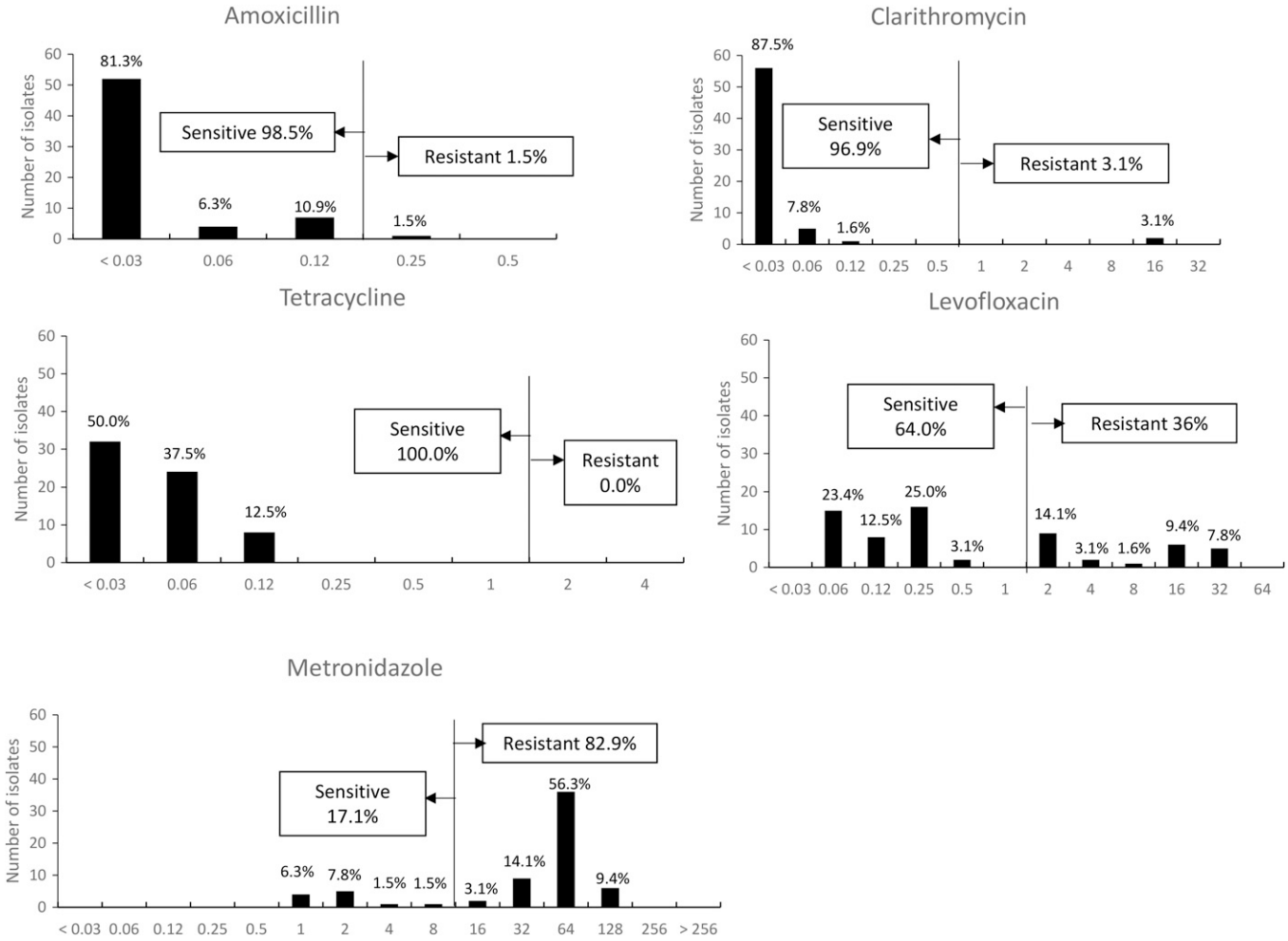

FIGURE 1. Antibiotic minimum inhibitory concentration values. The frequencies of resistance to metronidazole and levofloxacin were high; in contrast, the frequencies of resistance to clarithromycin, amoxicillin, and tetracycline were very low.

of LVX resistance and the number of mutations in these two genes. Interestingly, there were no mutations in either *gyrA* or *gyrB* in three strains, including in the strain with the highest LVX MIC.

Based on 23S rRNA sequencing of the two CAM-resistant strains, one strain had a point mutation resulting in the A2142G substitution. In addition, a T to G substitution at site 1958 was observed in both strains (Table 7). In contrast, there were no nucleotide mutations in the CAM-sensitive strains.

Nucleotide sequencing. Nucleotide sequence data from this study are available under DDBJ accession numbers LC176131–LC176133 (23S rRNA), LC176134–LC176161 (*gyrA*), LC176162–LC176189 (*gyrB*), LC176190–LC176238 (*rdxA*), LC200542-LC200585 (*frxA*), LC199398-LC199405 (*rpsU*), LC199406-199413 (*rpsD*), LC199414-LC199421 (*dppA*), LC199422-LC199429 (*dppB*), LC199430-LC199437 (*rnc*), LC199438-LC199445 (*dapF*), LC199446-LC199453 (*recA*), LC199454-LC199461 (*fdxA*), LC199462-LC199469 (*fdxB*), LC199470-LC199477 (*trxA*).

DISCUSSION

In this study, we revealed the low prevalence of CAM and AMX resistance. In addition, there were no TCN-resistant strains, suggesting that the standard CAM-based triple therapy may still be useful as an initial treatment of *H. pylori* infection in the Dominican Republic. Our results agree with those of a meta-analysis, which reported that the overall prevalence (13%) of CAM resistance in Latin America was below the prevalence reported in some European and Asian countries.[17] The authors suggested that empirical use of CAM might not be appropriate in Peru and perhaps in Colombia.[17] These differences of resistance frequency may be associated with local utilization of antibiotics.[28,29] In fact, the 10-year sales trends for macrolides showed large increases in Peru, Brazil, and Argentina.[30] In general, macrolides are frequently used for a number of infectious diseases in the Dominican Republic (M. Cruz and J. A. Jiménez Abreu, personal communication). However, there is no reliable report regarding CAM utilization in the Dominican Republic.

Interestingly, although A2142G substitution associated with *rdxA* is responsible for 90% of primary CAM-resistant *H. pylori* cases in western countries,[31] we observed a T to G substitution at site 1958 in all CAM-resistant strains obtained ($N = 2$). We need more evidence to conclude that this mutation was directly responsible for or synergistically associated with other CAM-resistance mutations. Point mutations resulting in substitutions other than A2142G

TABLE 4
Mutations in *rdxA* and *frxA* that were associated with metronidazole resistance

No.	Strains	MIC (mg/L)	Profile	*rdxA* mutations	*frxA* mutations
1	DM5	2	Sensitive	None	None
2	DM20	1	Sensitive	None	None
3	DM63	4	Sensitive	P44T, K168E, W209C	V34A
4	DM92	2	Sensitive	E194Q	Poor sequence quality
5	DM95	1	Sensitive	H25R	None
6	DM4	64	Resistant	None	Q27E, N111H, N129T, S130D
7	DM7	64	Resistant	A68T, V85A, 150 frameshift	Poor sequence quality
8	DM8	128	Resistant	R16C, S108A, D205A	A70V
9	DM10	64	Resistant	S43W	Q27E, A70G, A85V, N111H, N129T, S130D, E176K, N182D
10	DM14	64	Resistant	R10K, P44L, K168R, K198I, R200*	Q27E, M126I, N129T, S130D, A153V, E176K, N182D
11	DM32	64	Resistant	G170S	G45R
12	DM39	64	Resistant	K198I, K201E	Q27E, N111H, N129T, S130D, E176K
13	DM43	32	Resistant	41frameshift	Q27E, N111H, N129T, S130D, N182D
14	DM44	64	Resistant	E75*	Q27E, N111H, N129T, S130D, E176K
15	DM50	64	Resistant	Q50*	Q27E, A32E, N111H, N129T, S130D, E176K
16	DM51	32	Resistant	G145R, E174G,	A70V
17	DM52	64	Resistant	120frameshift	Q27E, A70G, N111H, N129H, S130D, E169A, E176K
18	DM57	64	Resistant	3frameshift	Q27E, A70G, 86frameshift
19	DM60	64	Resistant	R16C, P96L	None
20	DM62	64	Resistant	A68T, G189S	Q27E, P41L, A70G, N111H, N129T, S130D, M146I
21	DM64	64	Resistant	9frameshift	Q27E, A32E, N111H, N129T, S130D, E176K, N182D
22	DM73	64	Resistant	L188F, K190*	None
23	DM75	64	Resistant	K190Y, A193*	Q27E, A32E, T110A, N111H, N129T, S130D, E176K
24	DM76	128	Resistant	122–149del, D205A	Poor sequence quality
25	DM79	64	Resistant	S30G, E38*	Q27E, N111H, N129T, S130D, E176K
26	DM81	128	Resistant	42frameshift	V103I
27	DM84	64	Resistant	G122S	Q27E, A32E, N111H, N129T, S130D, E176K, N182D
28	DM86	64	Resistant	T31H, V57A, L62V, K64N, P106S, G155*	NONE
29	DM87	64	Resistant	V85G, G145E, V151A, V192Q, A193G, E194R, Q197E, R200*	Q27E, N111H, N129T, S130D, E176K
30	DM88	32	Resistant	R16H, R200M, S202I, 204frameshift	None
31	DM89	64	Resistant	65frameshift	Q27E, N111H, N129T, S130D, E176K, N182D
32	DM96	64	Resistant	E35*	Q27E, N111H, M126I, N129T, S130D, E176K, N182D
33	DM100	32	Resistant	S108A, K190E	A153V, E176K
34	DM103	32	Resistant	C19*	Poor sequence quality
35	DM104	64	Resistant	R16H, 207del	22frameshift
36	DM105	16	Resistant	P51S, K203Q	18frameshift
37	DM112	64	Resistant	27frameshift	Q27E, N111H, N129T, S130D, E176K, N182D
38	DM115	64	Resistant	R16H, H97Y, V182A, R200*	A85V, N111H
39	DM117	32	Resistant	H69P, V85G, V86E, Q139H, L153*	Q27E, L72I, N111H, M126I, N129T, S130D, E169A, E176K, N182D
40	DM118	32	Resistant	S18F, S43L, K63Q, G155R, K198T, R200*	Q27E, N111H, M126I, N129T, S130D, E176K, N182D
41	DM126	64	Resistant	V85G, F117V, G122A, I147L, K198I, 208frameshift	Q27E, N111H, N129T, S130D, E169A, N182D
42	DM142	64	Resistant	R16H, G122A, P180T, 197frameshift	Q27E, N111H, N129T, S130D, N182D
43	DM143	64	Resistant	Q50*	18frameshift
44	DM146	64	Resistant	T31H, 192frameshift	Poor sequence quality
45	DM147	64	Resistant	R16Y, H25Y, E35D, L62F, Q146H, R200*	18frameshift
46	DM148	128	Resistant	R16C, R166S, 198frameshift	A153V
47	DM150	16	Resistant	Q197H, 200frameshift	R38I
48	DM151	128	Resistant	C140Y, Q197*	N111H, N129T, S130D, E176K
49	DM152	64	Resistant	E35*	Q27E, N111H, N129T, S130D, N182D

MIC = minimum inhibitory concentration. None: no specific mutations were identified; A68T means threonine replaced alanine in amino acid position 68; R200* means a stop codon replaced arginine in amino acid position 200; 150frameshift means there was a frameshift mutation in amino acid position 150. We ignored mutations that were present in both sensitive and resistant strains.

detected in this study have been described previously. The substitutions T2183C and A2223G have been more frequently associated with CAM resistance in Asian countries than in Europe and North America.[32] The A2143G substitution has much stronger impact on CAM resistance compared with A2142G or A2142C.[33]

In agreement with reports from most Latin American countries,[17] the frequency of resistance to AMX in isolates from the Dominican Republic is low, despite AMX being one of the most commonly used antibiotics around the world. Previous reports have indicated that a low detected frequency of resistance was associated with the phenotypic loss of *pbp1A* after storage or freezing of isolates.[34,35] However, Hu and others showed that resistance to AMX was stable before and after the storage at –80°C for 3 months or even years.[36] Indeed, *H. pylori* resistance to AMX does not necessarily decrease treatment efficacy.[37] These could compromise the utilization of AMX in most regimens, where it is commonly used owing to its adequacy and low cost. AMX-based triple or quadruple therapies that include TCN may be a useful alternative first-line regimen in the Dominican Republic.

The extremely high frequency of resistance to MNZ found in the Dominican Republic in this study could be attributable

TABLE 5

Mutations in other genes that were associated with metronidazole resistance

No.	Strains	MIC (mg/L)	Profile	*rpsU* (*hp0562*)	*rps4* (*hp1294*)	*dppA* (*hp0298*)	*dppB* (*hp0299*)	*rnc* (hp0662)	*dapF* (*hp0566*)	*recA* (*hp0153*)	*fdxA* (*hp0277*)	*fdxB* (*hp1508*)	*trx1* (*hp0824*)
1	DM30	1	Sensitive	None	None	35del, 36del, 37del, 179H	None	None	None	None	None	None	E9G
2	DM116	1	Sensitive	None	None	None	None	None	None	None	None	None	None
3	DM76	128	Resistant	None	A48T, V170A	E473G	I322V	P15L	V16A, A84N, I109V, K117E, E161G, G162L, R221C, G248E, A257V, R266G, E269K	None	N32S, S79I	V64I, M65L, P245A, I304T, A428I, A439T	S14N
4	DM151	128	Resistant	A34T	None	T32A, E473G	R124C	1del, N152K,	K50N, N164F, E177A, R221C, G248E, A257I, R266G, G271H	None	43del, 48del, S79I	F125L, A227V, N231K, P245A, R307H, M321L, A325T	S14N
5	DM4	64	Resistant	None	None	None	K199R, I322V	1del	V16A, K50R, D51N, A84N, K117E, E161G, G162L, G248E, A245V, R266G, G271H	None	N32S, S79I	A38V, V64I, M65L, A227V	None
6	DM64	64	Resistant	C32Y	None	E473G	I322V	P15S	V16A, K50R, D51N, A84N, I109V, K117E, E161G, G162L, G248E, A257V, R266G, E269K	None	N32S, S79I	V64I, M65L, P245A, H311A, N395S, N424H	None
7	DM18	32	Resistant	None	None	E473G	I21V, V127M, I322V	I13A, R109H	V16A, A84N, I109V, K117E, E161G, G162L, G248D, A257V, R266G, E269K	None	N32S, S79I	V64I, M65L, M163V, P245T, S417G, A428I	N102S
8	DM51	32	Resistant	None	None	Q383E	None	None	K129N, A254T, V267I	S40D, I191V	N32S, S79I	S122R, P123S, D170E, I173V, P245A, N424S	I40V

MIC = minimum inhibitory concentration; None: no specific mutations were identified; 35del means there was a deletion at amino acid position 35; I79H means histidine replaced isoleucine in amino acid position 79. We ignored mutations that were present in both sensitive and resistant strains.

TABLE 6
Mutations in *gyrA* and *gyrB* that were associated with levofloxacin resistance

No.	Strain	MIC (mg/L)	Profile	*gyrA* mutations	*gyrB* mutations
1	DM8	0.25	Sensitive	None	None
2	DM32	0.25	Sensitive	None	None
3	DM57	0.25	Sensitive	None	None
4	DM86	0.25	Sensitive	None	None
5	DM95	0.25	Sensitive	None	None
6	DM9	16	Resistant	None	None
7	DM10	32	Resistant	N87I	None
8	DM14	2	Resistant	D91G, E193D, I194F, A197F	None
9	DM18	16	Resistant	N87T, D91N	None
10	DM43	2	Resistant	N87T, D91N	None
11	DM44	16	Resistant	N87T, A97V	None
12	DM49	32	Resistant	N87A	D435N, S479G
13	DM50	2	Resistant	E58G, G75V, D91G, Q98L	None
14	DM51	2	Resistant	D91N	None
15	DM60	2	Resistant	D91Y, A134V, D145N	None
16	DM62	2	Resistant	D91N	S479G
17	DM71	32	Resistant	N87I	S479G
18	DM73	32	Resistant	None	None
19	DM75	32	Resistant	N87I	None
20	DM96	4	Resistant	None	None
21	DM103	16	Resistant	N87I, D91N, I162T	None
22	DM115	2	Resistant	N87K	None
23	DM142	4	Resistant	N87T, D91N	None
24	DM146	16	Resistant	N87T	None
25	DM147	2	Resistant	D91Y	None
26	DM148	2	Resistant	None	None
27	DM150	16	Resistant	N87T, D91N, F149V	None
28	DM152	8	Resistant	N87I	Y514F

MIC = minimum inhibitory concentration. None: no specific mutations were identified; N87I means isoleucine replaced asparagine in amino acid position 87. We ignored mutations that were present in both sensitive and resistant strains.

to widespread over-the-counter use of this drug. MNZ is a drug with a modest cost that is used to treat not only *H. pylori* infections but also many other diseases; for example, intestinal parasites and periodontal and gynecologic diseases that are common in developing countries such as the Dominican Republic.[28,38] Therefore, regimens that include MNZ should not be chosen as a first-line treatment therapy in this population. However, in vitro resistance to MNZ may not accurately reflect in vivo resistance; therefore, MNZ resistance should be confirmed by determining concentrations of the drug in the blood of treated patients.[39] In addition, because most MNZ-resistant isolates showed MNZ MICs > 32 mg/L, this might represent an appropriate breakpoint, although clinical trials are required to confirm MNZ resistance in the Dominican Republic. Importantly, MNZ resistance is not more clinically relevant than CAM resistance. Furthermore, resistance to MNZ can be overcome by prolonging treatment and adding bismuth to the treatment regimen.[40]

By comparing the Dominican Republic MNZ-sensitive and MNZ-resistant strains, we recognized a high number of *rdxA* mutations in the MNZ-resistant strains. We also confirmed some mutations that could be explained by resistance structure (class mutations).[25] In contrast with results of a previous study, we found other mutations (E35, V85, G122, K190, and K198) that might be important to resistance in the Dominican strains. This agrees with a previous report, which showed that *rdxA* mutations were not uniform across all geographical regions.[41] Additionally, several *frxA* mutations were associated with MNZ resistance. We observed one strain without a mutation in *rdxA*, but that contained an *frxA* mutation (MNZ MIC of 64 mg/L), suggesting a role for *frxA*, irrespective of *rdxA*, although the combination of mutations in these two genes was not associated with higher-level resistance. Finally, we identified novel mutations in *dppA*, *dppB*, *fdxA*, and *fdxB* that were not associated with mutations in *rdxA* and *frxA*. Importantly, mutations in *trx1* and *dapF* were associated with difference levels of MNZ resistance, indicated by MIC values. Unlike *dapF*, which is associated with lysine and peptidoglycan biosynthesis,[42] *trx1* has a role as an electron donor for alkyl-hydroperoxide reductase, which is associated with MNZ resistance in vitro.[43]

LVX has recently been prescribed as a second-line drug to eradicate *H. pylori* in patients who experienced failed

TABLE 7
Mutations in the 23S rRNA that were associated with clarithromycin resistance

Strain	MIC (mg/L)	Profile	Mutations
DM44	≤ 0.03	Sensitive	None
DM62	16	Resistant	T1958G
DM105	16	Resistant	A1957G, T1958G, G1964T, A1968T, A2142G*

MIC = minimum inhibitory concentration.
*A recognized mutation; T1958G means guanine replaced thymine at amino acid position 1958.

first-line therapies.[44,45] However, the frequency of LVX resistance is expanding worldwide, including Latin America.[17] A similar expansion of resistance is observed in urinary tract *Escherichia coli* isolates from Latin America.[46] This may lead to cross-resistance with fluoroquinolone of different generations, including nalidixic acid, ciprofloxacin, and ofloxacin. Furthermore, the frequency of LVX resistance in the Dominican Republic is much higher than the average frequencies of fluoroquinolone resistance reported in Latin America (15.0%),[17] Europe (14.1%),[28] and Asia (11.6%).[24] According to European, Asia-Pacific, and American guidelines, LVX should be used in rescue treatments only after antibiotic susceptibility testing.[1,19,47]

We observed substitutions at Asn-87 and Asp-91 that were associated with LVX resistance as previously reported.[41,48,49] In addition, few mutations and the co-occurrence of the Ser-479 substitution in the *gyrB* subunit with substitutions in *gyrA* suggested that *gyrB* mutations had minimal influence on LVX-resistant strains in this study. Three resistant strains showed no changes in *gyrA* or *gyrB*. This suggests that substitutions at other sites might act synergistically in LVX resistance in the Dominican Republic strains.

Although only two strains were resistant to three antibiotics, 31.3% of the strains showed dual antibiotic resistance: this resistance was exclusive to MNZ and LVX. The Dominican Republic has a high prevalence of *H. pylori* infections,[18] and increased resistance to antibiotics used to treat these infections might result in an increased frequency of disease recurrence. With high rates of morbidity and mortality due to *H. pylori* infection–associated pathologies, prevention is the ultimate solution. Our results are very important as a guide for antibiotic use as follow-up to first-line treatment regimen failures. Mutations associated with antibiotic resistance (e.g., 23S rRNA point mutations at A2143G and A2144G) was determined using a fully automated rapid genetic analyzer within 60–120 minutes, compared with the 7–10 days required for testing by culture.[50] These results also suggest that alternative strategies such as bismuth or non-bismuth-based four-drug regimens or sequential therapies may be more effective in cases of CAM-based triple therapy failure in the Dominican Republic.

The number of samples analyzed here was relatively low, which presents a limitation to this study. In addition, we did not consider the socioeconomic status, ethnicity, or residence of patients. The Digestive Disease Center is the only specialized public medical center for digestive diseases in the Dominican Republic, with half of patients coming from the countryside and most patients living at a low-middle socioeconomic level. Further studies are required to determine the association of sociodemographic data with antibiotic resistance patterns in the Dominican Republic.

In conclusion, the frequencies of resistance to MNZ and LVX were high in the Dominican Republic, and resistance to these antibiotics was associated with genetic mutations. It has been suggested that MNZ- and LVX-based triple therapies are not useful as a first-line treatment regimen in the Dominican Republic. Fortunately, the prevalence of CAM resistance was found to be low in this study, suggesting that the CAM-based triple therapy may still be useful as an initial treatment of *H. pylori* infection in the Dominican Republic. Epidemiological surveillance of antibiotic resistance in *H. pylori* strains will be required to determine optimal treatment strategies for use in the Dominican Republic.

Received September 8, 2016. Accepted for publication December 16, 2016.

Published online February 13, 2017.

Financial support: This work was supported in part by grants from the National Institutes of Health (DK62813) and the Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan (25293104, 26640114, 15H02657, and 221S0002) (Yoshio Yamaoka). It was also supported by the Japan Society for the Promotion of Science (JSPS) Institutional Program for Young Researcher Overseas Visits (Yoshio Yamaoka), Strategic Funds for the Promotion of Science and Technology from the Japan Science and Technology Agency (JST) (Yoshio Yamaoka). In addition, it was supported in part by a grant from The National Fund for Innovation and Development of Science and Technology (FONDOCYT) from the Ministry of Higher Education Science and Technology (MESCyT) of the Dominican Republic (2012-2013-2A1-65 and 2015-3A1-182) (Modesto Cruz). Muhammad Miftahussurur was a PhD student and Phawinee Subsomwong is a PhD student supported by The Japanese Government (MEXT) Scholarship Program for 2012 and 2013, respectively.

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REFERENCES

1. Fock KM, Katelaris P, Sugano K, Ang TL, Hunt R, Talley NJ, Lam SK, Xiao SD, Tan HJ, Wu CY, Jung HC, Hoang BH, Kachintorn U, Goh KL, Chiba T, Rani AA, Second Asia-Pacific Conference, 2009. Second Asia-Pacific Consensus Guidelines for *Helicobacter pylori* infection. *J Gastroenterol Hepatol 24:* 1587–1600.
2. Malfertheiner P, Megraud F, O'Morain CA, Atherton J, Axon AT, Bazzoli F, Gensini GF, Gisbert JP, Graham DY, Rokkas T, El-Omar EM, Kuipers EJ; European Helicobacter Study Group, 2012. Management of *Helicobacter pylori* infection: the Maastricht IV/ Florence Consensus Report. *Gut 61:* 646–664.
3. Papastergiou V, Georgopoulos SD, Karatapanis S, 2014. Treatment of *Helicobacter pylori* infection: meeting the challenge of antimicrobial resistance. *World J Gastroenterol 20:* 9898–9911.
4. Miftahussurur M, Yamaoka Y, 2015. Appropriate first-line regimens to combat *Helicobacter pylori* antibiotic resistance: an Asian perspective. *Molecules 20:* 6068–6092.
5. Suzuki RB, Lopes RA, da Camara Lopes GA, Hung Ho T, Speranca MA, 2013. Low *Helicobacter pylori* primary resistance to clarithromycin in gastric biopsy specimens from dyspeptic patients of a city in the interior of Sao Paulo, Brazil. *BMC Gastroenterol 13:* 164.
6. Stone GG, Shortridge D, Flamm RK, Versalovic J, Beyer J, Idler K, Zulawinski L, Tanaka SK, 1996. Identification of a *23S rRNA* gene mutation in clarithromycin-resistant *Helicobacter pylori*. *Helicobacter 1:* 227–228.
7. Goodwin A, Kersulyte D, Sisson G, Veldhuyzen van Zanten SJ, Berg DE, Hoffman PS, 1998. Metronidazole resistance in *Helicobacter pylori* is due to null mutations in a gene (*rdxA*) that encodes an oxygen-insensitive NADPH nitroreductase. *Mol Microbiol 28:* 383–393.

8. Jeong JY, Mukhopadhyay AK, Dailidiene D, Wang Y, Velapatino B, Gilman RH, Parkinson AJ, Nair GB, Wong BC, Lam SK, Mistry R, Segal I, Yuan Y, Gao H, Alarcon T, Brea ML, Ito Y, Kersulyte D, Lee HK, Gong Y, Goodwin A, Hoffman PS, Berg DE, 2000. Sequential inactivation of *rdxA* (HP0954) and *frxA* (HP0642) nitroreductase genes causes moderate and high-level metronidazole resistance in *Helicobacter pylori*. *J Bacteriol 182:* 5082–5090.
9. Binh TT, Suzuki R, Trang TT, Kwon DH, Yamaoka Y, 2015. Search for novel candidate mutations for metronidazole resistance in *Helicobacter pylori* using next-generation sequencing. *Antimicrob Agents Chemother 59:* 2343–2348.
10. Li AQ, Dai N, Yan J, Zhu YL, 2007. Screening for metronidazole-resistance associated gene fragments of *H. pylori* by suppression subtractive hybridization. *World J Gastroenterol 13:* 1847–1850.
11. Chang KC, Ho SW, Yang JC, Wang JT, 1997. Isolation of a genetic locus associated with metronidazole resistance in *Helicobacter pylori*. *Biochem Biophys Res Commun 236:* 785–788.
12. Mukhopadhyay AK, Jeong JY, Dailidiene D, Hoffman PS, Berg DE, 2003. The *fdxA* ferredoxin gene can down-regulate *frxA* nitroreductase gene expression and is essential in many strains of *Helicobacter pylori*. *J Bacteriol 185:* 2927–2935.
13. Kwon DH, El-Zaatari FA, Kato M, Osato MS, Reddy R, Yamaoka Y, Graham DY, 2000. Analysis of *rdxA* and involvement of additional genes encoding NAD(P)H flavin oxidoreductase (*FrxA*) and ferredoxin-like protein (*FdxB*) in metronidazole resistance of *Helicobacter pylori*. *Antimicrob Agents Chemother 44:* 2133–2142.
14. Baker LM, Raudonikiene A, Hoffman PS, Poole LB, 2001. Essential thioredoxin-dependent peroxiredoxin system from *Helicobacter pylori*: genetic and kinetic characterization. *J Bacteriol 183:* 1961–1973.
15. Jones KR, Cha JH, Merrell DS, 2008. Who's winning the war? Molecular mechanisms of antibiotic resistance in *Helicobacter pylori*. *Curr Drug Ther 3:* 190–203.
16. Rimbara E, Noguchi N, Kawai T, Sasatsu M, 2012. Fluoroquinolone resistance in *Helicobacter pylori*: role of mutations at position 87 and 91 of *GyrA* on the level of resistance and identification of a resistance conferring mutation in *GyrB*. *Helicobacter 17:* 36–42.
17. Camargo MC, Garcia A, Riquelme A, Otero W, Camargo CA, Hernandez-Garcia T, Candia R, Bruce MG, Rabkin CS, 2014. The problem of *Helicobacter pylori* resistance to antibiotics: a systematic review in Latin America. *Am J Gastroenterol 109:* 485–495.
18. Shiota S, Cruz M, Abreu JA, Mitsui T, Terao H, Disla M, Iwatani S, Nagashima H, Matsuda M, Uchida T, Tronilo L, Rodriguez E, Yamaoka Y, 2014. Virulence genes of *Helicobacter pylori* in the Dominican Republic. *J Med Microbiol 63:* 1189–1196.
19. Malfertheiner P, Megraud F, O'Morain C, Bazzoli F, El-Omar E, Graham D, Hunt R, Rokkas T, Vakil N, Kuipers EJ, 2007. Current concepts in the management of *Helicobacter pylori* infection: the Maastricht III Consensus Report. *Gut 56:* 772–781.
20. Malfertheiner P, Megraud F, O'Morain CA, Gisbert JP, Kuipers EJ, Axon AT, Bazzoli F, Gasbarrini A, Atherton J, Graham DY, Hunt R, Moayyedi P, Rokkas T, Rugge M, Selgrad M, Suerbaum S, Sugano K, El-Omar EM; European Helicobacter and Microbiota Study Group and Consensus Panel, 2017. Management of *Helicobacter pylori* infection: the Maastricht V/Florence Consensus Report. *Gut 66:* 6–30.
21. Kwon DH, Pena JA, Osato MS, Fox JG, Graham DY, Versalovic J, 2000. Frameshift mutations in *rdxA* and metronidazole resistance in North American *Helicobacter pylori* isolates. *J Antimicrob Chemother 46:* 793–796.
22. Wang LH, Cheng H, Hu FL, Li J, 2010. Distribution of *gyrA* mutations in fluoroquinolone-resistant *Helicobacter pylori* strains. *World J Gastroenterol 16:* 2272–2277.
23. Ho SL, Tan EL, Sam CK, Goh KL, 2010. Clarithromycin resistance and point mutations in the *23S rRNA* gene in *Helicobacter pylori* isolates from Malaysia. *J Dig Dis 11:* 101–105.
24. De Francesco V, Giorgio F, Hassan C, Manes G, Vannella L, Panella C, Ierardi E, Zullo A, 2010. Worldwide *H. pylori* antibiotic resistance: a systematic review. *J Gastrointestin Liver Dis 19:* 409–414.
25. Martinez-Julvez M, Rojas AL, Olekhnovich I, Espinosa Angarica V, Hoffman PS, Sancho J, 2012. Structure of RdxA: an oxygen-insensitive nitroreductase essential for metronidazole activation in *Helicobacter pylori*. *FEBS J 279:* 4306–4317.
26. Jenks PJ, Ferrero RL, Labigne A, 1999. The role of the *rdxA* gene in the evolution of metronidazole resistance in *Helicobacter pylori*. *J Antimicrob Chemother 43:* 753–758.
27. Kwon DH, Hulten K, Kato M, Kim JJ, Lee M, El-Zaatari FA, Osato MS, Graham DY, 2001. DNA sequence analysis of *rdxA* and *frxA* from 12 pairs of metronidazole-sensitive and -resistant clinical *Helicobacter pylori* isolates. *Antimicrob Agents Chemother 45:* 2609–2615.
28. Megraud F, Coenen S, Versporten A, Kist M, Lopez-Brea M, Hirschl AM, Andersen LP, Goossens H, Glupczynski Y; Study Group Participants, 2013. *Helicobacter pylori* resistance to antibiotics in Europe and its relationship to antibiotic consumption. *Gut 62:* 34–42.
29. Boyanova L, Mitov I, 2010. Geographic map and evolution of primary *Helicobacter pylori* resistance to antibacterial agents. *Expert Rev Anti Infect Ther 8:* 59–70.
30. Wirtz VJ, Dreser A, Gonzales R, 2010. Trends in antibiotic utilization in eight Latin American countries, 1997–2007. *Rev Panam Salud Publica 27:* 219–225.
31. Oleastro M, Ménard A, Santos A, Lamouliatte H, Monteiro L, Barthélémy P, Mégraud F, 2003. Real-time PCR assay for rapid and accurate detection of point mutations conferring resistance to clarithromycin in *Helicobacter pylori*. *J Clin Microbiol 41:* 397–402.
32. Ierardi E, Giorgio F, Losurdo G, Di Leo A, Principi M, 2013. How antibiotic resistances could change *Helicobacter pylori* treatment: a matter of geography? *World J Gastroenterol 19:* 8168–8180.
33. De Francesco V, Margiotta M, Zullo A, Hassan C, Troiani L, Burattini O, Stella F, Di Leo A, Russo F, Marangi S, Monno R, Stoppino V, Morini S, Panella C, Ierardi E, 2006. Clarithromycin-resistant genotypes and eradication of *Helicobacter pylori*. *Ann Intern Med 144:* 94–100.
34. Dore MP, Osato MS, Realdi G, Mura I, Graham DY, Sepulveda AR, 1999. Amoxycillin tolerance in *Helicobacter pylori*. *J Antimicrob Chemother 43:* 47–54.
35. Yakoob J, Fan X, Hu G, Liu L, Zhang Z, 2001. Antibiotic susceptibility of *Helicobacter pylori* in the Chinese population. *J Gastroenterol Hepatol 16:* 981–985.
36. Hu CT, Wu CC, Lin CY, Cheng CC, Su SC, Tseng YH, Lin NT, 2007. Resistance rate to antibiotics of *Helicobacter pylori* isolates in eastern Taiwan. *J Gastroenterol Hepatol 22:* 720–723.
37. Kim N, Kim JM, Kim CH, Park YS, Lee DH, Kim JS, Jung HC, Song IS, 2006. Institutional difference of antibiotic resistance of *Helicobacter pylori* strains in Korea. *J Clin Gastroenterol 40:* 683–687.
38. Megraud F, 2004. *H. pylori* antibiotic resistance: prevalence, importance, and advances in testing. *Gut 53:* 1374–1384.
39. Farshad S, Alborzi A, Japoni A, Ranjbar R, Hosseini Asl K, Badiee P, Amin Shahidi M, Hosseini M, 2010. Antimicrobial susceptibility of *Helicobacter pylori* strains isolated from patients in Shiraz, southern Iran. *World J Gastroenterol 16:* 5746–5751.
40. Malfertheiner P, Bazzoli F, Delchier JC, Celinski K, Giguere M, Riviere M, Megraud F; Pylera Study Group, 2011. *Helicobacter pylori* eradication with a capsule containing bismuth subcitrate potassium, metronidazole, and tetracycline given with omeprazole versus clarithromycin-based triple therapy: a randomised, open-label, non-inferiority, phase 3 trial. *Lancet 377:* 905–913.
41. Teh X, Khosravi Y, Lee WC, Leow AH, Loke MF, Vadivelu J, Goh KL, 2014. Functional and molecular surveillance of *Helicobacter pylori* antibiotic resistance in Kuala Lumpur. *PLoS One 9:* e101481.

42. Richaud C, Higgins W, Mengin-Lecreulx D, Stragier P, 1987. Molecular cloning, characterization, and chromosomal localization of dapF, the *Escherichia coli* gene for diaminopimelate epimerase. *J Bacteriol 169:* 1454–1459.
43. Trend MA, Jorgensen MA, Hazell SL, Mendz GL, 2001. Oxidases and reductases are involved in metronidazole sensitivity in *Helicobacter pylori*. *Int J Biochem Cell Biol 33:* 143–153.
44. Gisbert JP, Gonzalez L, Calvet X, 2005. Systematic review and meta-analysis: proton pump inhibitor vs. ranitidine bismuth citrate plus two antibiotics in *Helicobacter pylori* eradication. *Helicobacter 10:* 157–171.
45. Gisbert JP, 2009. Second-line rescue therapy of *Helicobacter pylori* infection. *Therap Adv Gastroenterol 2:* 331–356.
46. Andrade SS, Sader HS, Jones RN, Pereira AS, Pignatari AC, Gales AC, 2006. Increased resistance to first-line agents among bacterial pathogens isolated from urinary tract infections in Latin America: time for local guidelines? *Mem Inst Oswaldo Cruz 101:* 741–748.
47. Chey WD, Wong BC; Practice Parameters Committee of the American College of Gastroenterology, 2007. American College of Gastroenterology guideline on the management of *Helicobacter pylori* infection. *Am J Gastroenterol 102:* 1808–1825.
48. Nishizawa T, Suzuki H, Kurabayashi K, Masaoka T, Muraoka H, Mori M, Iwasaki E, Kobayashi I, Hibi T, 2006. Gatifloxacin resistance and mutations in *gyra* after unsuccessful *Helicobacter pylori* eradication in Japan. *Antimicrob Agents Chemother 50:* 1538–1540.
49. Garcia M, Raymond J, Garnier M, Cremniter J, Burucoa C, 2012. Distribution of spontaneous *gyrA* mutations in 97 fluoroquinolone-resistant *Helicobacter pylori* isolates collected in France. *Antimicrob Agents Chemother 56:* 550–551.
50. Sugimoto M, Furuta T, 2014. Efficacy of tailored *Helicobacter pylori* eradication therapy based on antibiotic susceptibility and CYP2C19 genotype. *World J Gastroenterol 20:* 6400–6411.