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Original Article

Clinical evaluation of a real-time PCR assay for diagnosis of *Helicobacter pylori* infection and antibiotic resistance

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Abstract: Objectives: *Helicobacter pylori* (*H. pylori*) is a globally prevalent bacterium that increases the risk of developing various gastrointestinal diseases, including gastric adenocarcinoma. This study aimed to evaluate the performances of real-time PCR assay in detecting *H. pylori* infection, as well as clarithromycin and levofloxacin resistance, in both stool and gastric biopsy specimens. Methods: Stool and gastric biopsy specimens were collected from patients within one to three days post-hospitalization. All patients were analyzed for *H. pylori* infection and resistance to clarithromycin and levofloxacin using a real-time PCR based molecular assay. Results: 169 patients (83 males) with a mean age of 43.6±13.1 years were included in the study. The prevalence of *H. pylori* was 89.9% (152/169) in stool and 90.5% (153/169) in gastric biopsy samples. The molecular diagnostics employed in this study exhibited a sensitivity of 99.3% and a specificity of 100%, resulting in a diagnostic accuracy rate of 99.6%. Resistance to clarithromycin was 36.1% (61/169) in stool and 44.4% (75/169) in gastric biopsy samples. The molecular tests for clarithromycin resistance demonstrated a sensitivity of 96.8% and a specificity of 86.8%, with an overall diagnostic accuracy of 90.5%. Furthermore, resistance to levofloxacin was 22.5% (38/169) and 26.6% (45/169) in stool and gastric biopsy samples, respectively. The molecular test demonstrated a sensitivity of 80.9% and a specificity of 94.3%, resulting in a diagnostic accuracy of 90.5%. Conclusion: The implementation of real-time PCR-based screening for *H. pylori* infection and resistance to clarithromycin and levofloxacin in the stool may enhance the success rate of eradication therapy.

Keywords: *Helicobacter pylori*, antibiotic resistance, clarithromycin, levofloxacin, real-time PCR

Introduction

Helicobacter pylori (*H. pylori*) infection is a common chronic bacterial infection in humans which affects more than half of the human population [1]. The prevalence of infection by *H. pylori* is declining in the developed countries; however, it remains a major cause of morbidity and mortality worldwide [2]. *H. pylori* is a major etiological factor of various gastroduodenal diseases, including chronic gastritis and peptic ulcer disease, also closely associated with the development of mucosa-associated lymphoid tissue lymphoma and even gastric adenocarcinoma [3]. Due to its carcinogenic potential, both the World Health Organization (WHO) in 1994 and the United States Department of Health and Human Services (HHS) in 2022 have classified *H. pylori* as a class I carcinogen [4, 5]. Consequently, there is a critical need for the development of low-cost, accurate, and non-invasive diagnostic methods to detect *H. pylori* infection for the implementation of effective therapeutic strategies and the primary prevention of gastric cancer.

Currently, the detection methods for *H. pylori* infection are primarily divided into invasive and non-invasive methodologies [6]. Recently, with the development of molecular biology technology, polymerase chain reaction (PCR) has been widely used to detect pathogenic bacteria. PCR-based molecular tests are highly reproducible, quicker than microbiological antibiotic susceptibility testing (AST) methods [7]. Real-time PCR is a breakthrough technique in the diagnosis of

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H. pylori, not only providing rapid and accurate detection, but also detecting point mutations associated with antibiotic resistance [8]. The fundamental principle is to monitor the increase in amplicon formation in real time. Primers that are specific for the target gene are designed, as well as a biprobe on the amplicon, the acceptor probe (sensor probe) labelled with LC-Red, and a donor probe (anchor probe) labelled with fluorescein [9]. When excited, the anchor probe fluorophore transfers energy to the sensor probe fluorophore, which emits a signal. Real-time PCR offers numerous advantages over standard PCR, including enhanced sensitivity, ease of implantation, and a reduced risk of contamination [10]. It is of significant importance to note that real-time PCR can be conducted in two distinct manners: firstly, in a culture-based manner when performed on cultured isolates, and secondly, in a culture-free manner when directly applied to a variety of biological specimens, including fresh, frozen or paraffin-embedded gastric biopsy samples, stool samples and gastric juice [11]. Nevertheless, the accuracy of real-time PCR may be influenced by the condition of the samples and the potential for contamination or degradation of the DNA.

A significant limitation of studies on *H. pylori* is the necessity of obtaining stomach biopsy material, which severely restricts the populations that can be sampled [12]. There is evidence that infected individuals excrete *H. pylori* in their feces, which is an easily accessible biosample and has been used to diagnose numerous infectious diseases [13]. A number of studies have employed PCR tests to identify *H. pylori* infection in stool samples from patients, showing an 82% sensitivity and a 99% specificity [14, 15]. In terms of cost, real-time PCR tests are competitive with the other tests usually performed, such as histologic examination, culture and susceptibility testing, but more costly than the rapid urease test, which has a lower sensitivity [16].

The prevalence of *H. pylori* antibiotic resistance has been on the rise globally, while the success rate of *H. pylori* eradication has been on the decline [17]. The primary reason for unsuccessful treatment outcomes has been attributed to antimicrobial resistance. It is therefore crucial for clinicians to be aware of the local prevalence of *H. pylori* resistance to clarithromycin and levofloxacin, in order to select the most appropriate *H. pylori* eradication regimen, whether in the first or second line of treatment [18]. Although real-time PCR is the most sensitive and specific method for detecting of *H. pylori* in gastric biopsy specimens, there is a lack of studies that have compared the performance of real-time PCR using different sample types. Therefore, the objective of this study is to evaluate the diagnostic accuracy of stool-based real-time PCR as an alternative approach for detecting *H. pylori* infection and antibiotic resistance, and to compare it to the gastric biopsy-based real-time PCR assay, which serves as the reference standard.

Materials and methods

Ethics approval

This study was conducted in accordance with the Consolidated Standards of Reporting Trials (CONSORT) statement. Written informed consent was obtained from all participants. The study protocol was approved by the Institutional Ethics Board of the Civil Aviation General Hospital, Beijing, China (No. 2023-L-K-05) and conducted in accordance with the Declaration of Helsinki. The trial was registered in the Chinese Clinical Trials Registration (www.chictr.org.cn) with the registration number ChiCTR-2300070267.

Study design and assessment

Between April 2023 and August 2023, patients aged 18-75 years with confirmed *H. pylori* infection by ^{13}C-urea breath test (UBT) were eligible for enrollment. A total of 169 patients who met the eligibility criteria were enrolled in outpatient clinics. The inclusion criteria included: (1) Male or female patients aged 18 to 75 years; (2) Patients diagnosed with *H. pylori* infection by positive ^{13}C-UBT; (3) Patients diagnosed with upper gastrointestinal symptoms, such as epigastric pain, acid reflux, heartburn, epigastric distention, and nausea; (4) Patients who signed the informed consent form. Exclusion criteria were as follows: (1) Patients who had taken antibiotics, bismuth, and PPIs within four weeks; (2) Patients who had recurrent or long-term use of macrolides and penicillin; (3) Patients with a serious primary disease; (4)

Table 1. Demographic and clinical data of patients

Characteristic	*H. pylori*-infected patients (*n* = 169)
Age, (y, mean ± SD)	43.6±13.1
Gender	
Male	83 (49.1%)
Female	86 (50.9%)
BMI (kg/m^2)	23.6±3.7
Alcohol drinking	
No	137 (81.1%)
Yes	32 (18.9%)
Smoking	
No	134 (79.3%)
Yes	35 (20.7%)
Endoscopy diagnosis	
Chronic superficial gastritis	63 (37.3%)
Chronic atrophic gastritis	71 (42.0%)
Gastroduodenal polyps	35 (20.7%)

Percentage calculated based on total number of patients. *H. pylori*: *Helicobacter pylori*, BMI: body mass index.

Patients with clinically significant liver or kidney insufficiency; (5) Pregnant or lactating women.

Both stool and gastric biopsy samples were collected from each patient between one and three days after hospital admission. A molecular test based on real-time PCR was employed to screen for *H. pylori* infection and resistance to clarithromycin and levofloxacin. Both stool and gastric biopsy samples were used, and the test kit employed was the Clarithromycin, Quinolone-Resistant and Non-Resistant *Helicobacter Pylori* Nucleic Acid Amplification Test Kit (Jiangsu Mole Bioscience Co., Ltd., Taizhou, Jiangsu, China).

Statistical analysis

Statistical analyses to ascertain predictive factors were executed using SPSS 26.0 (SPSS, Chicago). The quantitative data were expressed as mean, standard deviations (SD), range, or percentages. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of each test were investigated. The agreement between the stool and gastric biopsy sample real-time PCR test was estimated using the kappa coefficient. A *P*-value of less than 0.05 was considered statistically significant.

Results

Patient characteristics

The clinical characteristics are presented in **Table 1**. A total of 169 patients were enrolled in this study, with a mean age of 43.6±13.1 years. Approximately half of the subjects were male (n = 83, 49.1%). Endoscopic examination revealed that 63 (37.3%) patients had chronic superficial gastritis, 71 (42.0%) had chronic atrophic gastritis, and 35 (20.7%) had gastroduodenal polyps. **Figure 1** presents the study design and flowchart of this study.

Diagnostic performance for H. pylori

Accurate and reliable diagnosis of *H. pylori* infection is important for the effective management of infected patients. As shown in **Table 2**, *H. pylori* infection was identified in 152 patients (89.9%) *via* stool samples and in 153 patients (90.5%) through gastric biopsy samples using a real-time PCR-based molecular test. The real-time PCR test demonstrated 99.3% sensitivity and 100% specificity, with a diagnostic accuracy of 99.6%. The concordance between stool and gastric biopsy samples' real-time PCR results was almost perfect (Kappa = 0.963, *P*<0.001), indicating that stool samples could be a reliable non-invasive alternative to gastric biopsies for diagnosing *H. pylori* infection.

Diagnostic performance for clarithromycin resistance

As shown in **Table 3**, the presence of clarithromycin resistance was observed in 61 patients (36.1%) from stool samples and 75 patients (44.4%) from gastric biopsy samples. The molecular test demonstrated 96.8% sensitivity and 86.8% specificity, with a diagnostic accuracy of 90.5%. There was a strong concordance (Kappa = 0.805, *P*<0.001) between the results of the real-time PCR tests on stool and gastric biopsy samples, confirming the reliability of the real-time PCR-based test for stool samples.

Diagnostic performance for levofloxacin resistance

As shown in **Table 3**, the prevalence of levofloxacin resistance was found to be 22.5% (38/169) in stool samples and 26.6% (45/169) in gastric biopsy samples. The real-time PCR test exhibit-

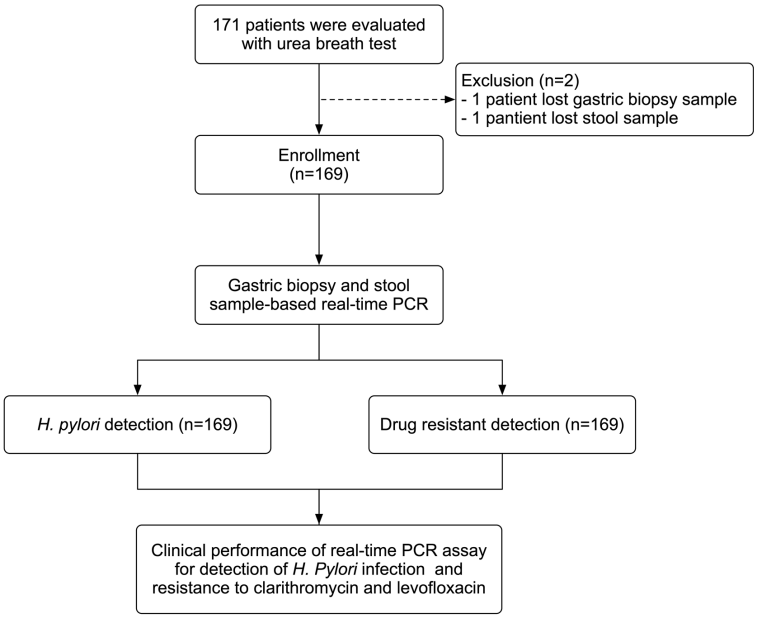

Figure 1. Study design and flowchart. A total of 171 patients were enrolled in the study. Following the exclusion of two patients, 169 patients were included and stool and gastric biopsy specimens were collected to detect *H. pylori* infection and resistance to clarithromycin and levofloxacin.

Table 2. Clinical performance of real-time-PCR detection of *H. pylori* on stool and gastric biopsy samples

Tests performances	*H. pylori* detection
Positive in stool samples, n	152 (89.9%)
Positive in gastric biopsy samples, n	153 (90.5%)
Sensitivity	99.3%
Specificity	100%
Misdiagnosis rates	0%
Missed rate	0.9%
Accuracy	99.6%
Youden's index	99.3%
Positive predictive value	100%
Negative predictive value	91.3%
Kappa	0.963*

The kappa coefficient was classified as follows: >0.90, almost perfect; 0.80 to 0.90, strong; 0.60 to 0.79, moderate; 0.40 to 0.59, week; 0.21 to 0.39, minimal; 0 to 0.20, no agreement. Youden's index was calculated as sensitivity + specificity - 1. *P<0.001. *H. pylori*: *Helicobacter pylori*, real-time PCR: real-time polymerase chain reaction.

ed 80.9% sensitivity and 94.3% specificity, with a diagnostic accuracy of 90.5%. Moderate concordance (Kappa = 0.761, P<0.001) between the results of the real-time PCR tests on stool and gastric biopsy samples was observed. These findings provided evidence to support the use of non-invasive stool testing in clinical practice, offering a reliable alternative to invasive gastric biopsies.

Discussion

This study is the first to evaluate the analytical performance of real-time PCR assays detecting *H. pylori* infection and antibiotic resistance in stool samples. This side-by-side comparison aims to expand diagnostic methodologies beyond traditional gastric biopsies, offering non-invasive options with high diagnostic accuracy. Our findings demonstrate the feasibility and high efficacy of using real-time PCR assay on stool samples to detect *H. pylori* and assess antibiotic resistance. This approach represents a compelling alternative to more invasive gastric biopsy, particularly in settings where endoscopy is less accessible or contraindicated.

The urea breath test (UBT) is an effective method of determining the presence or absence of *H. pylori* infection [19]. In our study, all patients with *H. pylori* infection confirmed by ^{13}C-UBT, identified in 89.9% of stool samples and 90.5% of gastric biopsy samples, had a positive result on a real-time PCR-based molecular test. Possible causes include not following the manufacturer's instructions and being slow to read the UBT. A false-positive UBT may be obtained in the presence of other urease-producing organisms, such as members of the *Enterobacterales* or *Staphylococcus* aureus [20]. In particular, the administration of antibiotics prior to UBT has been implicated in false negative results. Similarly, the use of proton pump inhibitors (PPIs) prior to endoscopy can lead to negative results on both the UBT and histological exami-

Table 3. Clinical performance of real-time-PCR detection of clarithromycin and levofloxacin resistance on stool and gastric biopsy samples

Tests performances	Clarithromycin	Levofloxacin
Drug-resistant in stool samples, n	61 (36.1%)	38 (22.5%)
Drug-resistant in gastric biopsy samples, n	75 (44.4%)	45 (26.6%)
Sensitivity	96.8%	80.9%
Specificity	86.8%	94.3%
Misdiagnosis rates	13.2%	5.7%
Missed rate	3.2%	19.1%
Accuracy	90.5%	90.5%
Youden's index	83.6%	75.2%
Positive predictive value	81.3%	84.4%
Negative predictive value	97.9%	92.7%
Kappa	0.805*	0.761*

The kappa coefficient was classified as follows: >0.90, almost perfect; 0.80 to 0.90, strong; 0.60 to 0.79, moderate; 0.40 to 0.59, week; 0.21 to 0.39, minimal; 0 to 0.20, no agreement. Youden's index was calculated as sensitivity + specificity - 1. *P<0.001.

nation. However, these limitations are not typically observed with molecular techniques, which offer more reliable detection under these conditions.

The main obstacle in the management of *H. pylori* infection is the direct impact of antimicrobial resistance, making the detection of such resistance crucial [5]. Clarithromycin plays a central role in the triple therapy regimen commonly used to eradicate *H. pylori* [21]. However, escalating rates of resistance to this antibiotic in various countries have made it the main factor in the up to 70% failure rate of first-line eradication regimens [22]. Recent international guidelines have discouraged empirical clarithromycin-based triple therapy in geographical areas with a significantly high prevalence (15%) of clarithromycin resistance [23]. Antimicrobial susceptibility testing is highly recommended and has been shown to be significantly more effective than empiric treatment [24]. Typically performed on gastric biopsy samples, this approach requires approximately 20 to 72 hours to yield results. However, its low yield and extensive laboratory requirements limit its accessibility and pose challenges for widespread use [25]. Because of these limitations, culture methods are gradually being replaced by molecular biology methods such as PCR [26]. Using non-invasive sample types could improve patient compliance and therapeutic outcomes, thereby optimizing the time and cost implications of disease management, especially in the context of future increases in resistance rates [27]. Recent European guidelines advocate clarithromycin susceptibility testing, using molecular techniques or culture methods where available, prior to the administration of any clarithromycin-based regimen [28]. PCR methods facilitate the use of optimized triple therapy, which is effective in 60 to 90% of patients.

Both invasive and non-invasive tests are available to accurately diagnose infection, but none can be considered the gold standard by itself [29]. Research on the PCR detection of both *H. pylori* and clarithromycin resistance in faecal samples has been limited. The few existing studies have used poorly defined criteria and small patient cohorts, resulting in wide confidence intervals for their performance characteristics [30]. In particular, stool-based PCR assays such as GenoType *Helico*DR (Hain Lifescience, Nehren, Germany) and *H. pylori* ClariRes (Ingenetix, Vienna, Austria) were originally developed for biopsy specimens and have not undergone extensive validation in faecal samples [31]. A non-invasive molecular approach using faecal samples could facilitate the implementation of customized eradication protocols. However, the literature generally indicates that the sensitivity of real-time PCR for the detection of *H. pylori* in stool samples is typically low [32]. In our study, *H. pylori* infection was detected in 152 patients (89.9%) from stool samples and in 153 patients (90.5%) from gastric biopsy samples. The results of the real-time PCR test are promising, with a sensitivity of 99.3% and a specificity of 100%, and a diagnostic accuracy of 99.6%. The real-time PCR results from stool and gastric biopsy samples showed excellent concordance (Kappa = 0.963, P<0.001), suggesting that the stool-based real-time PCR test is reliable for the diagnosis of *H. pylori* infection.

Clarithromycin, a bacteriostatic antibiotic of the macrolide family, has a primary role in the treatment of *H. pylori* by preventing protein transla-

tion [33]. Studies have shown that empirical first-line treatment with clarithromycin has a very low success rate, with only 18% achieving over 85% eradication and around 60% failing to achieve 80% eradication [34]. Recent studies have reported high resistance prevalence rates among *H. pylori* strains to clarithromycin (20-50%), metronidazole (40-70%) and levofloxacin (20-50%) [35]. Our study showed clarithromycin and levofloxacin resistance rates of 36.1% and 22.5% in stool samples and 44.4% and 26.6% in gastric biopsy samples, respectively, suggesting that the use of standard empirical triple therapy should be modified. Our data correlate with a recent meta-analysis showing an increase in resistance to clarithromycin and levofloxacin worldwide [21].

The exceptional performance of the real-time PCR-based test for stool samples in our study supports its recommended use in circumstances where a histological examination of the gastric mucosa is not essential. This test is particularly advantageous for various indications related to *H. pylori* testing and targeted treatment. Furthermore, this test is suitable for screening for *H. pylori* infection in patients for whom endoscopy and biopsy are initially indicated. The feasibility of the molecular assay was very good and time-saving (5 min for extraction and 10 min for amplification, with a total turnaround time of 3 h, including validation of the result). The total cost per patient was estimated to be less than $40.

This study has several limitations. First, the sample size was relatively small to analyze the predictive values and accuracy of real-time PCR. Second, this study did not investigate the phenotype of clarithromycin and levofloxacin resistance with *H. pylori* culture. PCR can detect resistance mutations that do not always translate into actual phenotypic resistance, potentially leading to misinterpretation of a strain's resistance profile, which could affect the reliability of using PCR as a stand-alone test for antibiotic resistance.

Conclusion

The main advantage of the real-time PCR assay is its ability to accurately diagnose *H. pylori* infection non-invasively and simultaneously detect clarithromycin and levofloxacin resistance. This benefits patient compliance and promotes early and appropriate treatment, ultimately leading to successful *H. pylori* eradication and reduced risk of associated complications.

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Disclosure of conflict of interest

None.

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