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ORIGINAL ARTICLE

Point Mutations at gyrA and gyrB Genes of Levofloxacin Resistant *Helicobacter pylori* Strains and Dual Resistance with Clarithromycin

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SUMMARY

Background: **Spontaneous point mutations in genes encoding gyrA/B subunits of DNA gyrase are responsible for fluoroquinolone resistance. We aimed to determine the clarithromycin and levofloxacin resistance phenotypically in *H. pylori* strains and to investigate the mutations responsible for levofloxacin resistance and the effects of these mutations on dual antibiotic resistance.**

Methods: **A total of 65 *H. pylori* isolates were included. The E-test method was used for the clarithromycin and levofloxacin antimicrobial susceptibility test. Real-time PCR was used to detect the point mutations.**

Results: **Twenty-four (36.9%) of 65 *H. pylori* strains were phenotypically resistant to clarithromycin and 14 (21.5%) to levofloxacin. The phenotypic levofloxacin resistance rate of strains with Asn87Lys and Asp91Asn mutations were significantly higher (*gyrA* gene) ($p < 0.05$). The phenotypic levofloxacin resistance rate of strains with Arg484Lys and Asp481Glu mutations were significantly higher (*gyrB* gene) ($p < 0.05$). The Asn87Lys mutation increased the risk of phenotypes being resistant to levofloxacin 70.156 times and Asp91Asn mutation increased 125,427 times higher. Seven (10.8%) of 65 *H. pylori* strains showed dual resistance to both levofloxacin and clarithromycin. The rate of being dual resistant with A2143G mutation (clarithromycin resistance) was found to be significantly higher ($p < 0.05$).**

Conclusions: **The Asn87Lys and Asp91Asn mutations in the *gyrA* gene had a phenotypically enhancing effect on levofloxacin resistance, while the presence of Asp481Glu and Arg484Lys mutations in the *gyrB* gene did not. The existence of dual resistance was developed with the increase in clarithromycin and levofloxacin resistance rates.**
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KEY WORDS

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INTRODUCTION

Helicobacter pylori (*H. pylori*) can colonize the antrum and corpus region of the stomach despite the acidic environment and thick mucin layer [1,2]. *H. pylori* causes the development of important gastroduodenal pathologies such as ulcer, gastric cancer and is defined as a first class carcinogen by the World Health Organization/International Agency for Research on Cancer Working Group [3]. While a dual antibiotic combination (clarithromycin + amoxicillin or clarithromycin + metronidazole) was used in addition to proton pump inhibitor (PPI) as the standard treatment regimen (first line treatment regime), alternative new treatment protocols have been introduced with the decrease in eradication rates due to increasing antibiotic resistance in recent years (such as "sequential" or "concomitant" non-bismuth quadruple regimens). Accordingly, it is recommended to use bismuth quadruple therapy (PPI + bismuth subcitrate or bismuth subsalicylate + tetracycline + metranidazole) or levofloxacin triple therapy (PPI + levofloxacin + amoxicillin) as salvage therapy in regions where clarithromycin resistance is > 15% [4].

While resistance to amoxicillin is still low, resistance to clarithromycin and fluoroquinolones is increasing. *H. pylori* acquires antibiotic resistance not only through plasmids, but through mutations in chromosomes [5]. Clarithromycin is a macrolide antibiotic that inhibits protein synthesis by binding to ribosomes from the peptidyl transferase region of 23S rRNA [6]. Clarithromycin resistance in *H. pylori* mainly results from point mutations in the V region of 23S rRNA. These mutations cause structural changes by the inhibition of clarithromycin interaction with 23S ribosomal RNA, leading to the development of resistance [7], and A2142G, A2142C, A2143G are the most common mutations for the development of clarithromycin resistance [8,9].

Levofloxacin is a quinolone antibiotic and blocks DNA synthesis by inhibiting DNA gyrase and topoziomerase IV enzymes. DNA gyrase and DNA topoziomerase IV are tetrameric proteins that are structurally similar and consist of repetitions of two subunits. DNA gyrase consists of *gyrA* and *gyrB*, and topoziomerase IV, which is their homologue, consists of *parC* and *parE* subunits [10,11]. Spontaneous point mutations (quinolone resistance determining region) occurring in genes encoding *gyrA/B* subunits of DNA gyrase are responsible for fluoroquinolone resistance [11,12]. Point mutations at ASN87 and ASP91 positions in the *gyrA* gene have been associated with fluoroquinolone resistance. Amino acid substitutions occurring positions 87 (Asn to Lys) and 91 (Asp to Gly, Asp to Asn, Asp to Tyr) of the *gyrA* are mutations which are held responsible for fluoroquinolone resistance [13-15]. *gyrB* gene mutations usually occur together with *gyrA* gene mutations [15,16]. It has been shown that mutations such as S479G, D481E, and R484K occurring in the *gyrB* gene may be associated with resistance [17,18].

In this study, we aimed to determine the phenotypical clarithromycin and levofloxacin resistance in *H. pylori* strains isolated from biopsy samples and to investigate the mutations responsible for levofloxacin resistance by molecular methods and the effects of these mutations on dual antibiotic resistance.

MATERIALS AND METHODS

This study was conducted between June 2014 and June 2017, with 65 *H. pylori* strains isolated from gastric antrum and corpus biopsy samples of patients with dyspeptic complaints. Patients who are 18 years of age and above, without previous gastric surgery and *H. pylori* eradication therapy, did not use any antibiotics or antisecretory drugs, bismuth salts or sucralfate in the last 2 weeks, and had no history of bleeding-clotting disorder were included.

Consent was obtained from all patients according to the World Medical Association Declaration of Helsinki. The study was approved by the Clinical Research Ethics Board of Cerrahpasa Faculty of Medicine, and all patients gave their informed consent before participating in the study. Ethical approval No. A-15.

H. pylori isolation and antimicrobial sensitivity testing

Biopsy samples were cultured on *Helicobacte*r Agar (Salubris, Turkey) (Brain heart infusion (BHI) agar supplemented with 10% defibrinated horse blood and *H. pylori* selective antibiotic supplement (Oxoid, UK) containing vancomycin 5 mg, trimethoprim 2.5 mg, cefsaludin 2.5 mg and amphotericin B 2.5 mg). Cultured biopsy specimens were incubated for 5 - 7 days at 37°C in a 5% CO_2 incubator in microaerophilic environment. Gram staining was performed from bacteria in the form of transparent water droplets growing on *Helicobacter* Agar during the incubation period. Curved Gram-negative colonies on Gram staining were considered as suspected *H. pylori* strains. Bacteria with positive urease, oxidase, and catalase tests from these colonies were identified as *H. pylori*. Strains identified as *H. pylori* were tested for susceptibility to levofloxacin and clarithromycin using the E-test method. A suspension of 3 McFarland turbidity bacteria was prepared from *H. pylori* strains and inoculated on Mueller Hinton Agar medium containing 10% horse blood. Clarithromycin and Levofloxacin E-Test (BioMeriex, Marci, L'etoile France) strips were placed on the medium and incubated at 35 ± 2°C in microaerophilic medium for 72 hours. At the end of this period, the results were evaluated according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria. *H. pylori* ATCC 43504 origin was used as a standard control in our study.

PCR and DNA sequencing

Extraction of *H. pylori* DNA

Freshly grown bacteria were diluted with distilled water to 0.5 McFarland. High Pure PCR Template Preparation Kit (Roche Diagnostics GmBH, Germany) kit was used for *H. pylori* DNA extraction. The H. pylori DNA extraction process was carried out in accordance with the manufacturer's instructions.

PCR amplification

While the mutations in *gyrA* (582bp) and *gyrB* (465bp) gene regions in all *H. pylori* strains were investigated using PCR method, in addition to these gene regions point mutations in 23 S rRNA (425 bp) gene were determined using real-time PCR method. Primers belonging to these specific gene regions were used in the study are shown in Table 1 [15,19].

Determination of point mutations in *gyrA* and *gyrB* gene regions

A mix containing 1 µL forward primer, 1 µL reverse primer, 2.5 µL 10 x PCR Gold buffer, 2 µL 25 mM MgCl2, 2 µL dNTP, (10 mM each deoxynucleoside triphosphate) (Fermentase®, Lithuania), 0.3 µL of Taq DNA polymerase (Fermentase®, Lithuania), and 11.2 µL of nuclease-free water was prepared. Then, 5 µL of extraction product was added to 20 µL of the mix. Amplification was performed in a thermal cycler (MJ Research, Inc., Waltham, MA, USA) under the following conditions: After 10 minutes denaturation at 95℃; 40 cycles were performed at 94℃ for 40 seconds, at 55℃ for 40 seconds, at 72℃ for 2 minutes. Final extension was done at 72℃ for 15 minutes.

Determination of point mutations in 23S rRNA

Real-time PCR method was used to detect the A2143G point mutation in all strains included in the study. In order to apply this method, LightCycler 480 Probe Master (Roche Diagnostics Mannheim, Germany) kit was used in line with the manufacturer's recommendations.

The reaction mixture had a final volume of 20 µL and contained 3 µL Nuclease-free water, 2 µL Primer/Probe Mix, 10 µL LC480 Probe Master Mix, and 5 µL of DNA.

Real-time PCR amplification consisted of an initial denaturation step at 95℃ for 10 minutes, followed by 45 amplification cycles consisting of 95℃ for 10 seconds, annealing at 60℃ for 15 seconds, and extension for 1 minute, and a final step of cooling to 40℃ for 1 minute. Results were evaluated using the Melting Curve analysis module in LightCycler 480 1.5 software.

Sequencing analysis

Amplified products of DNA samples were run on 2% agarose gel stained with ethidium bromide and were visualized by ultraviolet transilluminator. Amplification products were purified from agarose gel using the QIAquick PCR Purification Kit (Qiagen GmBH, Hilden Germany) in accordance with the manufacturer's recommendations. Pure amplification products were sequenced bidirectionally using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) with the ABI PRISM 3130 Genetic Analyzer (ABI, ThermoFisher, USA) with the 3130POP7_BD-Tv3 protocol which was the default program on the analyzer. The sequences were analyzed with Sequencing Analysis 5.3.1 (ABI, ThermoFisher, USA). Alignment of single consensus sequences, 23S rRNA gene (GenBank Accession number: U27270.1), and *gyrA/gyrB* genes (Gen Bank Accession numbers: KY807298-KY 807417) were compared using cluster W. and FASTA files.

Statistical analysis

Statistical Package for Social Sciences v. 25.0 software (IBM SPSS Statistics for Windows; IBM Corp, Armonk, NY, USA) was used for statistical analysis of the research data. Socio-demographic characteristics of the participants were determined by frequency analysis, and Pearson's chi-squared test was used to compare resistance status according to whether there was a mutation or not. In the related comparisons, Fisher's exact test was used when the assumptions of Pearson's chi-squared test were not provided. Logistic regression analysis was used to determine the resistance risk analysis of the participants according to mutations, and the Cox & Snell R2 value was taken into account in determining the variance explained for the dependent variable in the said analysis.

RESULTS

A total of 65 people, including 33 (50.77%) women and 32 (49.23%) men, were included. The average age was 47.30 ± 12.17, and 36.9% of 65 *H. pylori* strains were phenotypically resistant to clarithromycin and 21.5% to levofloxacin The distribution of the patients according to their sociodemographic characteristics and phenotypic resistance results is shown in Table 2.

Levofloxacin and clarithromycin MICs of *H. pylori* strains were evaluated according to EUCAST criteria. Fourteen (21.5%) of 65 *H. pylori* strains were resistant to levofloxacin (> 1 mg/L), while 51 (78.5%) (< 1 mg/L) were found to be susceptible. Twenty-four (36.9%) of the strains (> 0.5 mg/L) were resistant to clarithromycin, and 41 (63.1%) (< 0.25 mg/L) were susceptible to clarithromycin.

Distribution of point mutations in the *gyr* A gene

The presence of Asn87Lys (N87K), Asp91Tyr (D91Y), Asp91Asn (D91N), and Asp91Gly (D91G) point mutations in the *gyrA* gene region was investigated and the effect of these mutations on phenotypic levofloxacin resistance was investigated. While 6 (85.7%) of the strains with the Asn87Lys mutation were found to have levofloxacin resistant phenotype, 8 (13.8%) of the strains without mutation were found to be resistant to

Table 1. Primers used in the study.

Gene Regions	Sequences	Product size	References
gyr A	(F): 5'AGC TTA TTC CAT GAG CGT GA 3' (R): 5'TCA GGC CCT TTG ACA AAT TC 3'	582 bp	25
gyrB	(F): 5' CCC TAA CGA AGC CAA AAT CA 3' (R): 5' GGG CGC AAA TAA CGA TAG AA3'	465 bp	25
23S rRNA	(F): 5' CCA CAG CGA TGT GGT CTC AG 3' (R): 5'CTC CAT AAG AGC CAA AGC CC 3'	425 bp	29
HPY-S	5-AGGTTAAGAGGATGCGT CAGTC-3	267 bp	29
HPY-A	5-CGCATGATATTCCCATTAGC AGT-3		
Red640	5-GGCAAGACGGAAAGACC-3	16 bp	29
5Flour	5-TGTAGTGGAGGTGAAAATTCCTCCTACCC-3	28 bp	29

Table 2. Sociodemographic characteristics and phenotypic resistance results of the patients.

	n	%	Clarithromycin				Levofloxacin			
			Susceptible 41 (63.1%)		Resistant 24 (36.9%)		Susceptible 51 (78.5%)		Resistant 14 (21.5%)	
Gender										
Female	33	50.77	21	63.6	12	36.4	26	78.8	7	21.2
Male	32	49.23	20	62.5	12	37.5	25	78.1	7	21.9
Age ($\bar{x}$±s = 47.3 ± 12.17)										
< 55	47	72.31	27	57.4	20	42.6	36	76.6	11	23.4
55 ≥	18	27.69	14	77.8	4	22.2	15	83.3	3	16.7

Table 3. The comparison of levofloxacin sensitivity *H. pylori* strains according to the presence of point mutations in *gyrA* gene region.

		Levofloxacin susceptible		Resistant		Total	p	OR	95% CI	
		n	%	n	%				Lower	Upper
Asn87Lys mutation										
Asn87Lys	-	50	86.2	8	13.8	58	0.000 *	37.5	3.973	353.912
	+	1	14.3	6	85.7	7				
Asp91Tyr mutation										
Asp91Tyr	-	49	77.8	14	22.2	63	0.613	-	-	-
	+	2	100.0	0	0.0	2				
Asp91Asn mutation										
Asp91Asn	-	50	87.7	7	12.3	57	0.000 *	50	5.325	469.483
	+	1	12.5	7	87.5	8				
Asp91Gly mutation										
Asp91Gly	-	50	78.1	14	21.9	64	0.785	-	-	-
	+	1	100.0	0	0.0	1				

*** p < 0.05.**

Table 4. Phenotypic levofloxacin resistance risk variables due to the point mutations in *gyrA* gene region by binary logistic regression analyses.

	B	S.E.	Wald	df	Sig.	Exp (B)	95.0% CI for Exp (B)	
							Lower	Upper
Asn87Lys	4.189	1.263	11.004	1	0.001 *	65.966	5.551	783.920
Asp91Asn	4.412	1.243	12.592	1	0.000 *	82.433	7.207	942.826
Constant	-2.795	0.595	22.094	1	0.000 *	0.061		

Table 5. The comparison of levofloxacin sensitivity of *H. pylori* strains according to the presence of point mutations in *gyrA* gene region.

		Levofloxacin susceptible		Resistant		Total	p	OR	95% CI	
		n	%	n	%				Lower	Upper
S479G mutation										
S479G	(-)	51	78.5	14	21.5	65	-	-	-	-
Asp481Glu mutation										
Asp481Glu	(-)	47	88.7	6	11.3	53	0.000 *	15.667	3.600	68.176
	(+)	4	33.3	8	66.7	12				
Arg484Lys mutation										
Arg484Lys	(-)	46	88.5	6	11.5	52	0.000 *	12.267	3.012	49.961
	(+)	5	38.5	8	61.5	13				

Table 6. Phenotypic levofloxacin resistance risk variables due to the point mutations in *gyrA* and *gyrB* gene region by binary logistic regression analyses.

	B	S.E.	Wald	df	Sig.	Exp (B)	95.0% CI for Exp (B)	
							Lower	Upper
Asn87Lys(1)	4.251	1.347	9.964	1	0.002 *	70.156	5.009	982.513
Asp91Asn(1)	4.832	1.820	7.051	1	0.008 *	125.427	3.544	4438.816
Asp481Glu(1)	-1.665	1.918	0.753	1	0.386	0.189	0.004	8.125
Arg484Lys(1)	2.005	1.424	1.983	1	0.159	7.429	0.456	121.050
Constant	-2.988	0.652	21.008	1	0.000 *	0.050		

*** $p < 0.05$.**

levofloxacin. The phenotypic levofloxacin resistance rate of strains with Asn87Lys mutation was found to be significantly higher than strains without mutations ($p < 0.05$). While 7 (87.5%) of the strains with Asp91Asn mutation were phenotypically resistant to levofloxacin, the phenotypic levofloxacin resistance rate of those without Asp91Asn mutation was found to be 1 (12.3%). The phenotypic levofloxacin resistance rate of strains with Asp91Asn mutation was significantly higher than those without mutation ($p < 0.05$). There was no statistically significant difference between the levofloxacin resistance rates of the phenotype according to the status of Asp91Tyr and Asp91Gly mutations in *H. pylori* strains ($p > 0.05$) (Table 3).

According to the binary logistic regression analysis, the presence of Asn87Lys and Asp91Asn mutations had a significant effect on levofloxacin phenotypic resistance ($p < 0.05$). Asn87Lys mutation had a 65.966-fold higher

risk for the development of levofloxacin resistance. Asp91Asn mutation had a 82,433-fold higher risk for the development of levofloxacin resistance (Table 4).

Distribution of point mutations in the *gyr* B gene

The presence of S479G, Asp481Glu(D481E) and Arg-484Lys(R484E) point mutations in the *gyrB* gene region and the effects of these mutations on phenotypic levofloxacin resistance were investigated. Eight (66.7%) of *H. pylori* isolates with Asp481Glu mutation and 6 (11.3%) of isolates without the mutation were found phenotypically resistant to levofloxacin ($p < 0.05$). The phenotype of 8 (61.5%) of *H. pylori* strains with Arg-484Lys mutation was found to be resistant, while 6 (11.5%) of strains without Arg484Lys mutation were found to be resistant to levofloxacin. The rate of phenotypic levofloxacin resistance of isolates with Arg484Lys mutation was found to be significantly higher than isolates without mutations ($p < 0.05$) (Table 5).

According to the binary logistic regression analysis, the presence of Asp481Glu and Arg484Lys mutations did not have a statistically significant effect on phenotypic levofloxacin resistance ($p > 0.05$). According to binary logistic regression analysis, Asn87Lys and Asp91Asn mutations were found to have a significant effect on levofloxacin phenotypic resistance ($p < 0.05$), while Asp-481Glu and Arg484Lys mutations did not have a significant effect ($p > 0.05$). The risk of phenotypes being resistant to levofloxacin in isolates with Asn87Lys mutation was 70.156 times higher, and the risk of phenotypes being resistant to levofloxacin in isolates with Asp91Asn mutation was 125,427 times higher (Table 6).

Distribution of point mutations in both levofloxacin and clarthyromycin-resistant *H. pylori* strains (Dual Resistance)

Seven (10.8%) of 65 *H. pylori* strains showed dual resistance to both levofloxacin and clarithromycin. Accordingly, A2143G mutation associated with clarithromycin resistance in 7 isolates with dual resistance (phenotypically resistant to both agents), Asn87Lys, Asp91-Asn, Asp481Glu, and Arg484Lys mutations associated with levofloxacin resistance were examined. Accordingly, A2143G mutations were found in 3 (42.9%) of 7 strains with dual resistance and Asn87Lys, Asp91Asn, Asp481Glu and Arg484Lys mutations in 2 (28.6%) of 7 strains. When the existence of mutations and the relationship of dual resistance are examined, the rate of being dual resistant in isolates with A2143G mutation was found to be significantly higher than isolates without A2143G mutation ($p < 0.05$). There was no statistically significant difference between the dual resistance rates of the strains with and without Asn87Lys, Asp91Asn, Asp491Glu, and Arg484Lys mutations ($p > 0.05$).

The median MIC values of levofloxacin in phenotypically levofloxacin resistant *H. pylori* strains are as follows: the Asp91Asn point mutation had the highest median MIC value (32 m/L, range 1.5 - 256 mg/L) and the Asn87Lys point mutation had a lower median MIC value (12 mg/L). For the other point mutations in gyrB gene, Asp481Gly, and Arg484Lys had the same median MIC value (12 mg median MIC value, range 1.5 - 256 mg/L).

DISCUSSION

H. pylori is classified in the high-risk category by the World Health Organization [20]. International organizations such as the European *Helicobacter* pylori study group developed guidelines for diagnosis and treatment modalities. High cure rates can no longer be achieved with empirical triple therapies due to the rapid increase in antimicrobial drug resistance rates [5]. Today, new guideline recommendations limit empirical triple eradication treatment choices depending on the prevalence of regional antibiotic resistance and previous use of an antibiotic of the same class [4,21]. Although the resistance rates of *H. pylori* strains against macrolides, nitroimidazoles, and fluoroquinolones have increased rapidly, amoxicillin, tetracycline, and rifabutin still seem to be effective against *H. pylori* strains [22,23].

The development of resistance to clarithromycin is suggested to be related to the extensive use of macrolide antibiotics [23]. While the rate of clarithromycin resistance in Europe was 9% in 1998, it has increased to 17.6% in 2008. This increase in resistance has continued over the years [7,24]. It is noteworthy that there are disparencies for clarithromycin resistance rates reported from Turkey with different geographical features. Clarithromycin resistance in *H. pylori* strains has been reported as follows: 36.7% by Caliskan et al. [25] and 38.1% by Kocazeybek et al. [26]. Our results were found to be consistent with the results of the studies reported by Kocazeybek et al. [26] and Caliskan et al. [25]. Today, triple therapy containing levofloxacin are being used as an alternative primary-line therapy, especially in areas with high clarithromycin resistance. As a result, this treatment including quinolones, showed an increase in bacterial resistance against quinolones [4, 27]. In a multicenter study by Megraud et al. [23] levofloxacin resistance rate reached 18%, especially in central European countries. The levofloxacin resistance rates of *H. pylori* strains were reported as 18.8% [28] in Germany, 15% in Japan [29], and 42.9% [30] in Nepal. In 2012, Cagdas et al. [31] reported levofloxacin resistance at a rate of 18.2% in Turkey, and levofloxacin resistance rate was reported as 29.5% by Caliskan et al. [25]. In a review by Kocazeybek et al. [32], levofloxacin resistance rate was found to be 23.77%. While the data obtained from this study are similar to the data of the studies reported from Turkey, they differ with the data from abroad. This difference is due to geographical differences and the fact that the frequency of quinolone use varies by region.

While the amino acid changes occurring in position 87 (Asn to Lys) and 91 (Asp to Gly, Asp to Asn, Asp to

Tyr) in the *gyrA* gene are responsible for fluoroquinolone resistance, Ser479Gly (S479G), Asp481Glu (D48 1E) point mutations and Arg484Lys (R484K) in the gyrB gene have also been reported to be associated with fluoroquinolone resistance [14-18]. The prevalence of each point mutation varies by geographic region. In a Nepal study, the Asp91 mutation of the *gyrA* gene was found in 50% of 18 levofloxacin resistant strains and the Asn87 mutation of the *gyrA* gene were found in 33.3% [30]. In a study by Garcia et al. [33], 33%, 30.9%, 7.2%, and 6.2% of 97 *H. pylori* strains with fluoroquinolone resistance had N87K, D91N, D91G, and D91Y point mutations, respectively. In an Algerian study, 33.8% of 68 levofloxacin resistant strains were found to have N87K mutations, 44.73% N87K, 23.68% D91N, and 7.89% D91G mutations [34]. Miyachi et al. [29], reported that 33.8% N87K, 2.9% D91N, 14.7% D91G, and 8.8% D91Y mutations were detected in *H. pylori* strains with levofloxacin resistance. Mutations in both *gyrA* and *gyrB* genes were detected in only 2.9% of the levofloxacin resistant *H. pylori* strains. In the same study, mutations in 50 levofloxacin susceptible *H. pylori* strains were also examined, and accordingly, 2% of N87K, D91N, and D91Y and 4% of D91G mutations were detected. In a Colombian study, 11.3% of 80 levofloxacin resistant isolates were found to have N87K and 28.8% had D91N mutations [21]. In a study conducted in Brazil, 16.6%, 34.8%, 18.1%, and 13.6% of 66 fluoroquinolone resistant *H. pylori* strains were found to have N87K, D91N, D91G, and D91Y, respectively [35]. In a study reported from Kuala Lumpur, 44.4%, 22.2%, and 11.1% of the *gyrA* genes of levofloxacin resistant *H. pylori* strains were found to have N87K, D91N/ D91G, and D91Y mutations, respectively, and 33.3% of *gyrB* genes of levofloxacin resistant *H. pylori* strains had D481E and R484K mutations [36]. In an Iranian study, D481E mutation was found in 12.12% of levofloxacin susceptible *H. pylori* strains, and R484K mutations were found in 6.01%. On the other hand, D481E and R484K mutations were found in 9.09% levofloxacin resistant *H. pylori* strains [37]. In our study, N87K mutation was found in 85.7% of the *gyrA* gene of levofloxacin resistant *H. pylori* strains, and D91N mutations were found in 87.5% of them, while no D91Y and D91G mutations were found in any levofloxacin resistant *H. pylori* strains. The presence of N87K and D91N mutations was found to be significantly higher in resistant strains, and it was found to affect phenotypic resistance, similar to many published data. In the analysis of the *gyrB* gene in levofloxacin resistant *H. pylori* strains, D481E mutations were detected in 66.7% and R484K mutations in 61.5%, and no S479G mutation was found. D481E and R484K mutations were detected significantly higher in the levofloxacin resistant *H. pylori* strains but it was determined that the presence of these mutations did not affect the phenotypic levofloxacin resistance. N87K, D91N, D91G, D91Y, D481E, and R484K mutations were detected in susceptible strains, but these mutations were not reflected in the phenotypic resistance.

The emergence of multiple antibiotic-resistant *H. pylori* strains with increasing prevalence, also causes the treatment failure [4,23,38]. In a study reported from Portugal, dual resistance both to clarithromycin and levofloxacin was found in 41 (22.8%) of 180 *H. pylori* strains [39], while in an Argentinian study, dual resistance was at a rate of 7.7% [40]. In another study, dual resistance was found in 4 (28.57%) of 14 clarithromycin resistant *H. pylori* strains. Hence, A2143G and T2128C mutations were detected in the 23S rRNA of all strains with dual resistance and N87K, D91Y, D91G, and D481E mutations were also found in the *gyrA* and *gyrB* genes regions [36]. In a study conducted by Caliskan et al. [25] from Turkey in 2015, the presence of dual resistance to both clarithromycin and levofloxacin was reported at a rate of 27.2%.

In our study including 65 *H. pylori* strains, dual resistance both to clarithromycin and levofloxacin was found only in 7 strains (10.8%). The A2143G mutation was detected in the 23S rRNAs of *H. pylori* strains with dual resistance, the rate of dual resistance of *H. pylori* strains with the A2143G mutation was found to be higher than the strains without the A2143G mutation. The Asp91Asn (D91N) point mutation had the highest median MIC value (32 mg/L). The Asn87Lys (N87K) point mutation had a lower median MIC value (12 mg/L) for levofloxacin. Asp91Asn (D91N) point mutation had a higher median MIC value than the median MIC value of all levofloacin resistant *H. pylori* strains. Dual resistance was found at a lower rate than the results of the study by Caliskan et al. [25] reported from the Marmara Region of Turkey.

CONCLUSION

The Asn87Lys and Asp91Asn mutations in the *gyrA* gene had a phenotypically enhancing effect on phenotypic levofloxacin resistance, while the presence of Asp481Glu and Arg484Lys mutations in the *gyrB* gene did not have a significant effect. We also demonstrated the existence of dual resistance development with the increase in clarithromycin and levofloxacin resistance rates. We believe that phenotypic antimicrobial resistance in *H. pylori* strains should be determined before the treatment and the antibiotics to be applied in the treatment should be selected in this direction. We suggest that it is necessary to carry out comprehensive studies from different regions in order to evaluate the coexistence of other mutations with large sample numbers.

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The authors declare that there is no conflict of interests regarding the publication of this paper.

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