Genetic diversity in the quinolone resistance-determining region of *gyrA* in *H. pylori*

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Summary

Objective: Levofloxacin (LEV) is the common quinolone antibiotic and a practical option in *H. pylori* eradication regimens. The LEV resistance can be acquired through variant sequences of a *gyrA*. This study investigated the correlation between gyrA genetic diversity and LEV resistance in *H. pylori*. *Subject and method*: We conducted a cross-sectional study that included 99 *H. pylori* strains isolated from patients with gastritis, peptic ulcer disease, and gastric cancer at the 108 Military Central Hospital from 2019 to 2022. The minimum inhibitory concentration (MIC) of each strain against levofloxacin was determined using the E-test method. Sanger sequencing was used for variant identification and analysis of *gyrA*. Mutations were found among both susceptible and non-susceptible strains. *Result*: Resistance to LEV was observed in 33.3% of the *H. pylori* isolates, significantly higher in female. Most LEV-resistant strains carry genetic mutations in the quinolone resistance-determining region (QRDR) of *gyrA* among LEV-resistant strains was remarkably higher than in LEV-sensitive strains. The presence of D91G/Y/N and N87K/I point mutations among non-susceptible was remarkably higher than among susceptible strains. A higher prevalence of mutations outside QRDR (R130K) in resistant strains was also noted. Multiple mutations in the *gyrA* are only found among resistant strains. All mutant strains exhibited a higher MIC for LEV than wild strains, but statistical significance was only found in strains with D91G/Y/N mutation. *Conclusion*: Mutation located in the QRDR region (D91G/Y/N, N87K/I) and outside that region (R130K) might result in the LEV-resistance of *H. pylori.*

Keywords: Helicobacter pylori, levofloxacin, resistance, *gyrA* point mutations.

I. BACKGROUND

Helicobacter pylori (*H. pylori*) is a gram-negative bacteria recognized as one of the most prevalent infectious agents worldwide and classified as a Group I carcinogen for gastric cancer[1]. This bacterium can cause many severe digestive diseases, and experts recommended that eradication of *H. pylori* has been shown to prevent gastritis and reduce the rate of recurrent gastric ulcers and gastric cancer[2]. In the recent decade, the standard first-line therapy for eradicating *H. pylori* contains proton pump inhibitors (PPIs), clarithromycin, and amoxicillin (or metronidazole), which is no longer appropriate in most areas with a high rate of antibiotic resistance, such as Vietnam. The use of second-line rescue triple therapy with levofloxacin after failure of quadruple "sequential" or "concomitant" treatment to eradicate *H. pylori* infection has been widely recommended[3]. However, resistance to quinolones is easily acquired and is increasing in most countries, which reduces the

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effectiveness of levofloxacin-based therapy in eradicating *H. pylori*[4].

Levofloxacin (LEV) is a fluoroquinolone antibiotic considered a promising alternative to standard treatment regimens[5]. It is effective against both Gram-negative and Gram-positive bacteria through the mechanism of action interfering with DNA replication by inhibiting bacterial type II topoisomerase enzymes, including DNA gyrase and topoisomerase IV. Further understanding antibiotic resistance mechanisms may also be crucial in investigating changes in fluoroquinolone resistance.

The most common mechanism of fluoroquinolone resistance is mutations in the gyrase and topoisomerase IV genes. These genes encode complex enzymes of four subunits: GyrA, GyrB (DNA gyrase), and ParC and ParE (topoisomerase IV). However, *H. pylori* does not have the gene encoded for topoisomerase IV, so fluoroquinolone resistance tended to be caused by mutations in the gyrase gene (*gyrA*), which encodes the A subunit of DNA gyrase (GyrA)[6]. The discovery of the point mutation in the *gyrA* gene provides valuable insights into the fluoroquinolone resistance mechanisms of *H. pylori*. Monitoring fluoroquinolone resistance and developing more effective diagnostic and treatment methods are essential for controlling *H. pylor*i infection.

II. SUBJECT AND METHOD

Sample collection

The duodenal-gastric biopsies were collected from patients who underwent endoscopy at the 108 Military Central Hospital. The specimens were preserved in a transport medium at -86°C until processing.

H. pylori isolation and culture

To obtain the *H. pylori* isolates, the specimens were homogenized in 1mL of the specific transport medium, aliquoted 200uL for spreading in *H. pylori* selective agar plates (bioMérieux, France), and incubated for 3-7 days in the microaerophilic environment (5% O_2, 10% CO_2, 85% N_2). The remaining sample was stored at -86°C until the next attempt. On the bacterial isolation medium, colonies suspected of *H. pylori* are small, colorless, or transparent; Gram stain has a curved, slightly twisted shape, Gram-negative; oxidase (+), catalase (+) and urease (+), identification on the Vitek-MS system.

Antimicrobial susceptibility assessment

The antimicrobial susceptibility assessment was performed using the E-test technique. The colonies on the culture plate identified as *H. pylori* were taken out and mixed until 3 McFarland suspension, then using a pipette to draw 200µl of the suspension onto the Muller Hinton agar plate (bioMérieux, France) containing 5% horse blood (MHF). Spread the suspension evenly over the agar with a cotton swab and let the agar surface dry for about 3-5 minutes. Place the plate in a Genbag microaerobic bag (bioMérieux, France) to create an atmosphere of 5% O_2, 10% CO_2, and 85% N_2, and incubate at 37°C for 72 hours. The results of the antibiotic susceptibility test for *H. pylori* bacteria were evaluated using an E-test according to the EUCAST Clinical Breakpoint 2022 standard. The strain was concluded to resist LEV when the minimum inhibitory concentration (MIC) is > 1 µg/mL.

Genomic DNA extraction, sequencing, and mutation analysis

According to the manufacturer's instructions, genomic DNA was isolated from biopsy homogenous suspension using the commercial Monarch Genomic DNA Purification Kit (NEB, USA). DNA quality was assessed using a spectrophotometer NanoPhotometer P300 (IMPLEN, Germany). Polymerase chain reaction (PCR) amplification of *gyrA* was performed with a set of primers: gyrA_F (5'-AGC TTA TTC CAT GAG CGT GA-3'), gyrA_R (5'-TCA GGC CCT TTG ACA AAT TC-3'), (product 582bp). The PCR amplicons were then purified, and Sanger sequenced. The DNA sequence data were analyzed using the Geneious R11.1 (https://www.geneious.com) with reference sequences of *H. pylori* 26695 (NC_018939) and *H. pylori* J99 (CP011330).

Statistical analysis

GraphPad Prism 10 for macOS (version 10.2.3 (347), April 21, 2024) was used to perform all statistical analyses. Chi-square and Fisher's exact tests were used to determine the differences between categorical variables. The comparison between multiple groups with a mean of minimum inhibitory concentration (MIC) was performed using Kruskal-Walis and Dunn's post hoc test. A p-value ≤0.05 was considered statistically significant.

III. RESULT

3.1. Characteristics of phenotypic LEV resistance of isolated H. pylori strains

From 2019 through 2022 at the 108 Military Central Hospital, the study collected 99 *H. pylori* strains with the fulfilled profile of antibiotic susceptibility values and *gyrA* gene Sanger sequencing results.

Table 1. Characteristics of the population studied

	LEV susceptible (n = 66)	LEV resistant (n = 33)	Total (n = 99)
Age (mean ± SD, range)	50.79 ± 14.63	50.95 ± 14.13	50.9 ± 14.22
	(18 - 81)	(18 - 75)	(18 - 81)
Sex, n (%)*			
Male	45 (76.27)	14 (23.73)	59 (59.6)
Female	21 (52.5)	19 (47.5)	40 (40.4)
Endoscopic finding, n (%)			
Gastritis	21 (31.82)	13 (39.4)	34 (34.34)
Duodenal-gastric ulcer	35 (53.03)	13 (39.4)	48 (48.48)
Gastric cancer	10 (15.15)	7 (21.2)	17 (17.17)
Proportion (%)	66.67%	33.33%	100%

** Fisher's exact tests: Significant difference between males and females with p-value = 0.0175. OR = 2.9 (CI 95%: 1.24-6.51).*

This study focused on the *H. pylori*-infected adult patients with a mean age of approximately 50 ± 14.22 years old (range: 18-81 years old). The results showed that 33.33% of the *H. pylori* strains in our study were resistant to levofloxacin. The prevalence of those isolated from women (47.5%) was significantly higher than that from males (42.42%) with p-value = 0.0175. There was no relationship between LEV-resistant *H. pylori* proportion with host progression diseases and average age in adult groups.

3.2. gyrA gene mutations associated with LEV resistance in H. pylori strains

Simultaneous evaluation with two reference genes from NCBI of 26695 strain (NC_018939) and J99 strain (CP011330), a total of 13 amino acid substitutions were identified and presented into 19 variants among the 99 obtained *gyrA* sequences, accounting for 46.46% (46/99) of the strains. Among that, 84.85% (28/33) sequences were extracted from LEV-resistant strains, separately representing 13 *gyrA* variants. There were 6/19 identified variants only found in LEV-susceptible isolates with 9% (6/66).

Table 1. *gyrA* variants associated with LEV-resistant phenotype

No	Genotypes of *gyrA*	LEV susceptible (n = 66)	LEV resistant (n = 33)	Total (n = 99)
I	Wildtype (n, %)	48 (72.73)	5 (7.58)	53 (80.3)
II	Mutant (n, %)	18 (27.27)	28 (84.85)	46 (46.46)

No	Genotypes of *gyrA*	LEV susceptible (n = 66)	LEV resistant (n = 33)	Total (n = 99)
1	N87K/I (n, %)*	5 (7.58)	6 (18.18)	11 (11.11)
2	D91G/Y/N (n, %)**	3 (4.55)	7 (21.21)	10 (10.1)
3	R130K (n, %)*	3 (4.55)	4 (12.12)	7 (7.07)
4	N87K/I+R130K (n, %)	1 (1.52)	1 (3.03)	2 (2.02)
5	N87K/I+D91G/Y/N (n, %)	0 (0)	2 (6.06)	2 (2.02)
6	D161N (n, %)	0 (0)	1 (3.03)	1 (1.01)
7	N87K/I + D161N (n, %)	0 (0)	1 (3.03)	1 (1.01)
8	V65I + N87K/I (n, %)	0 (0)	1 (3.03)	1 (1.01)
9	N87K/I + R140K (n, %)	0 (0)	1 (3.03)	1 (1.01)
10	N87K/I + I194T (n, %)	0 (0)	1 (3.03)	1 (1.01)
11	D91G/Y/N + R140K (n, %)	0 (0)	1 (3.03)	1 (1.01)
12	A66T + D91G/Y/N + R140K (n, %)	0 (0)	1 (3.03)	1 (1.01)
13	N87K/I + D155G + V172I (n, %)	0 (0)	1 (3.03)	1 (1.01)

** Fisher's exact tests: Significant difference between presence mutant and wildtype group with p-value < 0.01.*

*** Fisher's exact tests: Significant difference between presence mutant and wildtype group with p-value < 0.005.*

The most dominant variants shown in Figure 1A were respectively N87K/I (23.91%), D91G/Y/N (21.74%), and R130K (15.22%), statistically significant presented in the LEV-resistant group compared to the LEV-susceptible. In addition, with the reference sequence of *H. pylori* strain 26695, we recorded two more amino acid substitutions (Figure 1B) but shared the same characteristics with the reference sequence of *H. pylori* strain J99. That was M191I with a frequency of 93%, present in 92/99 isolates, and V199A accounting for 16% of all isolates, but 100% (16/16) strains carrying that showed a LEV-susceptible phenotype (data not shown in Table 1).

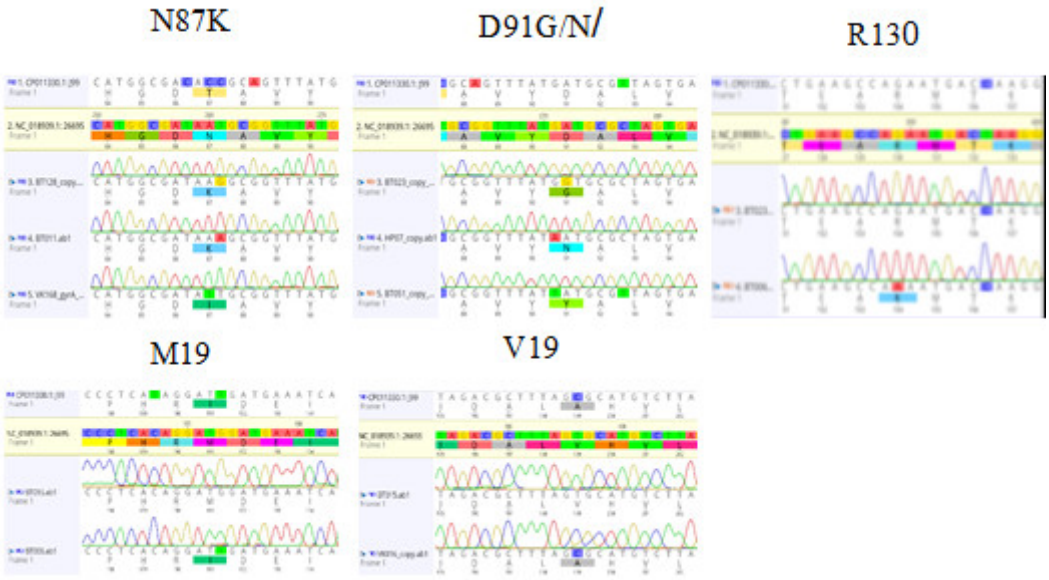

Figure 1. Sanger sequencing results of (A) the three most dominant variants in resistant strains and (B) the two variants shared genomic characteristics with the J99 sequence.

3.3. Effects of gyrA gene mutations on levofloxacin susceptibility level

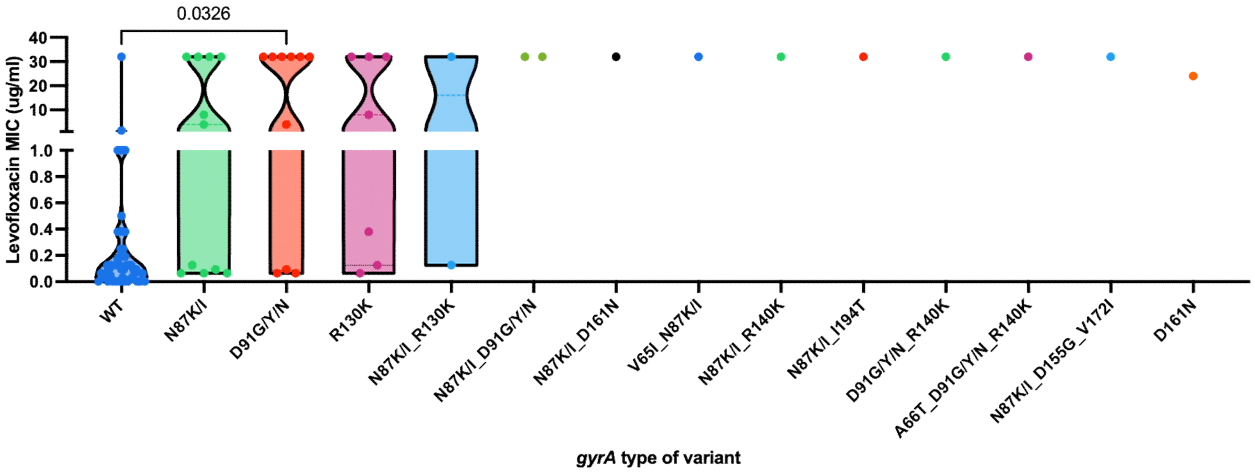

Figure 3. Levofloxacin-resistant MIC (ug/ml) distribution in correlation with gyrA variants

A comparison of levofloxacin susceptibility levels showed that the average LEV minimum inhibitory concentration of the group with the D91G/Y/N variant (19.62 ± 5.06ug/mL) was significantly higher than that of the wild-type group (0.85 ± 0.63ug/mL). The mean LEV MIC of other mutant groups with N87K/I variant (12.76 ± 4.65ug/mL) and R130K variant (14.94 ± 6.12ug/mL) were also out of the resistant breakpoint but the difference was not statistically significant.

IV. DISCUSSION

The prevalence of antibiotic-resistant *H. pylori* infection has shown complicated progression among regions of the world, which is one of the reasons leading to failure in eradication regimens, thereby affecting the effectiveness of treatment of related gastroduodenal diseases[7]. In Vietnam, the prevalence of primary resistance to clarithromycin and metronidazole of *H. pylori* strains has been noted in previous studies, demonstrating the importance of antibiotic selection in managing *H. pylori* infection. Even with this awareness, the latest report still shows that the alternative antibiotic in remedial therapy, levofloxacin, is also facing a complex increase in resistance rates. In particular, according to a study by Le Tran Thi Nhu (2022)[8], the LEV resistance rate in Tien Giang from 2020 to 2021 reached 60.1%, nearly 3 times higher than the national average reported in a review of Vu Van Khien (2019)[9]. Our study is recognized as the latest report on adults in the northern region, given that 2019-2022 showed a slight increase in LEV resistance of 33% compared to 27.1% published in 2019[9]. Our data show a significant difference from previous research in the same period. This comes not only from geographical differences but also related to investigated objects. While the earlier research focused on peptic ulcer patients, our study surveyed both patients with chronic gastritis and gastric cancer. Moreover, the rate of LEV-resistant strains isolated in women was significantly higher than in men, similar to the results described in previous studies[7]. However, additional studies are required to evaluate the relationship between patient gender and the emergence of LEV-resistant strains. The above phenotypic discussion has been reported in our previous study (reference). Therefore, in this study, the result we want to emphasize is that the variation in genotypic mechanism drives the strains to evolve in antibiotic conditions.

Levofloxacine, as quinolone, resistance is attributed to specific mutations in the quinolone

resistance-determining region (QRDR) of the *gyrA* gene, resulting in the lower ability of DNA gyrase to bind to antibiotics[10]. In our study, among a total of 19 *gyrA* variants assembled by 13 distinguish amino acid substitutions, not only the mutation at codon position of N87K/I (p = 0.0019), D91G/Y/N (p = 0.0001) located inside the QRDR were significantly associated with LVX resistance as shown in numerous studies[11, 12]. A codon of R130K located outside the target region also represents the association with 14.29% LEV-resistant strains (p = 0.0072), which was no longer significant in the previous studies. All strains not containing one of those three mutations were susceptible to LEV. Notably, there were 27.27% (18/66) of strains showing LEV-sensitive phenotype (MIC < 1ng/ml) recorded nine mutations in the *gyrA* gene, which is entirely different from the study of Lok (2020)[12]. It could be explained by the hypothesis known as heteroresistance, where multiple *H. pylori* strains are present, but one of these strains, upon isolation, has transitioned to a viable but non-culturable state, leading to hard-to-conclude the phenotype for that clinical case ultimately[13].

Regarding the modulated effect of genetic features, our study showed that all groups containing any one of three mutations N87K/I, D91G/Y/N, and R130K have a mean of LEV MIC higher than LEV-resistant breakpoint. However, only the mean MIC of strains carrying variant D91G/Y/N showed a statistically significant difference. Previous studies, while Vo Phuoc Tuan (2019) remarked that the resonance of 2 mutations in QRDR allowed strains to tolerate higher MIC levels of LEV than strains with only one mutation, Le Tran Thi Nhu (2023) indicated that some mutations outside the QRDR region can increase antibiotic affinity[11, 14]. Our study recorded ten strains carrying more than one mutation, including mutations in the QRDR, of which 9/10 strains showed resistance to LEV at the highest MIC (32ug/mL). However, each strain carried a different variant, and with a small sample size, the association of these variants with LEV resistance has not been evaluated.

V. CONCLUSION

Levofloxacin triple therapy was recommended as the rescue treatment for *H. pylori* infection. However, the major shortcoming of LEV-containing therapy is the low cure rate for eradicating levofloxacin-resistant strains. The mutation located in the QRDR region (D91G/Y/N, N87K/I) and outside that region (R130K) might be responsible for conferring high-level levofloxacin resistance of *H. pylori* and should be considered when evaluating the effectiveness of this therapy.

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