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Loop-Mediated Isothermal Amplification as a Fast Noninvasive Method of *Helicobacter pylori* Diagnosis

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Background: Helicobacter pylori infection is etiologically associated with some important health problems such as gastric cancer. Because of the high clinical importance of *H. pylori* infection, development of a noninvasive test for the detection of *H. pylori* is desirable. *Methods:* In this study, a loop-mediated isothermal amplification (LAMP) targeted *ureC* of *H. pylori* was evaluated on 100 stool specimens and compared with a stool antigen test. Culture and rapid urease test were considered as gold standards. *Results:* The overall detection rate of the fecal antigen test and LAMP was 58% and 82%, respectively. The analytical sensitivity of the fecal antigen test and LAMP was 500 and 10 *H. pylori* cells/g and 10 fg DNA/reaction, which is equal to six *H. pylori* genome. *Conclusion:* LAMP technique has been characterized by high sensitivity and low detection limit for the detection of *H. pylori* in stool specimen. Clinical diagnostic performance of LAMP was better than the stool antigen test. J. Clin. Lab. Anal. **30**: 464–470, 2016. © 2015 Wiley Periodicals, Inc.

Key words: clinical performance; *Helicobacter pylori*; limit of detection; loop-mediated isothermal amplification; sensitivity; stool antigen test

INTRODUCTION

Helicobacter pylori is a curved, microaerophilic, and Gram-negative rod bacterium. This bacterium is the most common human pathogen such that over half of the world's population is infected with this bacterium (1). In developed and developing countries, about 50% and 90% of adults, respectively, are infected with the bacteria (2). This bacterium is considered as the main cause of gastritis, gastric and duodenal ulcers, gastric adenocarcinoma, and MALT lymphoma (3).

In 1994, the International Agency for Research on Cancer introduced *H. pylori* as a class A carcinogens (definite carcinogen) (4). Gastric cancer, as a consequence of chronic gastric infection by *H. pylori*, is the fourth most common cancer and the second leading cause of cancer-related death in the world (5, 6).

Several methods can be used to diagnose *H. pylori* infection, which are divided into invasive and noninvasive methods based on the use of endoscopy. Histopathology, culture, and rapid urease test (RUT) are considered as the invasive tests. Fecal antigen test, urea breath test (UBT), and serology are known as the noninvasive tests (7, 8).

It is reported that mortality and morbidity rates at a single endoscopy were one in 2,000 and one in 200 people, respectively (9, 10). Therefore, great efforts have been performed to develop an accurate and reliable noninvasive method to diagnose *H. pylori*.

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On the basis of the Florence consensus report (Maastricht IV), endoscopy for diagnostic examination of the upper gastrointestinal tract (GI) with biopsies is recommended for old symptomatic patients and all patients with alarming features (such as weight loss, dysphagia, GI bleeding, abdominal mass, and iron deficient anemia). In dyspeptic patients younger than the age of 45 years and in populations where the prevalence of *H. pylori* is over 20%, test and treatment strategy is preferable. In these patients, *H. pylori* infections were assayed using a reliable noninvasive method and decision for the treatment of infection can be made. Test and treatment strategy is cost effective and efficient in patients with dyspepsia. Some of the noninvasive methods are proposed for the detection of *H. pylori* in test and treatment strategy including UBT, fecal antigen test, and serological test (11).

However, the culture of *H. pylori* from gastric biopsy specimen is the gold standard methods for diagnosis of *H. pylori* infection, but the culture of this bacterium from stool sample is extremely difficult due to overgrowth of other bacteria (12, 13).

Despite having a sensitivity of 88–95% and specificity of 95–100%, UBT is an expensive test and requires special device (13). Furthermore, the serological test has low specificity; therefore, this method cannot be used for following up the eradication (8, 14).

Today, the stool antigen test is more accessible and has been examined in numerous studies. The sensitivity and specificity of the test have been reported above 90%. But in polyclonal stool antigen, test sensitivity and specificity will drop. However, this test is very useful, as a noninvasive test but have limitations, such as the effect of stool consistency in the test result, and clinical limitations such as bleeding. Monoclonal stool antigen kits are also very expensive (15–17).

Molecular methods can also be used as a noninvasive test for detection of *H. pylori* in the stool of patients. One of the molecular techniques currently used in the diagnosis of various microorganisms is loop-mediated isothermal amplification (LAMP). In this method, an isothermal temperature (60–65°C) is applied for 60–90 min and amplification is done by Bst DNA polymerase. A positive reaction is indicated by the turbidity caused by an increase in quantity of magnesium pyrophosphate in the reaction as a by-product of nucleic acid amplification or the color change after adding an intercalating dye to the reaction solution (18, 19).

Detection of *H. pylori* in stool specimens with LAMP technique can be used as a noninvasive test for the diagnosis and appropriate treatment of infection. It can also be used in epidemiological studies on *H. pylori* infection in different populations.

The purpose of this study was to set up the LAMP reaction as a fast noninvasive method and for the evaluation of its diagnostic value for the detection of *H. pylori* in stool of patients suffering from gastroduodenal disorders referred to Imam Khomeini Hospital, Kermanshah, and compared with the stool antigen detection kit.

MATERIALS AND METHODS

Study Population and Sampling

This study was a descriptive cross-sectional survey that was carried out on 100 patients who were referred to Imam Khomeini Hospital in Kermanshah in 2013–2014. Patients were examined by a specialist through gastrointestinal endoscopy. Informed consent and patient data collection form were completed for each patient. Patients were excluded if they have been taking drugs for the treatment of *H. pylori* infection 2 weeks prior to the study (20). Two pairs of biopsy specimens were collected from the antrum and corpus of the stomach for RUT and culture. One pair of biopsy specimens were transported to the laboratory in Brucella broth containing 20% glycerol as transport medium at 4°C. The other one was used for RUT. Stool samples were also collected from all patients. Stool samples were transported to the laboratory at 4°C and stored at −70°C until analysis.

RUT and culture were considered as the gold standard in this study. Sensitivity and specificity of the stool antigen detection kit and LAMP were compared according to the result of gold standard tests. A positive result was defined as either positive culture or positive RUT, and a negative result was considered if both tests were negative.

Culture

In order to set up the LAMP method, extracted DNA from pure colonies *H. pylori* was used in each reaction. Therefore, *H. pylori* was cultured in columbia agar plates enriched with eggs containing 5 mg/l trimethoprim, 10 mg/l vancomycin, and 2.5 mg/l amphotericin B. The plates were incubated under microaerophilic condition at 37°C for 3–5 days.

For the primary isolation and subculture of the identified colonies, the culture conditions were used as initially described. Tiny translucent gray presumed colonies were confirmed by gram staining, and positive reaction for oxidase, catalase, and urease tests (21).

DNA Extraction

DNA of stool samples was extracted using a stool-specific DNA extraction kit (QIAamp DNA Stool Mini Kit; Qiagene, Hilden, Germany) according to the manufacturer's instruction. The quality of the extracted DNA was determined by the Nanodrop device (Thermo Scientific, Delaware, USA) and stored at −20°C.

Helicobacter pylori DNA of the pure colonies was extracted by using a kit (AccuPrep Genomic DNA Extraction Kit; Bioneer, South Korea) according to the manufacturer's protocol. The quality of extracted DNA was determined by the Nanodrop device. *H. pylori* clinical isolates were used as a positive control and approved by biochemical methods, the Gram stain, and *ureC* gene PCR test.

Analytical Sensitivity of Stool Antigen Test and LAMP

The analytical sensitivity of LAMP and stool antigen tests was determined using spiking of specific cell number of pure 3 days old culture of *H. pylori*. The bacterial cell number was quantified by Petroff-Hausser Counter. Cell counting was done three times, and the mean was considered as a real number of *H. pylori* in suspension. *H. pylori* suspension was used to make tenfold serial dilution in phosphate-buffered saline (PBS), and the appropriate number of *H. pylori* cells was spiked in 200 mg of fecal samples (22). It must be noted that fecal sample used in the analytical sensitivity testing was collected from a patient who was negative for *H. pylori* in RUT, culture, and PCR of gastric biopsy and also negative for stool PCR.

Then, DNA of *H. pylori* spiked stool samples was extracted using a kit, and LAMP was performed using urease C specific primers for *H. pylori* according to previous conditions.

To determine the minimum amount of *H. pylori* DNA detected by LAMP (limit of detection), the tenfold serial dilution of the purified *H. pylori* DNA with known concentration was prepared and subjected to the LAMP reaction. Different concentrations of a known concentration of purified *H. pylori* DNA were prepared including 10, 1, 0.1, 0.01 ng (10 pg); 1, 0.1, 0.01 pg (10 fg); and 1, 0.1, and 0.01 fg. To calculate the copy number in the reaction, *H. pylori* genome size was considered as 1.6×10^7 bp.

Determining Analytical Specificity of the LAMP Primers

To determine the analytical specificity of the primers, the LAMP reaction was carried out on pure DNA of several bacteria, which were present in the stool. These bacteria were *Staphylococcus aureu*s ATCC 25923, *Enterococcus faecalis* ATCC 29212, clinical isolates of *Shigella dysenteriae*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Yersinia enterocolitica*, and *Salmonella typhi*. All the bacteria were confirmed by biochemical tests.

Stool Antigen Test

The presence of *H. pylori* antigen in stool was determined using a specific commercial kit (*H pylori* Ag test; Intex Diagnostika, Switzerland) according to the manufacturer's instruction. All components of the kit and fecal samples were kept at room temperature (18–25°C) before running the test. Finally, the results of the test were compared on the basis of results obtained from the two gold standards.

LAMP Reaction

For designing the primers used in the LAMP method, first conserved region of *ureC* gene was determined by alignment of *ureC* sequence from multiple strains of *H. pylori* using ClustalW2 software. Alignment file was subjected to primer design using Primer explorer version 4. Three pairs of primers used in this study are shown in Table 1.

The LAMP reaction was carried out as described previously with some modifications (23). In brief, the optimal reaction was performed in a total volume of 30 μl, consisting of 20 mM Tris-HCl (pH 8.8 at 25°C), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 2 Mm dNTPs, 12 Mm $MgSO_4$, 0.8 M Betaine (Sigma-Aldrich), primers including 1.6 μM HP-ureCFIP and HP-ureCBIP, 0.2 μM HP-ureCB3 and HP-ureCF3, 0.8 μM HP-ureCLB and HP-ureCLF, 8U Bst DNA polymerase, and 3 μl DNA, extracted from stool samples. Initial heat denaturation of the target DNA was carried out at 96°C for 3 min, and then the reaction mixture was placed on ice for 30 s to add 8 U Bst DNA polymerase (New England Biolabs, Bishop's Stortford, UK) followed by incubation at optimal temperature of 65°C for 60 min. Finally, the reaction was terminated at 90°C for 2 min. Positive and negative controls were included in each sample run (24). Amplification was detected using visual detection of white turbidity of the amplification by-products (magnesium pyrophosphate salts) or a white pellet following centrifugation with the naked eyes.

Statistical Analysis

The clinical sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of LAMP and fecal antigen tests for diagnosis of *H. pylori* in stool sample of the patients suffering from gastroduodenal diseases were calculated using SPSS. Positive culture and/or positive RUT were considered as the "gold standard." Statistical analysis was conducted using StatsDirect 2.7.2 (StatsDirect Ltd, Cheshire, UK) and the McNemar's chi-square test. The value of $P < 0.05$ was considered statistically significant. The agreement between the

TABLE 1. Primers Used in the LAMP Method

Primer name	Sequences (5′ to 3′)
HP-ureCBIP	CTCGCCTCCAAAATTGGCTTGCGATTGGGGATAAGTTTG
HP-ureCFIP	GCATATCATTTTTAGCGATTACGCTCACTAACGCGCTCACTTG
HP-ureCB3	TCCCAAGATTTGGAATTGAAG
HP-ureCF3	GCTTACCTGCTTGCTTTC
HP-ureCLB	TCAATTGCATGCATTCGCTCA
HP-ureCLF	CAGGCGATGGTTTGGTGTG

methods was calculated using the Cohen's kappa coefficient.

RESULTS

Patients

The age of enrolled patients was between 17 and 87 years with a mean age of 50.5 ± 17.61 years. A total of 100 patients including 42 female (42%) with a mean age of 49.09 ± 18.44 and 58 men (58%) with a mean age of 50.64 ± 16.64 were included. Among the patients, 64 patients (64%) were outpatients and 36 patients (36%) were hospitalized in the internal medicine ward and other departments.

Culture and RUT

Among the 100 biopsy specimens taken from patients with gastroduodenal diseases, 53 patients (53%) were positive for *H. pylori* in RUT and 44 patients (44%) were positive for *H. pylori* in culture of biopsy specimens.

Analytical Sensitivity of LAMP and Stool Antigen Test

The analytical sensitivity of fecal antigen test for detecting *H. pylori* in fecal samples was 100 bacteria per 200 mg of feces (500 bacteria per gram of feces). The evaluation of analytical sensitivity of LAMP using spiked stool sample with the known number of *H. pylori* shown in this test was able to detect as low as two bacteria per 200 mg of feces or in other words ten bacteria per gram of feces. The detection limit of the *H. pylori* purified DNA was determined using the LAMP assay; the *ureC*-specific primers were able to detect as low as 10 fg DNA per reaction, which is equal to six copy number of *H. pylori* genome.

Analytical Specificity of the Primers

None of the extracted DNA from non-*H. pylori* microorganisms resulted in positive amplification in LAMP, and the positive result was shown only in *H. pylori* DNA. This means that analytical specificity of the LAMP assay for *H. pylori* diagnosis was 100%.

Fecal Antigen Test

The overall detection rate of the fecal antigen test was 58% (58/100), and 42% (42/100) of the samples were negative for *H. pylori*. It means that 16 and 25 samples, which have been negative in urease and cultures, were positive in the fecal antigen test.

The sensitivity, specificity, PPV, and NPV of the fecal antigen test were 79.3%, 71.4%, 79.3%, and 71.4%, respectively. The observed agreement between the fecal antigen test and gold standard was 76% and the κ coefficient of 0.5074 suggested a moderate agreement between two diagnostic tests.

LAMP Reaction

The rate of positivity for the LAMP assay was 82% (82/100). The data showed that the LAMP assay had a higher positive rate than that of fecal antigen test and gold standard methods. Twenty-nine out of 47 RUT were negative, whereas 38 out of 56 culture negative biopsy specimens were positive in the LAMP assay.

The sensitivity, specificity, PPV, and NPV of the LAMP assay were 100%, 42.8%, 70.7%, and 100%, respectively. The observed agreement between the LAMP test and gold standard was 76% and the κ coefficient of 0.4652 suggested a moderate agreement between two diagnostic tests.

DISCUSSION

H. pylori is a curved, microaerophilic, and Gram-negative rod bacterium, which is etiologically associated with gastritis, peptic ulcer disease, MALT lymphoma, and gastric cancer (3). The stomach cancer is the most common cancer among Iranian men and third common cancer in women (25). The age-standardized death rate (per 100,000 person year) of stomach cancer in Iran is 26.1 and 11.1 in male and female, respectively (5).

Because of the high clinical importance of *H. pylori* infection, numerous diagnostic methods based on nucleic

acid technology for its detection in clinical, biological, and environmental samples have been developed (22, 26–34).

Stool specimen has been considered and used as a sample for the development of a direct method of *H. pylori* detection by many investigators because it is easy to collect by noninvasive access (35). A lot of studies have been conducted for detecting *H. pylori* in stool samples based on PCR with target sequences such as *ureA*, *ureC*, and 16S rRNA genes from different populations. Different sensitivity and specificity have been reported ranging from 25% to 100% and 80% to 100%, respectively (33, 36–42). However, the result of these tests depend on the quality and amount of DNA recovered, the target sequences, differences in the specificity and sensitivity of the primers used, and the nature of the amplification protocol (43). As a consequence of the mentioned obstacles, the frequency of the *H. pylori* DNA detection in stool specimen also varies from 25% to 100%.

In addition to improved sensitivity and specificity, a diagnostic test for *H. pylori* detection must be rapid in performance, cost effective, and have potential of standardization and specially noninvasiveness. Since endoscopy as an invasive procedure is not necessary in many patients unless in patients with alarming symptoms, the development of diagnostic test for *H. pylori* detection has traditionally focused on a noninvasive test (11).

LAMP, as a novel nucleic acid isothermal amplification technique, has been described by Notomi et al. (44), and nowadays known as a rapid, specific, sensitive, cost-effective, easy-operating, and most promising molecular diagnostic test for infectious pathogens (23, 30, 44, 45).

The aim of this study was to evaluate the LAMP reaction as a fast noninvasive method and its diagnostic value for the detection of *H. pylori* in stool of patients.

The overall detection rate of the fecal antigen test was 58% (58/100). The sensitivity, specificity, PPV, and NPV of the fecal antigen test were comparable to other studies, which were 79.3%, 71.4%, 79.3%, and 71.4%, respectively. The sensitivity and specificity of the fecal antigen test, which have been reported in previous studies, range from 67% to 100% and 83% to 99%, respectively. While the sensitivity and specificity were significantly higher when the monoclonal fecal antigen test has been used (43, 46, 47), it must be emphasized that monoclonal kits are not affordable in terms of price (48, 49). In addition, the sensitivity of the stool antigen test will decrease in frozen fecal sample. The analytical sensitivity of the stool antigen test kit used in this study was 500 bacteria per gram of feces, whereas in other studies the analytical sensitivity was not checked.

The overall detection rate of the LAMP method was 82% samples (82/100). The sensitivity, specificity, PPV, and NPV were 100%, 42.8%, 70.7%, and 100%, respectively. The LAMP test was positive in 24 stool specimens, which were negative in the fecal antigen test. This is because the *H. pylori* is present at a low number in fecal samples and only a test with sufficient limit of detection or high analytical sensitivity can detect it. The LAMP method has a detection limit as low as ten *H. pylori* cell per gram of feces, which is 50 times more sensitive than the fecal antigen test. In addition, the inhibitors, which are present in extracted DNA from stool, were tolerated by Bst DNA polymerase in the LAMP reaction (35).

However, the analytical sensitivity was not evaluated in many studies, but the analytical sensitivity of the LAMP for detection of *H. pylori* in stool samples achieved in this study was more than earlier studies using other molecular tests (50).

The sensitivity and specificity that have been reported in previous studies ranged from 42.6% to 93.7% and 92.3% to 100%, respectively (42, 48, 50).

For example, in a study using a real-time PCR technique the sensitivity and specificity of *H. pylori* detection in feces have been reported as 69% and 100%, respectively (33). These values for EIA were 88.9% and 94.6% (50). Low specificity of the mentioned methods is due to low limit of detection or high analytical sensitivity. Thus, it is suggested that the gold standard for *H. pylori* diagnosis should be revised.

The limit of detection was also determined using pure DNA. The minimum amount of the pure *H. pylori* DNA, which was detected by the LAMP method, was 10 fg. This amount of DNA is approximately equal to six *H. pylori* genome. This means the limit of detection of pure DNA was equal to the one achieved by 16SrRNA-PCR (29, 42) and ten times more sensitive than the earlier study (30).

CONCLUSIONS

LAMP technique has been characterized by high sensitivity and low detection limits for the detection of *H. pylori* in stool specimens, and the clinical diagnostic performance was better than the stool antigen test.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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