Point Mutations at *gyrA* and *gyrB* Genes of Levofloxacin-Resistant *Helicobacter pylori* Isolates in the Esophageal Mucosa from a Venezuelan Population

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Abstract. The treatment of *Helicobacter pylori* infection is complicated by antibiotic resistance. A high levofloxacin (LVX) resistance rate was previously demonstrated in *H. pylori* isolates from gastric mucosa (40%) and esophagus (19%) in individual hosts of a Venezuelan population. We aimed to assess the molecular mechanisms of LVX resistance and susceptibility in isolates from the gastroesophageal mucosa, by studying point mutations in the quinolone resistance-determining region of *gyrA* and *gyrB* genes. Sequencing of *gyrA* and *gyrB* genes ($N = 120$) helped to identify point mutations in 60 isolates (30 from antrum and 30 from esophagus) of five dyspeptic patients. Double (Asn87Thr and Asp91Asn) and single (Asn87Ile or Asn87Thr) mutations in the *gyrA* gene were identified in the esophageal mucosa. These mutations have been commonly found in the stomach. Occurrence of a single (Asn87Ile) mutation was associated with high resistance (minimum inhibitory concentration ≥ 32 $\mu\text{g/mL}$) to LVX. Only a single (Ser479Gly) mutation was found in the *gyrB* gene in both mucosae. One patient presented isolates with no mutations in the two genes studied. Isolates with the same mutation pattern in individual hosts revealed identical genetic profiles for these genes, confirming that isolates identified in the esophageal mucosa come from isolates colonizing the stomach. *Helicobacter pylori* resistance to LVX in the esophagus is related to double- and single-point mutations in *gyrA* and *gyrB* genes, such as those found in the stomach. Levofloxacin should be applied with caution, because its antibiotic effect on *H. pylori* is decreasing in Latin America, perhaps owing to high prescription rates.

INTRODUCTION

Helicobacter pylori is a Gram-negative rod-shaped bacterium with microaerophilic metabolism, capable of colonizing the gastric mucosa, making it responsible for severe gastrointestinal disorders (peptic ulceration, chronic gastritis, gastric lymphoma, and gastric adenocarcinoma).¹ The esophagus represents a niche for *H. pylori* colonization due to multiple microenvironment conditions. However, few studies have corroborated its presence in the esophageal mucosa.^{2,3} In Venezuela, high prevalence of *H. pylori* in the esophagus (86%) and a heterogeneous distribution of *cag*-pathogenicity island (*cag*-PAI) genotypes have been demonstrated, indicating the coexistence of different strains in a single host.^{4,5} Reports provided by our research group demonstrated multiple *H. pylori* colonization in the esophageal mucosa by studying *cag*-PAI genotypes and antibiotic resistance profiles. *Helicobacter pylori* in the esophageal mucosa was characterized by a partial *cag*-PAI island and low antibiotic resistance, whereas a higher antibiotic resistance and a complete island were found in the gastric mucosa.⁶

Helicobacter pylori has a remarkable genetic variability and high allelic diversity, determining that isolates from different patients and/or same individual may have distinct genotypes; this is driven by an elevated mutation rate and frequent intraspecific recombination that contribute to host adaptation by the increment of antibiotic resistance probabilities.⁷ Antibiotic resistance is a major problem in the treatment of *H. pylori* as it is the principal reason for the failure of eradication regimens (20–30%).⁸ Different treatment schemes have been recommended at consensus, including the following: amoxicillin

and clarithromycin or metronidazole with a proton pump inhibitor given for a week as the first-line eradication treatment.⁹ Among other antibiotics, levofloxacin (LVX) has been evaluated in several studies and used for the treatment of patients failing to respond to the first-line scheme.^{9,10} Levofloxacin is a fluoroquinolone with broad activity against Gram-negative and Gram-positive bacteria, which inhibits the DNA gyrase and topoisomerase IV. The common mechanism for fluoroquinolone resistance comprises point mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* and *gyrB* genes from the DNA gyrase.^{11,12}

High resistance to LVX has been confirmed in *H. pylori* isolates from the gastroesophageal mucosa by E-test.⁶ The present study aimed to investigate point mutations in the QRDR of *gyrA* and *gyrB* genes of LVX-resistant *H. pylori* isolates from the gastric and esophageal mucosa in individual Venezuelan patients.

MATERIALS AND METHODS

Patients. Subjects were selected from those having matching *H. pylori*-positive cultures of biopsies of both gastric and esophageal mucosae from a previous study (8 out of 80 dyspeptic patients) taken at the gastroenterology unit of the Oncology Service Hospital of Instituto Venezolano de los Seguros Sociales (IVSS).^{5,6} All volunteers signed an informed consent form in accordance with the bioethics committees of Instituto Venezolano de Investigaciones Científicas (IVIC) and IVSS. During endoscopy procedure, antral and esophageal biopsy specimens were collected and submitted for *H. pylori* culture as previously described.⁶ Patients were selected from those *H. pylori*-positive in both gastroesophageal biopsies and culture.^{5,6} Antimicrobial susceptibility testing by E-test had previously demonstrated a high LVX resistance rate in 96 isolates of the eight patients: 48 isolates from the gastric mucosa (40%) and 48 isolates from the esophageal mucosa (19%).⁶ For this study, only

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60 isolates in both mucosae (5/8 patients) were selected to assess the molecular mechanisms of LVX resistance and susceptibility, as described in other sections.

Isolates. From each *H. pylori*-positive culture, six small translucent colonies were picked and identified by morphological characteristics, gram staining, and rapid urease test (Ure-IVIC).¹³ A total of 60 isolates were obtained from both mucosae in the five selected patients. Isolates were preserved in sterile *Brucella* broth with 15% glycerol at -80°C .⁶ DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions and stored at -20°C . All isolates obtained from the cultures were processed separately for *Helicobacter* genus and *H. pylori* species by polymerase chain reaction (PCR) assays amplifying the 16S rRNA, *glmM*, and *ureA* fragments of 399, 294, and 491 bp, respectively.^{14–16} Polymerase chain reaction and sequencing of the *gyrA* and *gyrB* genes were performed for mutation analysis and antimicrobial susceptibility and resistance analysis for LVX through the epsilometer test (Etest; AB bioMérieux, Askim, Sweden).

Antimicrobial susceptibility testing by E-test. The E-test for LVX was performed in 30 gastric isolates and 30 esophageal isolates, as previously described.⁶ The resistance breakpoint for LVX was defined as the minimum inhibitory concentration (MIC) of ≥ 1 $\mu\text{g/mL}$, according to the Clinical and Laboratory Standards Institute 2010, similar to EUCAST breakpoints (http://eucaast.org/clinical_breakpoints/). *Helicobacter pylori* isolates were classified according to a MIC range and defined as highly resistant ≥ 32 $\mu\text{g/mL}$ or resistant from 1 to 32 $\mu\text{g/mL}$.¹⁷ A strain of *H. pylori* ATCC43504 was used as a positive control for antimicrobial susceptibility testing.

***gyrA* and *gyrB* genes PCR assays.** The genes *gyrA* and *gyrB* genes were PCR amplified, sequenced, and analyzed for the detection of point mutations in the QRDR. Specific primers were used for each gene as previously described: *gyrA* primers F5'-TTT AGC TTA TTC CAT GAG CGT-3' and R5'-GCA GAC GGC TTG GTA AAA TA-3', *gyrB* primers F5'-TGC AAA AGC CAG AGA AGC CA-3' and R5'-ACA TGC CCT TGT TCA ATC AGC-3'.¹⁸ Polymerase chain reactions were performed using colorless Go Taq Hot Start Master Mix (Promega Corp., Madison, WI) in a thermal cycler model GeneAMP PCR System 9700 (Applied Biosystems, Hayward, CA). DNA from the strain *H. pylori* ATCC26695 was used as a positive control. The PCR thermal cycling conditions were performed at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, and extension at 72°C for 1 minute. A final extension at 72°C for 10 minutes, and finally at 4°C constant.¹² Subsequently, the amplicons were visualized by running 7 μL of the reaction mixture in a TBE agarose gel (2.0%), stained with ethidium bromide, and observed by means of photography under UV transillumination.

Sequencing analysis. Fragments of the *gyrA* (426 bp) and *gyrB* (444 bp) genes were purified for sequencing after the MacroGen protocol and sequenced at MacroGen Inc., Seoul, Korea. Sequences were compared with a compilation of the *gyrA* and *gyrB* gene sequences available in the GenBank nucleotide library by BLAST searching at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>) and the ATCC26695 as control; multiple alignments were performed using BioEdit v7.2.5 software package (<http://www.codoncode.com>). The corresponding amino acid sequences were obtained from the nucleotide

sequences using the ExPASy Translate tool. The *gyrA* and *gyrB* gene sequences (~400–450 bp) of the 60 isolates were deposited in GenBank under the accession numbers (KY807298–KY807417).

RESULTS

Antimicrobial susceptibility by E-test. All 60 isolates (30 obtained from antrum and 30 from esophagus) were positive for *H. pylori*. Overall, 47% (28/60) of all isolates were resistant to LVX, whereas 53% (32/60) were susceptible to LVX in the gastric and the esophageal mucosa. When the resistance to LVX was compared by individual mucosa in these 60 isolates, we found that 60% (18/30) of the isolates presented a higher resistance in the gastric mucosa than in the esophageal mucosa 33% (10/30) (Table 1).

***gyrA* PCR and sequencing analysis.** On the basis of the sequencing analysis of *gyrA* gene point mutations, we found amino acid changes in isolates from patients 2, 3, 4, and 5, but none in patient 1. Point mutations in the *gyrA* gene that do not change the amino acid composition (silent mutations) were common in all isolates (C571T, A619C, and T739C).

In the gastric mucosa, isolates from patients 2 and 3 showed double mutations at amino acid positions 87 (Asn87Thr) and 91 (Asp91Asn) in the QRDR of the *gyrA* gene, which were the most commonly found mutations (Table 2). Isolates from the patients 4 and 5 presented one single mutation, either Asn87Thr or Asn87Ile. In the esophagus, isolates from patient 2 also showed double mutations at positions 87 and 91, and the rest of the isolates presented one single mutation, either Asn87Thr (patients 3 and 5) or Asn87Ile (patient 4) (Table 2). When analyzing *gyrA* mutations in both mucosae, we found genetic profiles with single and double mutations in the gastric mucosa and the esophageal mucosa (Table 2).

***gyrB* PCR and sequencing analysis.** Amino acid changes were found in isolates from patients 2 and 3 at position Ser479Gly (Table 2). This amino acid is located close, but not within the *gyrB* QRDR (QRDR for *gyrB* in *H. pylori* 415–454).¹⁷ Isolates from patient 2 showed the mutation in both gastric and esophageal mucosae; however, isolates from patient 3 presented the change only in the gastric mucosa. No mutations were present in the remaining isolates (patients 1, 4 and 5). In addition, all isolates presented common silent mutations in the *gyrB* gene (C1314T, A1368G and G1545A). Isolates from patients 1 and 2 presented the same nucleotide composition profile for both *gyrA* and *gyrB* genes (99–100% identity at BLAST) in both mucosae. This suggests that the origin of *H. pylori* isolates colonizing the esophageal mucosa are related to isolates circulating in the stomach.

Relationship between mutations and LVX susceptibility. The antimicrobial susceptibility (MIC range values) and the presence/absence of mutations were analyzed. All 12 isolates from patient 1 presented no mutations and were susceptible to LVX, indicating that the absence of mutations makes them susceptible (Tables 1 and 2). When three amino acid changes occurred, two mutations in *gyrA* and one in *gyrB*, in 16 of 18 isolates, different resistance levels to LVX were found in both mucosae of patient 2 and in the gastric mucosa of patient 3. In the presence of the single mutation in *gyrA*, Asn87Thr, the 18 isolates were susceptible to LVX, indicating that this mutation alone does not confer resistance to the antibiotic (patient 4 in the gastric mucosa and patients 3 and 5 in the esophageal mucosa). Whenever the amino acid change Asn87Ile was

TABLE 1

LVX susceptibility and resistance profiles by E-test in 60 *Helicobacter pylori* isolates obtained from the gastric and esophageal mucosa of five patients

LVX against <i>H. pylori</i> isolates in the gastric mucosa				LVX against <i>H. pylori</i> isolates in the esophageal mucosa			
Patients (N = 5)	MIC (µg/mL)	Susceptible isolates n (%)	Resistant isolates n (%)	Patients (N = 5)	MIC (µg/mL)	Susceptible isolates n (%)	Resistant isolates n (%)
1*	< 1.00	6 (20) S	–	1*	< 1.00	6 (20) S	–
	–	–	–		< 1.00	2 (7) S	–
2	1.00–32	–	5 (17) R	2	1.00–32	–	2 (7) R
	≥ 32	–	1 (3) HR		≥ 32	–	2 (7) HR
3	1.00–32	–	3 (10) R	3	< 1.00	6 (20) S	–
	≥ 32	–	3 (10) HR		–	–	–
4	< 1.00	6 (20) S	–	4	≥ 32	–	6 (20) HR
5	≥ 32	–	6 (20) HR	5	< 1.00	6 (20) S	–
Total isolates (N = 30)	–	12 (40)	18 (60)	Total isolates (N = 30)	–	20 (67)	10 (33)

HR = highly resistance; LVX = levofloxacin; MIC = minimum inhibitory concentration; R = resistant; S = susceptible.

* Patient 1 susceptible to LVX in both mucosae.

present, the 12 isolates were highly resistant to LVX in the gastric and esophageal mucosa of patients 5 and 4, respectively (Tables 1 and 2). This suggests that the amino acid change in that position is associated with high resistance (MIC ≥ 32 µg/mL) to LVX in *H. pylori* isolates. These results indicate that the single mutation at Asn87Ile and the double mutations at Asn87Thr and Asp91Asn of *gyrA* have a large effect in the LVX resistance changes, whereas single mutation at Asn87Thr has no impact on *H. pylori*–resistance to the antibiotic. On the other hand, mutations of *gyrB* alone were not detected, but these were present simultaneously with double mutations of *gyrA*.

DISCUSSION

This is the first time that *H. pylori* resistance to a fluoroquinolone has been reported in dyspeptic Venezuelan patients, investigating its molecular mechanisms. Resistance to LVX was present in 47%, of all *H. pylori* isolates containing double and/or single mutations in the QRDR of *gyrA* and *gyrB* genes. There was a higher number of resistant isolates in the gastric mucosa (60%) than the esophageal mucosa (33%) as evaluated by E-test, in agreement to previous reports (40% in the gastric mucosa and 19% in the esophageal mucosa).⁶ Differences in fluoroquinolone resistance has been related to consumption rates among different regions worldwide. High-resistance rates to LVX have been reported in countries such

as Turkey (29.5%), China (26.6%), Portugal (26.3%), Egypt (23.2%), Senegal (15.0%), and Spain (14.5%), whereas fewer countries report lower resistant rates such as Israel (5.1%) and Western Australia (6%).^{11,19–25} In addition, high rates of resistance have been reported in Latin American countries such as Peru (37%), Argentina (32.7%), and Brazil (23%).^{26–28}

The QRDR is a region in the *gyrA* and *gyrB* genes of *H. pylori*, which promotes point mutations that contribute to fluoroquinolone resistance. Other mechanisms include the efflux pump activation which may be responsible for the selection of LVX-resistant *H. pylori* isolates.¹² Many studies have described either single or double mutations at positions Asn87 and Asp91 as the most frequent mutation sites in resistant isolates from the gastric mucosa.^{28,29} Position Asn87 has been described as the most frequent hotspot in the *gyrA* QRDR, resulting in single mutation rates at Asn87 higher than Asp91 or double mutations that always include position Asn87.^{11,17,25} Our study reports that mutations in Asn87 were more prevalent (80%) than mutations in any other site of the QRDR (20%) for isolates from each mucosa. Gastric strains isolated from patients with previous failure to *H. pylori* infection treatment in Japan were found to have point mutations in *gyrA* at the Asp87 and Asp91 sites. Another study in Egyptian patients reported higher prevalence of mutations at Asp91 (63.2%) than Asn87 (36.8%).^{17,18,21} In addition, double mutations with different amino acid changes (Asn87Lys and Asp91Asn, Thr87Ile and Asp91Tyr, or Asn87Lys and

TABLE 2

Point mutations in the quinolone resistance–determining region of *gyrA* and *gyrB* genes found in 60 *Helicobacter pylori* isolates in both mucosae

Mucosa	Patients	<i>gyrA</i> gene				<i>gyrB</i> gene		
		Mutation 690	Mutation 691	Amino acid change	Mutation 701	Amino acid change	Mutation 1435	Amino acid change
Gastric	1*	A→†	T→†	Asn87†	G→†	Asp91†	A→†	Ser479†
	2	A→ C	T→†	Asn87 Thr	G→ A	Asp91 Asn	A→ G	Ser479 Gly
	3	A→ C	T→†	Asn87 Thr	G→ A	Asp91 Asn	A→ G	Ser479 Gly
	4	A→ C	T→ C	Asn87 Thr	G→†	Asp91†	A→†	Ser479†
	5	A→ T	T→ C	Asn87 Ile	G→†	Asp91†	A→†	Ser479†
Esophageal	1*	A→†	T→†	Asn87†	G→†	Asp91†	A→†	Ser479†
	2	A→ C	T→†	Asn87 Thr	G→ A	Asp91 Asn	A→ G	Ser479 Gly
	3	A→ C	T→ C	Asn87 Thr	G→†	Asp91†	A→†	Ser479†
	4	A→ T	T→ C	Asn 87 Ile	G→†	Asp91†	A→†	Ser479†
	5	A→ C	T→ C	Asn87 Thr	G→†	Asp91†	A→†	Ser479†

Patients: six isolates/patient. The bold values show the mutation.

* Patient 1, susceptible to levofloxacin in both mucosae.

† No mutation, no amino acid change.

Asp91Try) have been described for LVX-resistant isolates in France.²⁹ We were able to differentiate isolates harboring single mutations at position Asn87, either changing Asn87Thr or Asn87Ile in the gastroesophageal mucosa.

Different amino acid changes at the same site have been described for *H. pylori* isolates in other studies (Asn87Lys, Thr87Ile, or Asp91Asn; Asp91Tyr and Asp91Gly).^{25,28,29} When comparing the mutation Asn87Ile with previous reports, this amino acid change has only been reported in countries such as South Africa, Senegal, and France.^{30,31} Curiously, Asn87Ile has been reported mostly in studies of Latin American subjects from Colombia and Argentina; supporting that amino acid variations confer resistance to antibiotics depending on the geographical origins.^{28,32,33} In contrast to our results, isolates from Colombian patients harboring Thr87Ile mutation showed low resistance.³³ Thus, this mutation requires further investigation in other Latin American countries.

The *H. pylori gyrB* gene has not been as thoroughly studied as the *gyrA* gene. In this study we found isolates in each mucosa with point mutation at Ser479Gly. Mutations in the *gyrB* gene have been reported in association with *gyrA* mutations being present in our study in 15% (18/60 isolates), which indicates that *gyrA* mutations at locations Asn87 and Asp91 have a more critical impact on LVX resistance than those on *gyrB*.^{12,17} Mutation Ser479Gly found in the studied isolates is located close to the QRDR (amino acids from 415 to 454), in accordance with Tankovic et al.,³⁴ the change did not happen in the QRDR of *H. pylori*, but close to the region in ciprofloxacin-susceptible isolates (another fluoroquinolone). This *gyrB* mutation of the QRDR has been reported as a nonresistance-related alteration. However, this mutation when combined with the above mentioned mutations in the *gyrA* gene, resulted in the occurrence of highly resistant isolates (Table 2).

The determination of the antimicrobial susceptibility can be performed by either phenotypic approaches such as the E-test or disc diffusion method, or DNA-based methods such as sequencing. The DNA-based approach is more rapid and accurate in contrast to phenotypic methods that need the culturing of *H. pylori* isolates, also known as fastidious microorganisms.²⁵ The phenotypic susceptibility character defined by MIC indicates that resistance to LVX is linked to presence/absence of amino acid changes in the QRDR of *gyrA/gyrB* genes in *H. pylori* isolates. The presence of a single mutation (Asn87Thr) in the *gyrA* gene does not turn on resistant isolates when using phenotypic methods such as E-test. Other studies have associated this mutation to either a low level or no fluoroquinolone resistance at all. Two or more mutations in the QRDR of *gyrA/gyrB* genes have been associated to high resistance levels to fluoroquinolones, as previously described.¹¹ Our study reported isolates with three changes in both genes of each mucosa, conferring high-resistance levels with MIC values greater than the LVX breakpoint of $\geq 1 \mu\text{g/mL}$.

Finally, this is the first report of LVX-resistant isolates with two or more mutations in the esophageal mucosa, such as those found in the stomach of individual hosts. Some *H. pylori* isolates in an individual host present identical clones (99–100% identity at BLAST) for both *gyrA* and *gyrB* genes, demonstrating that the identified isolates in the esophageal mucosa emerge from isolates circulating the gastric mucosa. Levofloxacin should be applied with caution, because its

antibiotic effect on *H. pylori* is decreasing in Latin America, perhaps due to high prescription rates.

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