

Using next-generation sequencing to analyze *Helicobacter pylori* clones with different levofloxacin resistances from a patient with eradication failure

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Abstract

The regimens containing levofloxacin (LVX) have been recommended as an alternate to standard triple therapy to treat *Helicobacter pylori* infections and *H pylori* mixed infection always lead to *H pylori* chronic infection. Although the molecular mechanism of LVX resistance with *gyrA* gene mutation has been clearly understood in *H pylori*, other genes involved in antibiotic resistance remain unclear. Efflux pump plays an important role in clinically relevant multidrug resistance. Furthermore, the relationship between the strains with different LVX level-resistances from individuals is also unknown.

Helicobacter pylori monoclonal strains were isolated from patients with eradication failure. *E* test was used to detect the minimal inhibitory concentration of LVX. One lower-level LVX-resistant clone and 2 higher-level LVX-resistant clones from the same patient were selected to sequence the complete genomes. Single-nucleotide variants (SNVs) and mutations were extracted and analyzed from *gyrA* and resistance-nodulation-division family efflux genes.

Two clones with higher-level resistance had the mutation pattern of Asn87Lys and one lower-level LVX-resistant clone had an Asp91Asn mutation. Compared to clones with higher-level resistance, the higher genetic variations were found in genes belonging to the resistance-nodulation-division family in *H pylori* strains with lower-level resistance to LVX. There were significantly more SNVs of Hp0970 (*hefE*) and Hp1329 (*hefI*) in the lower-level LVX-resistant clone than those in the higher-level LVX-resistant clones ($P = .044$).

The mutation pattern of the Asn87Lys of the *gyrA* gene confers a higher resistance to LVX than that of the Asp91Asn in *H pylori*. Increase in the number of SNVs of the Hp0970 (*hefE*) and Hp1329 (*hefI*) genes change the resistance to LVX. Twelve mutations verified by Sanger sequencing in Hp0970 (*hefE*) and Hp1329 (*hefI*) may decrease resistant levels to LVX.

Abbreviations: CLR = clarithromycin, *hefABC* = Hp0605-0607 gene clusters, *hefDEF* = Hp0969-0971 gene clusters, *hefGHI* = Hp1327-1329 gene clusters, InDels = insertions and deletions, LVX = levofloxacin, MDR = multidrug resistance, MIC = minimal inhibitory concentration, NGS = next generation sequencing, RND family = resistance-nodulation-division family, SNVs = single-nucleotide variants, WGS = whole-genome sequencing.

Keywords: *Helicobacter pylori*, higher-/lower-level resistance, levofloxacin, resistance-nodulation-division family, single-nucleotide variants

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Our research has been reviewed by the Medical Ethics Committee of Taizhou Hospital of Zhejiang Province. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

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1. Introduction

Helicobacter pylori, colonized in human gastric mucosa, infects more than 50% of the world's population and is confirmed to cause many serious diseases such as chronic gastritis, peptic ulcer diseases, mucosa-associated lymphoid tissue lymphoma, and gastric cancer.^[1,2] Many meta-analyses show that *H pylori* eradication can not only improve the cure rates of peptic ulcers but also prevents its recurrence and reduces the incidence of gastric cancer.^[3,4] The standard triple therapy using proton pump inhibitors, clarithromycin (CLR), and amoxicillin or metronidazole is recommended as the 1st line of therapy for *H pylori* treatment.^[5,6] Nevertheless, with the increasing prevalence of resistance to CLR and metronidazole, the eradication rate has dropped below 70% in numerous countries.^[7-9] In recent years, triple or quadruple therapy with levofloxacin (LVX) confers obvious advantages for clinical treatment of *H pylori* infections.^[10,11] However, the prevalence of LVX resistance in *H pylori* has increased because of the abuse of fluoroquinolone.

The majority of *H pylori* LVX-resistant clones have been identified to be linked to mutations in the fluoroquinolone resistance-determining region within the *gyrA* gene: Asn87 and Asp91.^[12] Simultaneously, some other mutations that have also been involved in LVX resistance include mutations at positions Ala88, Ala97, and Met191 of *gyrA* and Phe438, Glu463, Asp481, and Arg484 of *gyrB*.^[13-15] In addition, other mechanisms, such as the presence of efflux pumps, have been reported to explain the fluoroquinolone resistance in many gram-negative bacteria.^[16,17] Resistance to fluoroquinolones in gram-negative bacteria is acquired by active export of the agents via antibiotic efflux pumps. Using *Pseudomonas aeruginosa*, Oh et al found that an efflux pump is involved in fluoroquinolones resistance.^[18] Genome analysis suggests that *Campylobacter jejuni* contains at least 9 other putative efflux pumps, which influence antimicrobial resistance.^[17]

Additionally, the resistance-nodulation-division (RND) family, which is one of the efflux pumps, plays an important role in clinically relevant multidrug resistance (MDR) in *H pylori*.^[19] RND efflux pumps, including the AcrAB-TolC system, and 4 gene clusters, consisting of Hp0605-0607 (*hefABC*), Hp0969-0971 (*hefDEF*), Hp1327-1329 (*hefGHI*), and Hp1487-1489^[20,21] have been considered as RND family candidates in *H pylori*. Although, the molecular mechanism of efflux pumps leading to *H pylori* antibiotic resistance has been studied, such as CLR,^[22] studies on LVX resistance have not been previously reported.

In recent years, many studies have found that *H pylori*-mixed infections and heteroresistance is one of the reasons for refractory *H pylori* infection. Although *H pylori* clones from individual patients always have an antibiotic-susceptible or resistant phenotype, both of them could exist in a single population of *H pylori* in each patient.^[23] When the colonization of *H pylori* in the stomach is identified, susceptible or resistant clones could simultaneously be present in the same region or in different regions of the stomach.^[23] As early as 2010, mixed-infection of antibiotic susceptible and resistant *H pylori* clones were found in a single patient, which can lead to 34% with resistant strains being misclassified as susceptible if a biopsy of the antrum alone was used for antimicrobial susceptibility testing.^[24] To date, there are few studies on the drug-resistance mechanism of *H pylori*-mixed infections, especially the antibiotic resistance genes of different drug resistant strains in the same patient.

In this study, we applied Sanger sequencing to detect the genotype of *gyrA* and NGS to analyze the genomic variations in clinically isolated *H pylori* clones. The LVX susceptibility test was performed via the *E* test. Three *H pylori* monoclonal strains in the same patient meeting our requirements were applied to whole-genome sequencing (WGS), including one lower-level LVX-resistant clone and 2 higher-level LVX-resistant clones.

2. Methods

2.1. *Helicobacter pylori* clones and growth conditions

Gastric mucosal tissues were collected from the lesser curvature of the antrum, the greater curvature of the antrum and the gastric corpus from patients with eradication failure due to *H pylori* at Taizhou Hospital (Taizhou, Zhejiang Province). The following principles must be followed as a patient selection: Patients failed 2nd time or more times after a standardized treatment, such as standard quadruple therapy; Patients have completed the entire course of treatment each time and the treatment time is 10 to 14 days per treatment.^[25] In this study, a total of 31 patients participated in the 1st phase of the study. The collection and genomic analysis of the DNA were approved by the ethics committee of Taizhou Hospital, and written informed consent, in accordance with the declaration of Helsinki, was obtained. Isolation and culture of *H pylori* were performed at the laboratory of the Hangzhou Zhiyuan Medical Inspection Institute (Hangzhou, Zhejiang Province). Briefly, grinded gastric mucosa tissue was inoculated onto Columbia agar plates supplemented with 5% fresh defibrinated sheep blood. The plates were cultured at 37°C under microaerophilic conditions (5% O₂, 10% CO₂, and 85% N₂) for 3 to 7 days. Suspicious colonies were identified by gram staining and were confirmed to be positive by their urease, oxidase, and catalase traits. To ensure consistency of drug susceptibility results, we performed a monoclonal separation.

2.2. Monoclonal culture preservation

Multiple scattered monoclonal colonies were picked on the culture-positive plate and then inoculated on fresh blood-agar medium in sections. The medium was cultured in a microaerobic environment (5% O₂, 10% CO₂, and 85% N₂) for 2 days. Different monoclonal moss was again transferred and inoculated on different fresh blood-agar medium. The labeled strains were expanded and cultured according to the growth of the strain until the growth of the strain reached a certain level. The strains were collected and stored in a storage tube containing brain heart infusion for future study.

2.3. Antibiotic susceptibility testing

The minimal inhibitory concentration (MIC) of LVX for *H pylori* was determined using the *E* test in this study. Briefly, the concentration of *H pylori* was equivalent to a 2.0 McFarland standard (8.8×10^7) by adjusting the concentration of bacteria solution with physiologic saline. The suspensions (200 μ L) were spread on Columbia agar plates supplemented with 5% sheep blood. The plate was placed facing up in a microaerobic environment (5% O₂, 10% CO₂, and 85% N₂) for 15 minutes, making sure the bacterial solution penetrates into the medium. Subsequently, LVX *E* test strips were attached on the plates, with

the largest scale facing the edge of the flat plate and the test strip should be soaked with bacterial solution. The medium was cultured at 37°C for 3 to 5 days under microaerophilic conditions. The MIC of LVX for *H pylori* was determined according to the drug concentration value at the intersection of the inhibition zone and the *E* test strip. The *E* test strip has a series of 2-fold dilution gradients, with a concentration range of 0.016 to 32 µg/mL. Clones were considered resistant when the MIC value was more than 2 µg/mL.^[26] To understand *H pylori* mixed infection, we selected a monoclonal with the different susceptibility results for the next experiment. In this study, strains with different resistance levels were found in the same patient. So, the patient with one lower-level LVX-resistant clone and 2 higher-level LVX-resistant clones was the subject of a 2nd phase of the study.

2.4. DNA extraction and Sanger sequencing of *gyrA*

We used the Invitrogen Purelink Genomic DNA mini kit (Life Technologies, Carlsbad, CA) to extract *H pylori* genomic DNA following the manufacturer's instructions. The concentration of each extract was determined by the Qubit dsDNA HS assay kit (Life Technologies). According to the reference sequence, ATCC26695 (GenBank accession no: NC_000915), the HP-*gyrA* forward primer (5'-ACAGCGATGTGGTCTCAGC-3') and the HP-*gyrA* reverse primer (5'-CCACCAAG-CATTGTCCTGC-3') were employed to detect *gyrA* gene mutations at the 87 and 91 positions. Each 25 µL of reaction contained 5 µL of 5× PrimeSTAR buffer (Mg²⁺ plus), 2 µL of dNTP mixture (2.5 mM each), 0.5 µL each of 10 µM primer, 2 µL of genomic DNA, 0.25 µL of PrimeSTAR HS DNA polymerase (2.5 IU/µL) (Takara Bio Inc, Otsu, Japan), and 13.75 µL of sterilized water. The polymerase chain reaction (PCR) conditions were performed using 30 cycles of denaturing at 98°C for 10 seconds, annealing at 58°C for 15 seconds, and extending at 72°C for 40 seconds. The amplified PCR products were analyzed by 1.5% agarose gel electrophoresis to verify the size of 502 bp and subsequently sequenced with a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) using an ABI 3730XL DNA analyzer. The sequences of the *gyrA* gene were then aligned with the reference sequence of *H pylori* 26695 deposited in GenBank using MEGA 6.0 software.

2.5. WGS and analysis of the complete genome

Genomic DNA libraries were prepared and WGS was performed using Illumina HiSeq 2500 sequencing (Illumina, San Diego, CA), as described by Ni et al.^[27] The remaining clean reads were mapped against the reference genome with GenBank accession NC_000915 using BWA (version 0.7.12) after we trimmed the low-quality reads using Trimmomatic (version 0.30). Single-nucleotide variants (SNVs) and insertions and deletions (InDels) were named using the Genome Analysis Toolkit. A phylogenetic tree was constructed based on the identification results of the SNV to intuitively observe the phylogenetic relationships and relative genetic distances.

2.6. Variations in efflux pump genes

To validate the role of multidrug efflux transporter genes that contribute to antibiotic resistance and to assess their association with novel mechanisms of LVX resistance, variations of the RND

Table 1

The primers in Hp0970 and Hp1329.

Primers	Sequence (5' to 3')
Hp0970-F	CTGCCACGCTGTCTTAG
Hp0970-R	TTGAAGCGGGCGTTATTGTG
Hp1329-F1	CTCCTTACCACCTTATGCTACT
Hp1329-R1	TATAGCGGATCAGAGAATCG
Hp1329-F2	GTGTATGACAGGAGCGAATTGA
Hp1329-R2	CTTGTGAGCGTTCTTACCATC
Hp1329-F3	CTCATTAAAGGGCGGATTT
Hp1329-R3	TCTGTGCTTCTAAGCGTAA
Hp1329-F4	CGCATCTATTACGACAACTC
Hp1329-R4	ACGCTCCCTACCATTCTT

family were analyzed, including Hp0605-0607, Hp0969-0971, Hp1327-1329, and Hp1487-1489.

2.7. Verification experiment

We designed primers based on SNVs in Hp0970 (*hefE*) and Hp1329 (*hefI*) (Table 1). The lower-level LVX resistant and higher-level LVX resistant clones were prepared as a template for PCR amplification. To validate the SNVs, Sanger sequencing was performed with an ABI 3730XL DNA Analyzer (Applied Biosystems) using BigDye Terminator V3.1 according to the manufacturer's instructions.

2.8. Statistical analysis

All data analyses were performed using the SPSS statistical software package, version 19.0 for Windows (SPSS Inc, Chicago, IL). The relationships between variations of the MDR genes and higher/lower-resistance to LVX were determined by Chi-squared tests. Statistical significance was considered for *P* values of ≤.05.

3. Results

3.1. Genotype of *gyrA* and phenotype of *H pylori* that are resistant to LVX

To detect whether the patient was infected with mixed *H pylori* clones in this study, monoclonal strains from samples of the lesser curvature of the gastric antrum, the greater curvature of the gastric antrum, and gastric body were isolated and cultured. An LVX susceptibility test of the 3 clinically isolated monoclonal strains from the same patient indicated that 1 clone was lower-level LVX resistant and 2 clones were higher-level LVX resistant (Table 2). The gastric mucosal tissues of the same individual could isolate *H pylori* clones with different traits, such as negative or positive clones and resistant or sensitive clones.^[28] The genotype of *gyrA* was verified by Sanger sequencing. All of the 3 clones had mutations in the *gyrA* gene. Two of them had a mutation of Asn87Lys, and 1 clone had an Asp91Asn mutation (Table 2). Both mutation sites had been reported previously.^[14] The mutation pattern of Asn87Lys caused a higher resistance to LVX than that of Asp91Asn in *H pylori*.

3.2. Overview of WGS of the clinical *H pylori* clones

In this study, 3 *H pylori* genomes were successfully assembled by a HiSeq sequencer. After trimming the low-quality reads, clean reads ranged from 3.2 to 4.5 million (Table 3). Coverage depths

Table 2**Minimal inhibitory concentration and Sanger sequencing results.**

Name	Sampling site	Value of Mic, $\mu\text{g/mL}$	Nuc change	AA change
A	Lesser curvature of the gastric antrum	4	271G>A	Asp91Asn
B	Greater curvature of the gastric antrum	>32	261T>A	Asn87Lys
C	Gastric corpus	>32	261T>A	Asn87Lys

AA change = amino acid change, Nuc change = nucleotide change, Value of Mic = value of minimal inhibitory concentration of levofloxacin.

Table 3**Overview of whole genome sequencing for the 3 samples.**

Name	Clone ID	Total reads (clean reads)	Total mapped reads	Coverage, %	Total SNVs	Missense mutations	Total InDels
A	Asp91	4,198,905	4,027,650	95.92	60,802	19,297	873
B	Asn87	3,250,585	3,155,874	97.09	60,878	19,947	889
C	Asn87	4,533,108	4,408,950	97.26	60,694	19,918	874

A = lesser curvature of the gastric antrum, B = greater curvature of the gastric antrum, C = gastric corpus, InDels = insertions and deletions, SNVs = single-nucleotide variants.

were over 95% mapped to the reference genome. Therefore, the efficient reads were sufficient for subsequent analysis of the SNVs. The number of SNVs, InDels, and missense mutations is summarized in Table 2.

To understand the phylogenetic relationships of the 3 clones from different regions of the patient, we constructed a phylogenetic tree. As a result, 2 clones with the Asn87Lys mutation were in the same branch, and the Asp91Asn mutation was in another, indicating that the patient was infected with 2 different *H pylori* clones.

3.3. Identification of mutations in *gyrA* and *gyrB* genes

To analyze the relationship between LVX of lower-level-resistant and higher-level-resistant clones of *H pylori* with different mutations of the *gyrA* gene from the patient, mutations in the *gyrA* and *gyrB* genes were investigated. Consistent with Sanger sequencing, WGS showed that 2 of the clones had a mutation of Asn87Lys and 1 clone had a mutation of Asp91Asn. We did not find any mutations associated with the known LVX-resistant sites in the *gyrA* gene, except Asn87Lys or Asp91Asn, and the *gyrB*

gene. However, lower-level-resistant (Asp91Asn) clones have fewer missense mutations than those of higher-level (Asn87Lys)-resistant clones in both *gyrA* and *gyrB* genes, and the mutations of 2 higher-level LVX-resistant clones were exactly the same both in loci and numbers.

3.4. Identification of RND family efflux pump genes

To identify the mutations of the multidrug efflux transporter genes, a study of the RND family efflux pump was focused on *H pylori*. Prior to identification of the gene mutation in the RND family efflux pump genes, all mutations of these genes were presented after InDels mutations were filtered in the CDS region (Table 4).

Regardless of whether the clone is higher-level LVX-resistant or lower-level LVX-resistant, SNVs were detected in all 4 clusters of the RND family, and there were numerically more SNVs in the lower-level LVX-resistant clone than those in the higher-level LVX-resistant clones, without significantly difference ($P = .202$). There were significantly more SNVs of Hp0970 (*hefE*) and Hp1329 (*hefI*) in the lower-level LVX-resistant clone than those

Table 4**Single-nucleotide variants of the resistance-nodulation-division family efflux pump transporter genes.**

Clones genes	A: low-level LVX-resistant	B: high-level LVX-resistant	C: high-level LVX-resistant	Total base number
Hp 0605	94	90	90	1434
Hp 0606	25	33	33	705
Hp 0607	121	138	138	3087
Hp 0969	130	121	121	3063
Hp 0970*	58	38	38	1080
Hp 0971	56	53	53	1242
Hp 1327	61	67	67	1239
Hp 1328	47	36	36	1017
Hp 1329*	166	128	128	3108
Hp 1487	33	47	47	1098
Hp 1488	31	36	36	990
Hp 1489	61	72	72	1533
Total	883	859	859	19,616

A = clones from lesser curvature of the gastric antrum with lower-level LVX-resistant, B = clones from greater curvature of the gastric antrum with higher-level LVX-resistant, C = clones from gastric corpus with higher-level LVX-resistant, LVX = levofloxacin.

* Significant differences between different resistance level ($P < .05$).

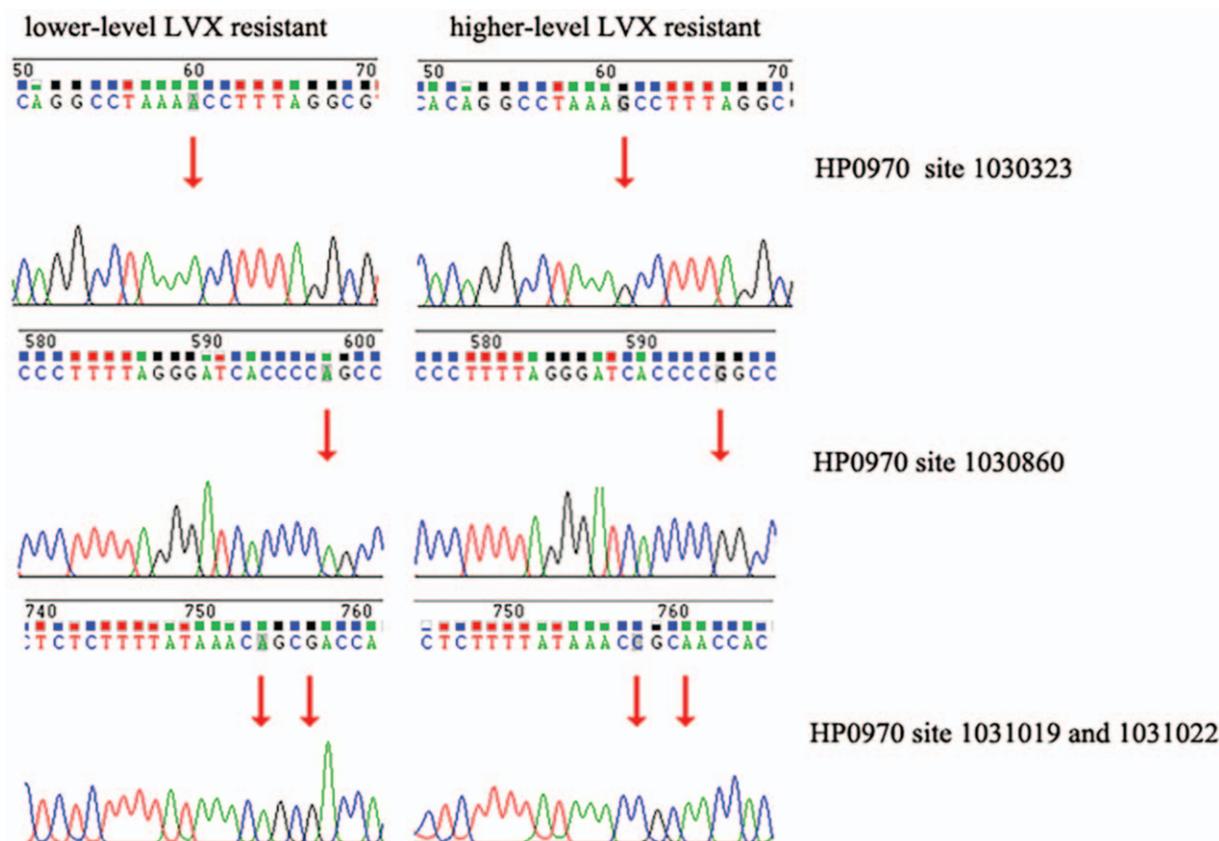


Figure 1. The results of Sanger sequencing for Hp0970 in lower-level and higher-level levofloxacin (LVX)-resistant clones. The mutation site is marked by an arrow.

in the higher-level LVX-resistant clones ($P = .044$). These results suggest that the SNVs of Hp0970 (*hefE*) and Hp1329 (*hefI*) may change resistant levels to LVX.

3.5. Verification of SNVs in Hp0970 (*hefE*) and Hp1329 (*hefI*)

To determine whether the predicted SNVs was mutated in clinical samples, we used Sanger sequencing to verify the lower-level and higher-level LVX-resistant clone in Hp0970 and Hp1329. According to mutation type (homozygous), base depth and mutation frequency ($=1$), we finally selected 9 SNVs of Hp0970 and 13 SNVs of Hp1329, which were present in lower-level LVX-resistant clone, but not in higher-level LVX-resistant clone. Through PCR identification and Sanger sequencing, we identified a total of 12 mutations, including 4 in Hp1970 (Fig. 1) and 8 in Hp1329 (Fig. 2). The positions of mutation are showed in Table 5.

4. Discussion

In recent years, due to a pre-existing antibiotic resistant *H pylori* clone or the generation of a new resistant clone derived from an original susceptible clone, treatment failure has been prevalent.^[29] It seems to be difficult to eradicate *H pylori* infections in patients using failed eradication therapies.^[30] The patient who was studied in this research failed 2nd eradication therapies, and 2 different *H pylori* clones of both the phenotypes and genotypes

were detected in different gastric regions. As a result, antibiotic susceptibility tests should be based on multiple sampling for patients infected with *H pylori* who have undergone failed eradication therapies.

Regimens containing fluoroquinolone have been proposed as an alternate to the classic standard triple therapy for the treatment of *H pylori* infections. Although the mechanism of LVX resistance of positions 87 and 91 mutations of *gyrA* has been reported,^[12] it is difficult to explain the different levels of the bacterial antibiotic resistance. A study in Latin America showed that the occurrence of the Asn87Ile mutation was associated with a high resistance (MIC $\geq 32 \mu\text{g/mL}$) to LVX.^[31] In agreement with a previous study,^[14] we found that the Asn87Lys mutation of the *gyrA* gene confers a higher resistance to LVX than that of the Asp91Asn mutation. We only investigated one type of mutation at each position, and double mutation sites, such as Asn87Lys with Asp91Asn, showed a high-level resistance to LVX in Chang's study.^[32]

The RND family is grouped into MDR efflux pumps in gram-negative bacteria. Many studies have been reported on the association of RND family efflux pumps with bacterial antibiotic resistance in some gram-negative bacteria, such as *Bacteroides fragilis*, *Escherichia coli*, and *Burkholderia cepacia*.^[33–35] In previous surveys, the role of RND efflux pumps in metronidazole and CLR resistance of *H pylori* has been studied.^[20,36] In LVX resistance, the studies only concentrated on *C jejuni*, *Campylobacter coli*, *P aeruginosa*, and *Streptococcus pneumoniae* strains.^[18,17,37] The influence of these pumps on LVX resistance

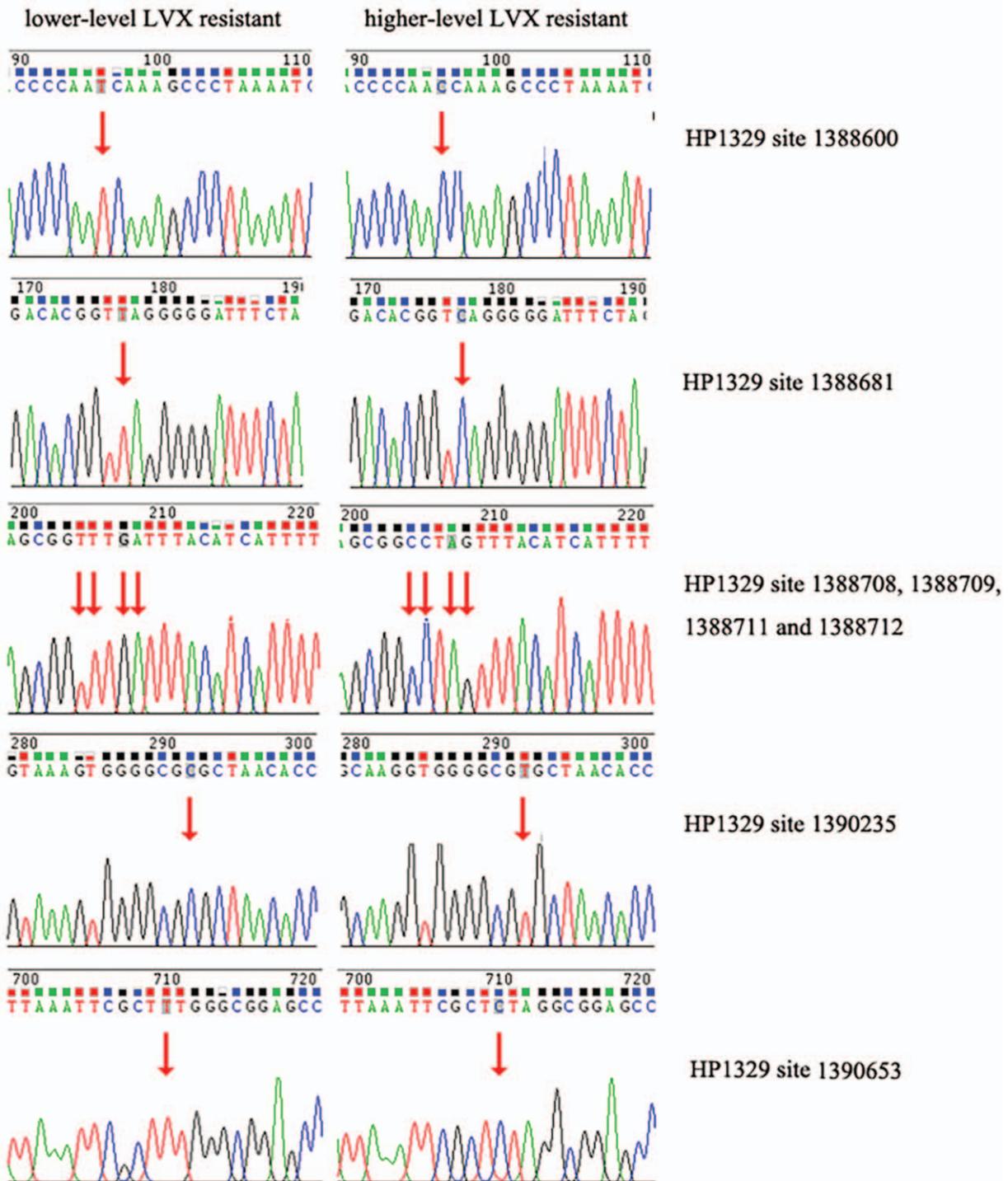


Figure 2. The results of Sanger sequencing for Hp1329 in lower-level and higher-level levofloxacin (LVX)-resistant clones. The mutation site is marked by an arrow.

to *H pylori* should be established. Efflux pumps have been confirmed to increase the resistance levels synergistically with other resistance mechanisms. In the current study, the SNVs of 4 RND efflux pump genes in *H pylori* were measured by WGS at different level resistances of LVX. The SNVs were detected in all 4 clusters of the RND family genes. In 2005, Ge et al,^[17] found that inactivation of efflux pumps gene could decrease the MIC and

increase the susceptibilities to fluoroquinolone. In *E coli* isolates, the inhibition of the efflux pump gene expression decreased the MIC of fluoroquinolone.^[38] Furthermore, in this study, there were significantly more SNVs of Hp0970 (hefE) and Hp1329 (hefI) in the lower-level LVX-resistant clone than those in the higher-level LVX-resistant clones. In other words, a large number of SNVs may increase the risk of gene inactivation or activation.

Table 5
The special variations were identified in Hp0970 and Hp1329.

Gene	Position of mutation*	Position of mutation†	Ref	Mutation
Hp0970	1030323	145	A	G
Hp0970	1030860	682	A	G
Hp0970	1031019	841	A	C
Hp0970	1031022	844	G	A
Hp1329	1388600	165	T	C
Hp1329	1388681	246	T	C
Hp1329	1388708	273	T	C
Hp1329	1388709	274	T	C
Hp1329	1388711	276	G	A
Hp1329	1388712	277	A	G
Hp1329	1390235	1800	C	T
Hp1329	1390653	2218	T	C

* Nucleotide position was determined by the respective reference sequence from GenBank: CP003904.1

† Nucleotide position was determined by the respective reference sequence from Hp0970 and Hp1329.

At the same time, the increased risk of gene inactivation increases the probability of MIC reduction. Through PCR identification and Sanger sequencing, we identified 4 mutations in Hp1970 and 8 mutations in Hp1329, which were present in lower-level LVX-resistant clone, but not in higher-level LVX-resistant clone. Therefore, we speculated that the 12 mutations of Hp0970 (hefE) and Hp1329 (hefI) may decrease resistant levels to LVX.

Our research also has several limitations. Firstly, the clones we isolated in this study were all monoclonal strains, and the susceptibility results of each monoclonal in the same site are consistent, which led to a small sample size for this study. There were only 3 *H pylori* clones, including 1 lower-level LVX-resistant clone and 2 higher-level LVX-resistant clones in 1 patient in our study. Secondly, we only study from genomics perspective to speculate the role of efflux pump genes, especially in a different number of SNVs in RND efflux pump genes between lower-level and higher-level resistant strains. Despite certain data support for other strains, such as *E coli*, *C jejuni*, and *C coli*, but lacks clear biologic validation. Future studies are needed to collect more *H pylori* monoclonal with same drug susceptibility. We believed that these genes may be responsible for regulating the susceptibility of *H pylori* to fluoroquinolone.

5. Conclusion

In this study, we successfully isolated 3 monoclonal strains with the different susceptibility results (1 lower-level LVX-resistant clone and 2 higher-level LVX-resistant clones) from the same *H pylori* patient with eradication failure. Compared with Asp91Asn of the gyrA gene, the mutation pattern of the Asn87Lys might make a higher resistance to LVX in *H pylori*. SNVs from RND family efflux genes change resistance to LVX, especially Hp0970 (hefE) gene and Hp13289 (hefI) gene. Twelve mutations verified by Sanger sequencing in Hp0970 (hefE) and Hp1329 (hefI) may decrease resistant levels to LVX.

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