Am J Digest Dis 2019;6(1):1-8
www.ajdd.us /ISSN:2329-6992/AJDD0093769

Original Article

A novel detection method for *GyrA* gene mutations based on real-time PCR in *helicobacter pylori* resistant to levofloxacin

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Received March 13, 2019; Accepted April 11, 2019; Epub April 15, 2019; Published April 30, 2019

Abstract: *Helicobacter pylori*, an important pathogen that colonizes the gastric mucosa, can cause peptic ulcers and gastric cancer in infected individuals. First-line treatment for *H. pylori* is combination antibiotic therapy, but drug resistance has become a major factor affecting the eradication rate. Whereas the clinical detection of clarithromycin resistance based on 23S rRNA evaluation is well established, reports on the detection of all levofloxacin resistance sites are lacking. In this study, we have established a method based on the principle of probe melting curve to distinguish mutations of the levofloxacin resistance gene *gyrA* at multiple sites with only one probe. In order to simultaneously distinguish between multiple resistance gene mutations and synonymous mutations at amino acids 87, 88, and 91 of levofloxacin, a series of bioinformatic tools were used to design the primers and probes, from which results can be predicted. One hundred forty-four clinical samples were simultaneously detected using our method and Sanger sequencing. By comparison, our method was more sensitive, less expensive, and easy to carry out in any diagnostic laboratory. More importantly, we use as few as 10 copies of DNA per reaction to obtain more precise results.

Keywords: Real-time PCR, *helicobacter pylori*, levofloxacin resistance, GyrA

Introduction

Helicobacter pylori (*H. pylori)*, a gram-negative bacterium that colonizes the surface of the gastric mucosa, is the main cause of peptic ulcer, chronic gastritis, and gastric malignant tumors [1]. The current first-line treatment for HP is triple antibiotics (clarithromycin, amoxicillin, and metronidazole) alone or with a proton pump inhibitor (quadruple therapy) [2]. If first-line therapy fails, salvage therapy with levofloxacin is an effective alternative [3]. However, with the global increase in antibiotic resistance, antibiotic resistance to macrolides (clarithromycin) and fluoroquinolones (levofloxacin) has limited the success rate of antibiotic treatments [4]. The presence of clarithromycin resistance has reduced the success rate of clarithromycin-containing triple therapy by approximately 50% [5], whereas levofloxacin resistance has reduced the success rate of levofloxacin-containing treatment regimens by approximately 20%-40% [6].

Drug resistance is mainly caused by specific base mutations. The clarithromycin resistance of *H. pylori* is mainly caused by mutations involving A2142G, A2142C, and A2143G mutations of 23S rRNA [7]. However, the mutations that cause levofloxacin resistance are more complex, and mainly occur in specific regions of the DNA gyrase subunit A gene (*gyrA*), especially in the quinolone resistance-determining domain (QRDR) [8]. After mutations at these key sites, the enzyme is responsible for the function of DNA supercoiling, reducing the affinity of antibiotics and reducing the antibiotic effect [9]. Eleven mutations have been reported in this domain, including those at the sites encoding the codons for the amino acids asparagine (Asn) at position 87, alanine (Ala) at position 88, and aspartate (Asp) at position 91 (viz, Asn87LyS, Asn87Tyr, Ala88Val, Asp91Gly, Asp91Asn, and Asp91Tyr) and synonymous mutations at amino acid 87 [10].

Traditionally, the clinical detection of *H. pylori* resistance is through an antibiogram, obtained

using the commercially available diagnostic device Etest [11]. However, this method requires special transportation conditions and culture laboratories for culturing the bacteria. Owing to the long culture period and low success rate, the timely provision of clinical treatment guidance is difficult to accomplish. Compared with traditional methods, molecular biology methods can provide more expedient and accurate guidance on the appropriate clinical drugs to use in individual cases. After PCR amplification of the GyrA mutation, sequencing is the gold standard in clinical practice [12]. Liou [13] proved that the molecular method of sequencing had a better correlation with the clinical results [13]. The eradication rate of wild type was 82.7%, and the eradication rate of mutation was 41.7%.

Compared to sequencing, real-time PCR was a clinically preferred method. Following the addition of a fluorescent dye or probe to the PCR system, the fluorescence signal is increased as the DNA sequence of interest is amplified, and the entire amplification reaction can be monitored in real time [14]. This technique has been widely used, especially for the detection of clarithromycin-resistant mutation sites, and has been used to guide clinical treatment [15]. Detection of levofloxacin-resistant is not as easy as clarithromycin-resistant detection, there is only one commercially available *H. pylori* test, which was designed for Helicobacter pylori strains from Germany, using two pairs of hybridization probes to detect mutations in amino acids 87 and 91 [16]. However, this method cannot be distinguished whether a strain has both a synonymous mutation of 87 AAT and a resistance mutation of Asp91Asn/Asp91Tyr.

In order to solve this problem and detect all eleven reported mutations, a novel RT-PCR technique was established based on the probe-based melting curve to distinguish all above-mentioned mutations in levofloxacin resistance gene GyrA using only one probe. We constructed multiple plasmid standards and diluted them to different gradients, demonstrating that the method was sensitive. The detection of 144 actual clinical samples proved the accuracy of the method, and the test results were consistent with Sanger sequencing.

Materials and method

H. pylori strains

The wild-type *H. pylori* standard strain (ATCC26695) was obtained from American Type Culture Collection cellbank. *H. pylori* DNA samples, extracted from the gastric mucosa of a total of 144 patients with peptic ulcer disease, were obtained from Shanghai Xinchao Biotechnology Company (Shanghai, China). The sequences of all the different mutations of the *H. pylori gyrA* gene were downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov).

Standard plasmids construction

Plasmid standards for the 87AAT wild type, 87AAC wild type, and 87AAA-resistant genotypes were constructed separately. Plasmids were extracted by TIANprep Mini Plasmid Kit (TiangenBiotech, Shanghai, China). After the concentration was measured using Nanodrop 2000, the plasmids were diluted to 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 10 copies/μL for sensitivity detection.

Probe design

The probe was designed on nucleic sites that covered 87, 88 and 91 resistant mutations after sequence alignment of all GyrA mutation types by ClustalX software. The melting temperature (Tm) and other parameters for hybridization of different mutation types were predicted by the online Unafold software (http://unafold.rna.albany.edu). To simultaneously detect different mutations at multiple sites, we finally designed a hybrid probe with FAM, 5'-CGATAATGCGGTTTATGATG, which matches the wild type of amino acid 87 AAT. In the software prediction results, 87 AAT and 87 AAC sensitive types had the highest melting temperature (62.4°C and 59.4°C), and all drug-resistant melting temperature were lower than 57°C, as shown in Table S1.

Primer design and optimization

The sequences from different mutations of the G*yrA* gene were downloaded from NCBI. All sequences were aligned using ClustalX software. The primers were designed in the most conserved regions by Oligo7 software. Different types of clinical *H. pylori* DNA were used for

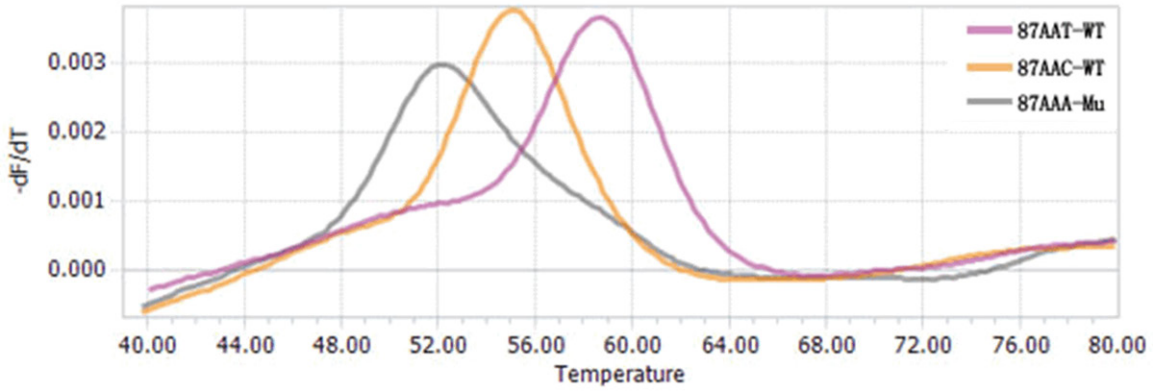

Figure 1. Melting curves of wild type and mutant types. The Tm value of the levofloxacin-sensitive standard, 87AAT and 87AAC, was 58.5°C and 54.9°C. The Tm value of the drug-resistant standard, 87AAA, was 52.8°C.

PCR validation to screen for the most conservative pair of primers. One paired specific primer (forward, 5'-AGGCGTATTTTGTATGCGATGC; and reverse, 5'-CGTTATCGCCATCAATAGAGCCA) hat covered most types of *H. pylori*, was selected in this study.

PCR and melting curve analysis

The reaction solution contained HpH buffer [17], 3 mM $MgSO_4$, 0.2 mM dNTPs, 1 U HS Taq DNA polymerase, 0.1 μM probe, 0.05 μM forward primer, 0.5 μM reverse primer, 1 μL DNA template.

Amplification and annealing were performed on Lightcycle 96 (Roche) using the following procedure: 95°C for 5 min; 40 cycles of 95°C for 20 s, 58°C for 30 s, 72°C for 20 s; 72°C for 5 min; 95°C for 3 min, 40°C for 3 min; The temperature was raised from 40°C to 80°C, 5 readings/°C, and the rate was 0.05°C/s. The fluorescence intensity was measured on the FAM channel, and the genotype was determined by the melting curve Tm value.

Result

Typical mix results of standard plasmids

The diluted plasmid standards were subjected to melting curve analysis by fluorescence-based real-time PCR in order to obtain the plasmid samples of 87AAT wild type, 87AAC wild type, and 87AAA resistant gtype on the Roche Lightcycle 96 instrument (**Figure 1**). Two peaks with different Tm values indicate the detection results of two kinds of wild types, which mean sensitivivity to levofloxacin. The left peak was mutation type 87AAA and the clinical significance was resistance.

Sensitivity of detection

Using a 10-fold gradient of plasmid DNA as a template, the sensitivity of the entire assay system was analyzed and the results showed that the minimum detection limit of all sites reached 10 copies/reaction (**Figure 2**). The melting curves of the samples with different Ct values all showed the same Tm value, indicating the high sensitivity of the detection system, and the detection results of different concentration templates were highly consistent. The peak height of the melting curve of the sample above 100 copies did not change until a significant decrease was observed at 10 copies, but was still clearly distinguishable from the negative control.

Typical sample detection

We selected different typical clinically types for melting curve analysis, which were confirmed by Sanger sequence results (**Figure 3**). The probe design was completely matched with the synonymous mutation sensitivity of C261T, thus the Tm value of the synonymous mutation sample 01 was 58.5°C in **Figure 3A**. The Tm value of wild type sample 09 was 54.8°C in **Figure 3B**. Taking the Tm value of the wild type sample as reference, the Tm values of the other resistant mutations were lower than the wild type above 2°C, such as C261A and A259T resistance in **Figure 3C** and **3D**. In addition to C261A, sample 21 also showed another muta-

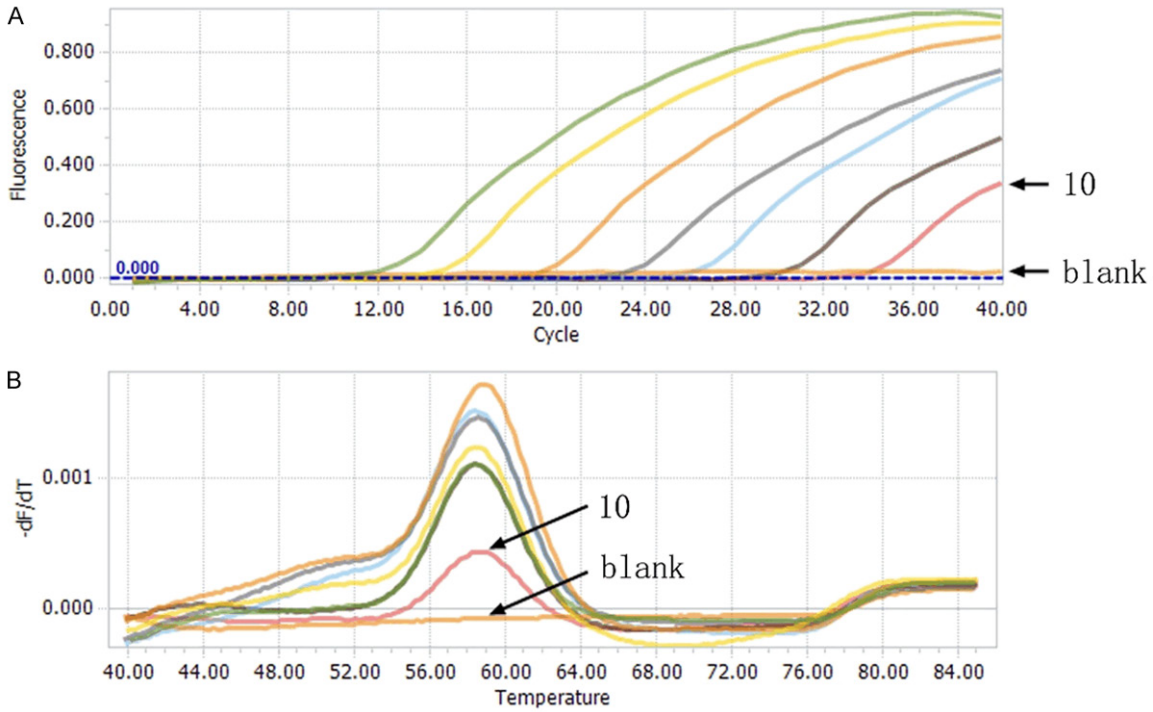

Figure 2. The minimum detection limit for real-time PCR reactions was 10 copies, and the lines of 8 different colors in the figure represent plasmid templates with a concentration from 1×10^7 to 10^1, and a negative control for water. (A) The amplification curve shows the difference in template concentration, and the Cycle value of the reaction decreases with the decrease in template concentration, and is still discernible until approximately 35 cycles. (B) The melting curve peak, each color corresponding to the (A).

tion in G264A, so the Tm value decreased significantly to 45.5°C in **Figure 3E**. Sample 31 was a rare mix of resistant and mutant, showing a double peak at 58.5°C and 51.5°C in **Figure 3G**. These samples were clearly confirmed by sequence results from **Figure 3H-N**. All typical samples are also shown in **Table 1**.

Clinical sample detection

H. pylori DNA samples were extracted from 144 human gastric mucosa specimens. The detection and statistical analysis are shown in **Table 2**. These samples were verified by Sanger sequencing, which showed that our method results were consistent with the sequencing results and indicated that the system was accurate and specific. Different instruments and reaction conditions will cause the sample Tm value to deviate from the predicted result. To eliminate this deviation, we corrected the Tm value of each wild type standard and calculated the average and standard deviation according to the difference (ΔTm) from the standard Tm value, as shown in **Table 2**. A total of 73 cases with levofloxacin resistance were detected, and the drug resistance rate has reached 50%.

Discussion

In our study we used a melting curve method to distinguish the sensitive and resistant types of the levofloxacin resistance gene, *GyrA*, by their difference Tm values of the melting curve. Compared with the existing detection methods, this method was the first report detecting all 11 kinds of point mutations by RT-PCR requiring only one probe, and the operation was simple, accurate and rapid. Our method also solved the problem of multi-drug resistance detection of *H. pylori*, and can conveniently detect other drug resistance or virulence genes simultaneously in multiple fluorescent channels.

The traditional drug resistance test method for *H. pylori* was to conduct drug susceptibility experiments after 2 weeks of culture. In contrast, molecular methods can provide timely clinical guidance. There were a number of molecular methods for the detection of Helico-

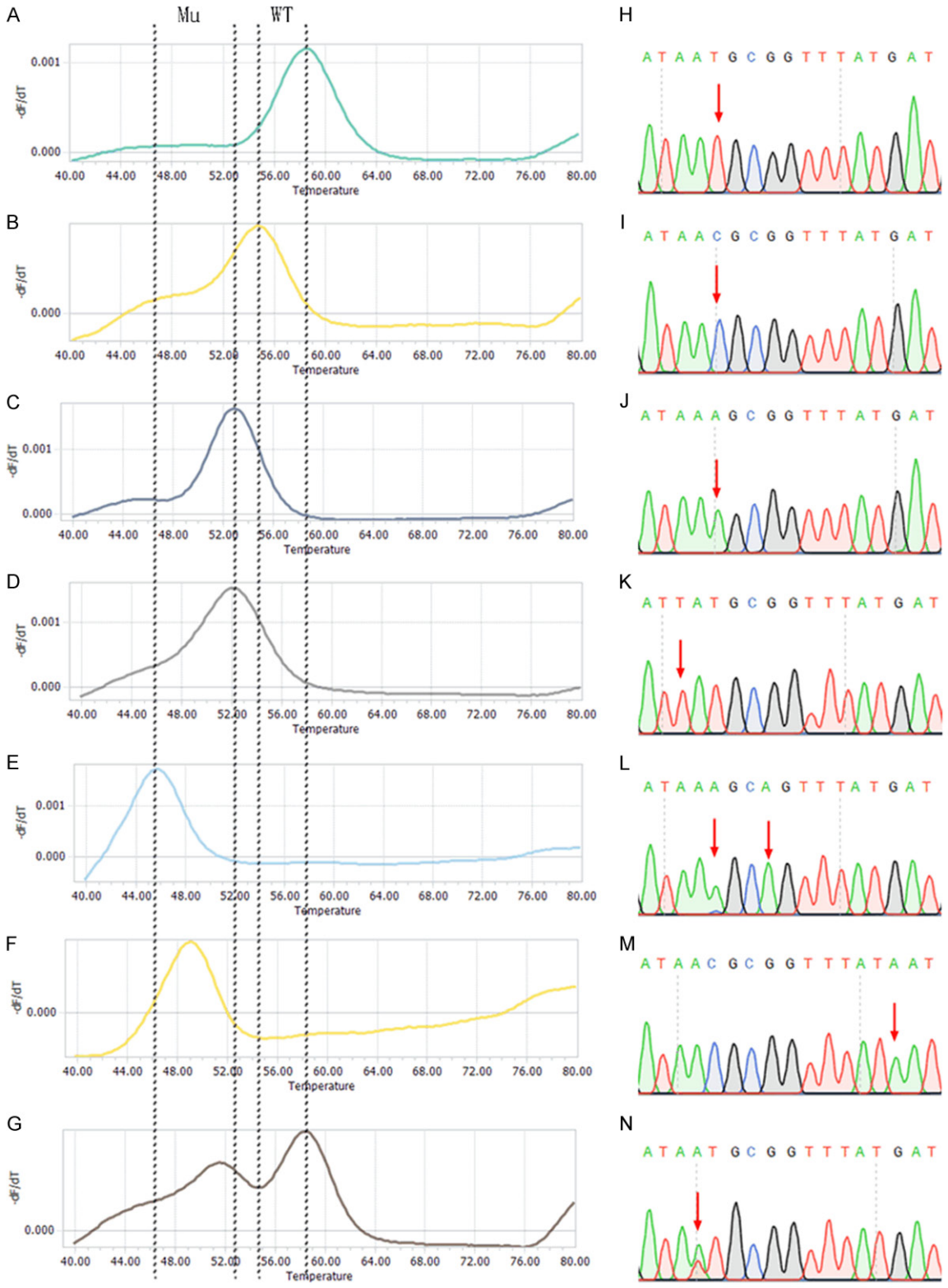

Figure 3. Melting curves of typical samples detection were proved by Sanger sequence results. The Tm value of the melting peaks were wild types in the dotted line to the right of the standard, and rest on the left were resistant mutations. Sequence results (H-N) correspond to the detection results (A-G) accurately. (A) 87AAT Synonymous wild type (B) 87AAC wild type (C) 87AAA resistant (D) 87TAT resistant (E) 87AAA and 88GCA two mutations resistant (F) 91AAT resistant (G) 87AAT Synonymous wild type mixed with 87ATT resistant.

Table 1. Typical sample detection and sequence results were consistent

Sample ID	Tm value	Detection result	Sequence result
01	58.5	Sensitive	87AAT synonymous wild type
09	54.8	Sensitive	87AAC wild type
06	52.8	Resistant	87AAA resistant
20	52.1	Resistant	87TAT resistant
21	45.5	Resistant	87AAA and 88GCA resistant
27	49.0	Resistant	91AAT resistant
31	51.5, 58.5	Mix	87AAT synonymous wild type mixed with 87ATT resistant

Table 2. Clinical samples detection and statistical results

Sample type	Tm	Mean (ΔTm)	SD	Detection result	Total
Synonymous wild type	58.4	-3.467	0.427	Sensitive	27
Wild type	54.9	-0.025	0.109	Sensitive	44
One mutation resistant	52.3	2.556	0.478	Resistant	55
Two mutation resistant	47.9	6.907	1.93	Resistant	18

bacter pylori resistance to clarithromycin. One of the most effective methods was real-time PCR. It was more sensitive than other detection methods and can detect the mixture of resistant and sensitive bacteria [18]. This method was also performed by Matsumura et al. [19] using different primers and probes. Accumulating evidence has shown a strong relationship between these molecular methods and sensitivity tests [20, 21]. However, molecular methods for detecting *H. pylori* resistance to levofloxacin are not as easy as detecting clarithromycin resistance, mainly because of the diversity of mutation types and the presence of synonymous mutations at amino acid 87, thus making traditional molecular detection methods difficult to distinguish.

The most critical feature of the multiplexed quantitative PCR system established in this project was the design of the probe. We used a Taqman probe with self-quenching label at both ends, and could choose any length and position design within the allowable range of the Tm value. Through exploration of the thermodynamic mechanism of molecular beacons in the past, we found complex changes in Tm values caused by different probe positions and different base changes. When the base of the probe was T and the base of the target sequence was C, ΔTm was significantly smaller than other types of mutations. We tried to cover probes of different lengths from amino acid 87-91, using unafold software to predict the Tm values for various mutants (Table S1). Finally, a probe was designed to distinguish between two synonymous mutations and all other resistant mutations.

In the molecular detection of pathogenic microorganisms, non-specific amplification and contamination of foreign DNA fragments have always been an important factor affecting the detection results. Non-specific amplification occurs primarily between homologous sequences of different microorganisms. For example, the *H. pylori* 23 s and GyrA gene detected in this subject also have homologous sequences in Escherichia coli and other common pathogenic microorganisms. The region in which the E. coli GyrA gene binds to the probe has less influence on the detection result because there are more than three base mismatches. However, some of the bases around the 2142 locus of the 23 s gene are perfectly matched. If non-specific amplification of the fragment occurs, the probe will bind, making the test result impossible to judge. To solve the problem, we designed multiple primers for the conserved and specific region of *H. pylori*, ensure that all different types of *H. pylori* can be precisely detected.

The clinical DNA samples were extracted from the gastric mucosa in patients with *H. pylori*. Our results of 144 samples in clinical trials showed that the levofloxacin resistance rate was more than 50%. All samples were verified by Sanger sequencing, which was the gold standard for current molecular detection methods [22, 23].

Resistant strains caused by single mutation can still be killed by increasing the dose of antibiotics, but double mutations cause a 7-fold

increase in levofloxacin resistance based on MIC levels [24]. The method we have established can also determine the double-site mutation by melting peak, but because there are few clinical studies in this area, more susceptibility tests are needed to verify the relationship between different types of double mutations and high levels of resistance.

In summary, our study established a reaction system for the detection of *H. pylori* levofloxacin resistance by RT-PCR. According to the melting peaks of different Tm values, levofloxacin drug resistance was detected. This accurate, rapid and sensitive detection method was more suitable for large-scale clinical applications.

Disclosure of conflict of interest

None.

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References

[1] Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, Sibley RK. Helicobacter-pylori infection and the risk of gastric-carcinoma. N Engl J Med 1991; 325: 1127-31.

[2] Xie Y, Zhang Z, Hong J, Liu W, Lu H, Du Y, Wang W, Xu J, Wang X, Huo L, Zhang G, Lan C, Li X, Li Y, Wang H, Zhang G, Zhu Y, Shu X, Chen Y, Wang J, Lu N; Chinese Society of Gastroenterology Chinese Study Group on Helicobacter pylori. Furazolidone-containing triple and quadruple eradication therapy for initial treatment for helicobacter pylori infection: a multicenter randomized controlled trial in China. Helicobacter 2018; 23: e12496.

[3] Chey WD, Leontiadis GI, Howden CW, Moss SF. ACG clinical guideline: treatment of helicobacter pylori infection. Am J Gastroenterol 2017; 112: 212-239.

[4] Savoldi A, Carrara E, Graham DY, Conti M, Tacconelli E. Prevalence of antibiotic resistance in Helicobacter pylori: a systematic review and meta-analysis in world health organization regions. Gastroenterology 2018; 155: 1372-1382, e17.

[5] Fischbach L, Evans EL. Meta-analysis: the effect of antibiotic resistance status on the efficacy of triple and quadruple first-line therapies for Helicobacter pylori. Aliment Pharmacol Ther 2007; 26: 343-57.

[6] Kuo CH, Hu HM, Kuo FC, Hsu PI, Chen A, Yu FJ, Tsai PY, Wu IC, Wang SW, Li CJ, Weng BC, Chang LL, Jan CM, Wang WM, Wu DC. Efficacy of levofloxacin-based rescue therapy for Helicobacter pylori infection after standard triple therapy: a randomized controlled trial. J Antimicrob Chemother 2009; 63: 1017-24.

[7] Ménard A, Santos A, Mégraud F, Oleastro M. PCR-restriction fragment length polymorphism can also detect point mutation A2142C in the 23S rRNA gene, associated with helicobacter pylori resistance to clarithromycin. Antimicrob Agents Chemother 2002; 46: 1156-7.

[8] Mégraud F, Bénéjat L, Ontsira Ngoyi EN, Lehours P. Molecular approaches to identify helicobacter pylori antimicrobial resistance. Gastroenterol Clin North Am 2015; 44: 577-96.

[9] Shi C, Zhang Y, Wang T, Lu W, Zhang S, Guo B, Chen Q, Luo C, Zhou X, Yang Y. Design, synthesis and biological evaluation of novel DNA gyrase-inhibiting spiropyrimidinetriones as potent antibiotics for treatment of infections caused by multidrug-resistant gram-positive bacteria. J Med Chem 2019; 62: 2950-2973.

[10] Moore RA, Beckthold B, Wong S, Kureishi A, Bryan LE. Nucleotide-sequence of the gyra gene and characterization of ciprofloxacin-resistant mutants of helicobacter-pylori. Antimicrob Agents Chemother 1995; 39: 107-11.

[11] Smith SM, O'morain C, Mcnamara D. Antimicrobial susceptibility testing for helicobacter pylori in times of increasing antibiotic resistance. World J Gastroenterol 2014; 20: 9912-21.

[12] Boyanova L. Prevalence of multidrug-resistant helicobacter pylori in bulgaria. J Med Microbiol 2009; 58: 930-5.

[13] Liou JM, Chang CY, Sheng WH, Wang YC, Chen MJ, Lee YC, Hung HW, Chian H, Chang SC, Wu MS, Lin JT. Genotypic resistance in Helicobacter pylori strains correlates with susceptibility test and treatment outcomes after levofloxacin- and clarithromycin-based therapies. Antimicrob Agents Chemother 2011; 55: 1123-9.

[14] Higuchi R, Dollinger G, Walsh PS, Griffith R. Simultaneous amplification and detection of specific DNA sequences. Biotechnology (N Y) 1992; 10: 413-7.

[15] Oleastro M, Ménard A, Santos A, Lamouliatte H, Monteiro L, Barthélémy P, Mégraud F. Real-time PCR assay for rapid and accurate detection of point mutations conferring resistance to clarithromycin in helicobacter pylori. J Clin Microbiol 2003; 41: 397-402.

[16] Glocker E, Kist M. Rapid detection of point mutations in the gyrA gene of helicobacter pylori conferring resistance to ciprofloxacin by a fluorescence resonance energy transfer-based real-time PCR approach. J Clin Microbiol 2004; 42: 2241-6.
[17] Bu Y, Huang H, Zhou G. Direct polymerase chain reaction (PCR) from human whole blood and filter-paper-dried blood by using a PCR buffer with a higher pH. Anal Biochem 2008; 375: 370-2.
[18] Gibson JR, Saunders NA, Burke B, Owen RJ. Novel method for rapid determination of clarithromycin sensitivity in helicobacter pylori. J Clin Microbiol 1999; 37: 3746-8.
[19] Matsumura M, Hikiba Y, Ogura K, Togo G, Tsukuda I, Ushikawa K, Shiratori Y, Omata M. Rapid detection of mutations in the 23S rRNA gene of helicobacter pylori that confers resistance to clarithromycin treatment to the bacterium. J Clin Microbiol 2001; 39: 691-5.
[20] Elviss NC, Lawson AJ, Owen RJ. Application of 3'-mismatched reverse primer PCR compared with real-time PCR and PCR-RFLP for the rapid detection of 23S rDNA mutations associated with clarithromycin resistance in Helicobacter pylori. Int J Antimicrob Agents 2004; 23: 349-55.
[21] Lehours P, Siffre E, Megraud F. DPO multiplex PCR as an alternative to culture and susceptibility testing to detect helicobacter pylori and its resistance to clarithromycin. BMC Gastroenterol 2011; 11: 112.
[22] Yamade M, Sugimoto M, Uotani T, Nishino M, Kodaira C, Furuta T. Resistance of Helicobacter pylori to quinolones and clarithromycin assessed by genetic testing in Japan. J Gastroenterol Hepatol 2011; 26: 1457-61.
[23] Liou JM, Chen CC, Chang CY, Chen MJ, Fang YJ, Lee JY, Chen CC, Hsu SJ, Hsu YC, Tseng CH, Tseng PH, Chang L, Chang WH, Wang HP, Shun CT, Wu JY, Lee YC, Lin JT, Wu MS; Taiwan Helicobacter Consortium. Efficacy of genotypic resistance-guided sequential therapy in the third-line treatment of refractory helicobacter pylori infection: a multicentre clinical trial. J Antimicrob Chemother 2013; 68: 450-6.
[24] Mori H, Suzuki H, Matsuzaki J, Masaoka T, Kanai T. Acquisition of double mutation in gyrA caused high resistance to sitafloxacin in helicobacter pylori after unsuccessful eradication with sitafloxacin-containing regimens. United European Gastroenterol J 2018; 6: 391-397.

Table S1. Probe design and prediction

Protein	Mutation	Nucleotide	Detection	Tm prediction	ΔTm
87N	Wild type	AAC	Sensitive	59.4	0
87N	C261T	AAT	Sensitive	62.4	-3.0
N87Y	A259T	TAT	Resistant	56.3	3.1
N87T	A260C	ACC	Resistant	57.3	2.1
N87I	A260T	ATC	Resistant	54.4	5.0
N87K	C261G	AAG	Resistant	56	3.4
N87K	C261A	AAA	Resistant	57	2.4
N88V	C263T	GTG	Resistant	52.3	7.1
D91G	A272G	GGT	Resistant	56.8	2.6
D91A	A272C	GCT	Resistant	56.9	2.5
D91N	G271A	AAT	Resistant	56.6	2.8
D91Y	G271T	TAT	Resistant	56.4	2.6