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Article *in* Journal of Pure and Applied Microbiology · January 2014

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JOURNAL OF PURE AND APPLIED MICROBIOLOGY, APRIL 2014. Vol. **8**(2), p. 1079-1086

A Comparison of Loop-Mediated Isothermal Amplification (LAMP) with PCR and Rapid Urease Test (RUT) to Detect *Helicobacter pylori* in Biopsy Samples

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(Received: 18 July 2013; accepted: 31 September 2013)

***Helicobacter pylori* is one of the most successful pathogens and the source of morbidity of about half of the worldwide population. Detection of these bacteria using traditional methods has some difficulties due to existence of some coccoid forms. The aim of this study is to compare different diagnostic methods such as LAMP, RUT and PCR in the patient biopsy samples. In this research, DNA of standard *H. pylori* extracted by DNG method. The LAMP and PCR were optimized and sensitivity and specificity of tests were studied. Then, 100 biopsy samples were obtained. RUT was done on all the biopsy samples. DNA extraction was done using DNP kit for all patient samples. Then the samples were studied using PCR and LAMP tests. The PCR test sensitivity was obtained 10 per reaction and LAMP test sensitivity was 5 per reaction. No undesirable products were observed in specificity test with DNA samples. 64% of the stomach biopsy samples were positive using RUT and 76% by PCR and 87% by the LAMP. The results indicate LAMP test has higher accuracy, sensitivity and specificity compared to PCR and RUT, therefore LAMP technique could be used as an alternative method in the *H. pylori* detection especially in developing country.**

Key words: *Helicobacter pylori*, RUT, PCR, LAMP.

H. pylori is a gram negative and microaerophilic bacillus which is often observed in stomach mucus layer as spiral form[1] and is observed in curve form[2] in culture. This bacterium is the cause of diseases such as gastritis, dyspepsia ulcers, stomach cancers and lymphoma[3].

Further, infection with *H. pylori* has been reported in non dyspepsia diseases such as brain vessels diseases and heart coroner vessel, hypertension, migraine headaches, chronic urticaria, pregnancy vomiting[4]. Rapid and accurate detection of this bacterium is very useful for treatment purposes. The diagnostic methods of these bacteria consist of the followings: A) invasive techniques such as histology, bacterial culturing, RUT and molecular method such as PCR. These methods are developed for the examination of biopsy from stomach endoscope and have very high sensitivity for infection evidence of *H. pylori* directly, which are used as the reference method (Gold standard). B) non invasive techniques such as Urease Breath Test (UBT) and stool antigen and serologic tests [4, 5]. The serologic tests are recommended for detecting *H. Pylori* among the non invasive techniques which are cost effective and rapid and recognized as primary screening tests. But, due to low sensitivity of serologic tests

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to evaluate the active infection they have been replaced with the other techniques. Further, the positive serologic results do not show the infection definitely. Therefore, we can not use these techniques after treatment [4, 5]. Also the UBT test is a time consuming process and requires expensive equipments. Furthermore, the false positive results are produced due to the existence of other Urease positive bacteria in the mouth and stomach cavities[6]. Among the invasive techniques frequent attempts have been done to culture *H. pylori*, but due to culturing difficulties, ineffectiveness and technical problems, the bacteria separation only was successful in few cases[7, 8]. To overcome these problems, different non-culturing techniques were examined to detect *H. pylori* in clinical samples[9, 10]. The pathology technique is very sensitive and specific, but in some cases the existence of some Metaplasia or atrophic injuries in human digestive system, utilizing some proton pump inhibitor chemicals or Bismuth and prolonged high costs limited application of this diagnostic technique[11].

The rapid Urease test (RUT) is based on the activity of *H. pylori* Urease enzyme compared to other methods such as culture and histology, this method is more rapid and less expensive, but RUT sensitivity depends on the organism density and concentration. Therefore, RUT has low value in duration treatment[12, 13]. In addition PCR technique has been proposed for detection biopsy samples. PCR has often significant advantages compared to culture, RUT and serology. Provided sample contains microbiota or low number this technique is wistful with high sensitivity. But on the other hand due to its complexity and requirement for specific equipment such as thermo-cycler is not widely applicable in small clinics[14, 15].

Loop-Mediated Isothermal Amplification (LAMP) is one the substitute methods. Target DNA is amplified in isothermal conditions. Since no time is needed to change temperature, LAMP technique is so rapid. This technique does not need an expert person after test optimization and it also does not require specific equipment such as thermo-cycler and electrophoresis; therefore it could be used even in deprived and far region's clinics [16-23]. Target genes to molecular detection in this case mainly triggered 16SrRNA and random chromosome sequences and antigen gene (SSA) gene the 26-kDa specific-species, the urease A (ureA) gene, the urease C (ureC) gene or its new name the phosphoglucosamine mutase (glmM) gene[24, 25]. The aim of this study was to compare LAMP technique to PCR and RUT tests for specificity and sensitivity detection of *H. pylori* in biopsy samples of the patients.

MATERIALS AND METHODS

Preparing the *H. pylori* strain and culturing method

H. pylori N:oC30 was obtained from liver and digestive disease research center of Shahid Beheshti University and was cultured in enriched Brucella blood Agar. The plates were incubated in microaerophilic with Anaerocult C (MERCK) for 5-7 days in 37º C incubator [26].

DNA extraction from standard strain

DNA was extracted using DNG (sinaclon) kit and PCR, LAMP tests were optimized on this strain.

PCR test optimization to detect *H. pylori*

The primers used for PCR test were used on the basis of glmM genes [25, 27] (Table. 1). PCR mixture was prepared as follows: DDW: 14 µl, 10X buffer: 2.5 µl, MgCl2 (50 mM): 0.75 µl, dNTP Mix(10 mM): 0.5 µl, (10 µM) Forward primer: 1µl, (10 µM) Reverse primer: 1 µl, Taq DNA Polymerase enzyme (5u/µL): 0.3 µl. Target DNA (from standard strain): 5 µl and total volume is 25 µl. Further, thermal profile was optimized as follows: The thermal cycles number were 35, including: Denaturation temperature: 30 sec at 93ºC, Annealing temp: 60 sec at 72ºC and Extension temp: 1 minute at 72°C and a final Extention 25 minutes at 72°C. PCR was done in optimized conditions and PCR products were electrophoresis in 2% Agaros gel containing cyber green in TBE 0.5X buffer.

PCR product cloning as positive control

The PCR product was purified by chloroform and ethanol precipitation methods. The purified product was ligated into the compatible sites of the T-Vector pTZ57R by cloning Thermo scientific (cat: K1214) kit.

Recombinant plasmids were confirmed using PCR and used as positive control in PCR tests.

LAMP test optimization to detect *H. Pylori*

LAMP primers were designed for glmM gene using primer explorer V4 software; (http://

primer explorer JP. /e/) (Table. 1)

LAMP reaction mixture was prepared as follows: DDW: 5.2 µl, Betaine 5Mol: 4µl, dNTP (10 mM): 3.5 µl, 10X buffer: 2.5 µl, MgSo4 (100 mM): 1.8 µl, Mix À: 1µl, Mix ÀÀ: 1µl, Bst DNA polymerase enzyme (New England BioLabs;Lot:33/110806): 1 µl, target DNA (extracted DNA from standard strain): 5 µl, and total volume is 25 µl. Mix À contaning FIP, BIP primers concentrations were 40, 10 µl DDW in 100 µl total volume respectively, and Mix ÀÀ containing LF, LB concentrations were 20 , 60 µl DDW in 100 µl total volume, respectively.

LAMP reaction evaluation

Thermal profile to glmM gene Amplification was in a simple heater block in 66ºC for 60 min. positive and negative control were used in each round of reaction. To evaluate reaction product 1 µl SYBR green (Invitrogen cat: 49753A) which was diluted 10 time more, was added to each tube and observed under transilluminator with 302 nm wave length. Microtube with positive reaction was observed as fluorescent green and negative reaction as orange color.

A suspension of fresh *H. pylori* culture was prepared which its concentration was 0.9×10^9 CFU/ml in OD=600 nm, and its DNA was extracted using DNG plus. Extracted DNA was diluted to 1 copy using dilution method. For specificity evaluation Human, Mouse, *Saccharomyces cerevisiae*, *Escherichia coli*, *Mycoplasma pneumonia*, Herpes Simplex Virus, *Mycobacterium tuberculosis* DNAs were extracted and were loaded in lanes accompanied by positive control.

Identification of LAMP test specification and sensitivity

After preparation of serial dilution of sample, SYBR green 0.1% was added to each tube and then observed under UV light. In order to LAMP test specificity, extracted DNAs of Human, Mouse, *Saccharomyces cerevisiae*, *Escherichia coli*, *Mycoplasma pneumonia*, *Herpes Simplex Virus*, *Mycobacterium tuberculosis* were used.

Sample preparation

In this study, 100 patients that referred to Baqiyatallah hospital and Booali Islamic Azad university hospital were studied. These patients had clinical symptoms of digestive dysfunction with ulcerou, stomach reflux symptoms and ulcer injuries.Twenty patients were already treated by antibiotics but recurred after 2 years. 100 biopsy samples of stomach tissues were obtained by Endoscopy surgery.

Rapid Urease test

To study rapid Urease activity Diagnostic Kits of Baharafshan Institute (www.bird-bahar.com) was used. The tube was filled with half of its volume by rapid Urease solution and a slice of stomach biopsy of each patient was placed in it and was shacked slightly then result was studied by color changing.

DNA extraction from tissue biopsy samples

Another slice of each patient tissue biopsy was carried to Iranian Gene Faravar (IGF) Institute in tubes containing physiologic serum to molecular examinations. Biopsy sample was sliced in the sterile tube and a homogenous suspension was obtained, then DNA was extracted from biopsy tissue using sinaclon kit (Cat:DN811530).

LAMP and PCR tests

PCR was done for entire 100 samples on the basis of glmM gene primers. Test results were studied on 2% Agarose gel and SYBR green and UV light using Transilluminatort. LAMP was done on all 100 samples as well. 1 µl SYBR green 0.1% was added to each tube and was observed under UV light.

Table 1. glmM gene based primers used in PCR and designed primers for LAMP

Primer	Sequence (5'…….3')
H.P-F	5' AAGCTT TTAGGGGTGTTAGGGGTT T 3'
H.P-R	5' AAGCTTACTTTCTAACACTAACGC 3'
F3	5´-ACACAATTTAAGACGTAGACTT-3´
B3	5´-GCTATGCGACAACATACGG-3´
FIP	5´- GAGGTAGGTAGGTAGGTAGGTAAGCGTTAAACAATTCAATGAGAG-3´
BIP	5´-ATCTGTGAGATGGAAGAATAAACCCAAAAAACACGAGGCACCG-3´
LF	5´-GTAGGTAGGTAGGTAGGTAGGTAAC-3´
LB	5´-CCGGGGGATCCATTTTTACG-3´

RESULTS

PCR and LAMP tests optimization

Amplicon of *H. pylori* (294 bp) observed in optimized PCR test on Agarose 2% (Fig. 1a). LAMP test was optimized in 66ºC for 1 hour (Fig. 1b).

PCR specificity and sensitivity tests

PCR sensitivity was done by preparing different serial dilutions of *H. pylori* DNA.The results showed that amplification is done with only 10 DNA copies. No amplification was seen in less than 10 copies of DNA which indicate is high sensitivity test (Fig. 2a). PCR Specificity test was done using DNAs of Human, Mouse, *Saccharomyces cerevisiae*, *Escherichia coli*, *Mycoplasma pneumonia*, Herpes Simplex Virus and *Mycobacterium tuberculosis*. PCR had very high specificity and only response with *H. pylori* DNA with specificity 100% (Fig. 2b).

LAMP test specificity and sensitivity

LAMP reaction was done in different DNA dilutions of *H. pylori* in 66ºC for 1 hour. The sensitivity results of LAMP test showed that amplification carried out with only 5 copies of DNA and green color was observed but in less than 5 copies it was not seen and tube remains in light orange which shows high test sensitivity (Fig. 3a). LAMP had very high specificity (Fig. 3b).

Results of RUT, PCR and LAMP

A study of 100 biopsy samples showed that 64% were positive using RUT test. DNA of 100 stomach tissue biopsy samples were extracted using DNP and were tested by PCR under optimized conditions, 76% showed positive results with PCR and 87% of samples showed positive

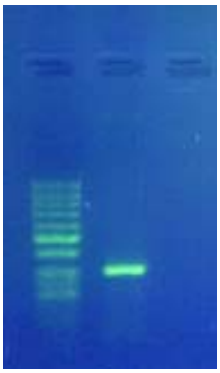

Fig. 1a. optimized PCR test for glmM gene of *H. pylori*. M: 50 bp DNA Ladder (Thermo scientific), 1; Amplicon (294 bp) of *H. pylori* (Positive control), 2; negative control

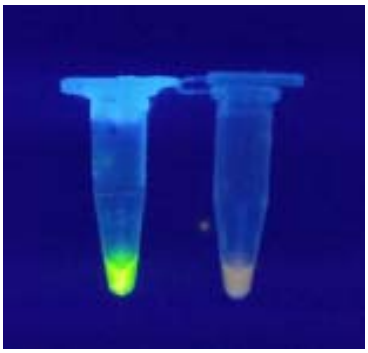

Fig. 1b. optimized LAMP test. 1; positive control, 2; negative control

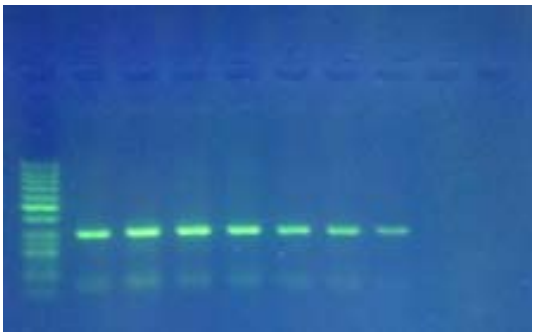

Fig. 2a. PCR sensitivity test using serial dilutions of *H. pylori* DNA, M: 50 bp DNA Ladder, 1; positive control, 2; 1000000 DNA per reaction, 3; 100000 DNA, 4; 10000 DNA, 5; 1000 DNA, 6; 100 DNA, 7; 10 DNA, 8; 1 DNA, 9; negative control

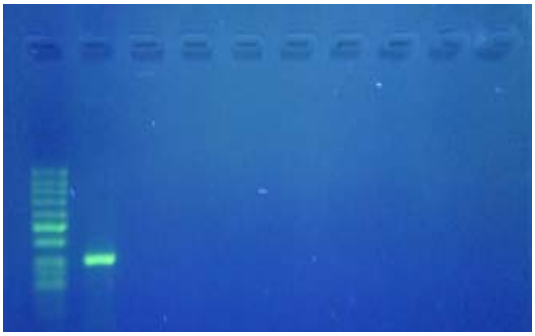

Fig. 2b. PCR specificity test. M: 50 bp DNA Ladder, 1; positive control, 2; Human DNA, 3; Mouse DNA, 4; *Saccharomyces cerevisiae* DNA, 5; *Escherichia coli* DNA, 6; *Mycoplasma pneumonia* DNA, 7; *Herpes Simplex Virus* DNA, 8; *Mycobacterium tuberculosis DNA, 9; negative control*

reaction with LAMP (Fig. 4).

Amplicon sequence was analyzed with forward and reverse primers and confirmed. The result of analysis showed that the size of amplified DNA was about 294 bp .

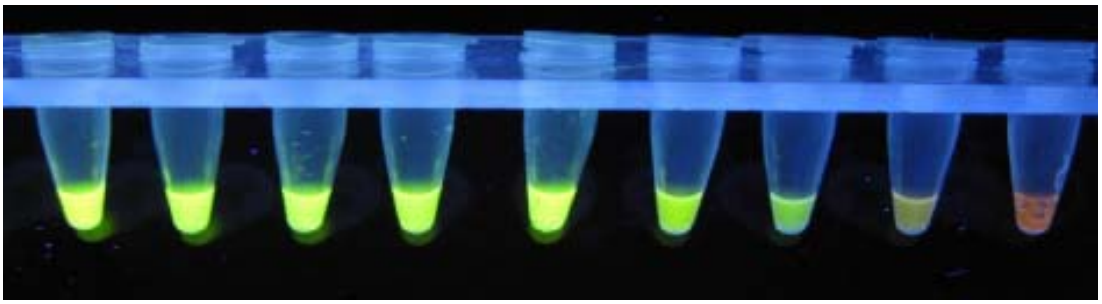

Fig. 3a. LAMP test sensitivity identification. C^+; positive control, 1; 1000000, 2; 100000, 3; 10000, 4; 1000, 5; 100, 6; 50, 7; 5, C^-; negative control

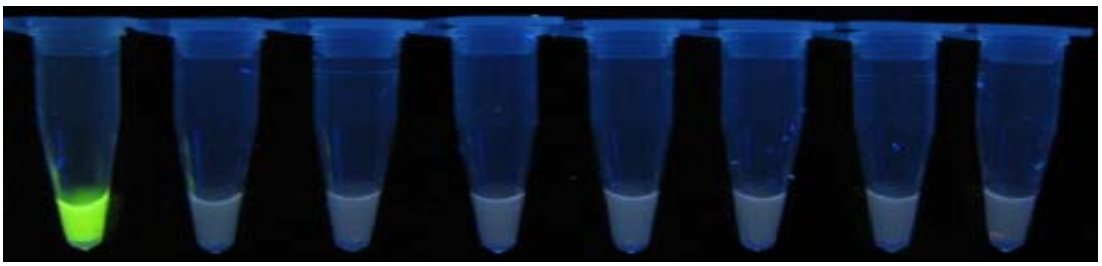

Fig. 3b. LAMP specificity test. C^+; positive control, 1; LAMP reaction with Human DNA, 2; Mouse DNA, 3; *Saccharomyces cerevisiae* DNA, 4; *Escherichia coli* DNA, 5; *Mycoplasma pneumonia* DNA, 6; *Herpes Simplex Virus DNA, 7; Mycobacterium tuberculosis* DNA, C^-: negative control

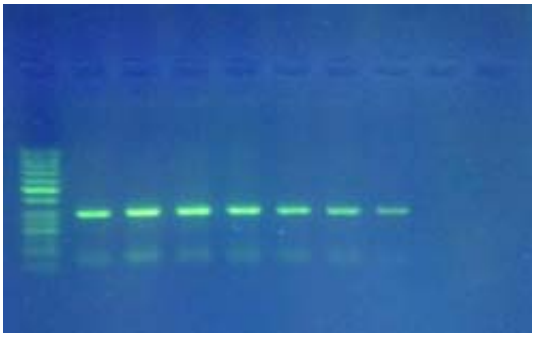

Fig. 4. PCR test of stomach tissue biopsy. M; Fermentas 50 bp marker size, 1; positive control, 2-7; positive samples, 8; negative samples, 9; negative control

DISCUSSION

Regarding *H. pylori's* high outbreak in the world and crucial consequences caused by it infection, rapid and accurate *H. pylori* diagnosis has significant importance. Jang-jih lu et al (1998) by PCR test on 5 different *H. pylori* DNA including 16SrRNA, random gene sequences, SSA gene, UreA gene, and UreC (glmM) gene found that glmM (UreC) gene with 96% specificity and 100% sensitivity is most suitable to study molecular detection of *H. pylori*[28]. Smith *et al.*, (2004), comparing 3 different PCR methods showed that UreC DNA is the most sensitive and specific for PCR technique to amplification of a 294 bp DNA[29]. The glmM (ureC) that was used in this research had very high specificity and sensitivity.

The RUT is one of the most common *H. pylori* detection tests among invasive techniques. It is easy to use in the office and/or endoscopy site but it has low sensitivity. The quality of biopsy sample may be cause of sensitivity and specificity regression of RUT test. For example biopsy pollution by blood, stomach acid or bile reflux may lead to sensitivity and specificity regression of RUT test or it may be caused by few numbers of bacteria exist in the sample or due to existence of other positive Urease microorganisms that yielded positive false results[15, 30]. Yakoob *et al.*, (2004), recognized 40% of patients with digestive

dysfunction symptoms in Pakistan by RUT[31]. In addition Tzang *et al.,* (2005) recognized 55.58% of 111 patients with digestive dysfunction in Taiwan using RUT technique[32].

PCR is one of the most rapid and a sensitive method to detect DNA and microorganism in very low amounts and simple technique and applicable in short time and decreases diagnostic errors. PCR technique improves sensitivity of diagnostic techniques. Fabre *et al.,* (1994) in a study on stomach biopsy samples claimed that PCR had more sensitivity and specificity compare to other techniques such as RUT[33]. PCR method has more sensitivity and specificity than RUT and culturing methods, but PCR also has some limitations such as frequent thermal cycles, high expensive, and lasting and laborious[30]. Therefore, there is a need to apply more rapid and simple method such as LAMP since many years ago. This technique is a one stage amplification reaction which could produce enormous number of copies (10^9) in less than 1 hour with a few number of DNA under isotherm conditions. The most important advantage of this technique is that it does not need denaturation of target DNA[16-20]. Therefore no require to change temperature. Amplification in LAMP technique is very high since reaction is isothermal[34]. Another advantage of LAMP technique is based on amplification of stem loop configuration, which leads to accumulation of high amount of products with different lengths and consequently makes the detection of amplified DNA very simple[35]. In an exclusive study on *H. pylori* detection in gastric biopsy samples using LAMP method and brushing technique conducted by Minami *et al.,* (2006), LAMP specificity was reported 100% and its sensitivity was 10^2 CFU[36].

PCR test sensitivity was 10 CFU and LAMP test was 5CFU in this study, where least amount of microorganisms were detectable using this technique. Since LAMP has 2 times more sensitivity compared to PCR, differences in results of 2 techniques is reasonable. Results studied using chi-Squared and mean tests. There is a significant difference between positive Urease (64%) and negative Urease (36%) results for detection *H. pylori* in gastric tissue biopsy (p<0/01). There is significant difference between positive PCR (76%) and negative PCR (24%) to detection *H. pylori* in gastric tissue biopsy samples (p<0/01). There is significant difference between positive LAMP (87%) and negative LAMP (13%) to detection *H. pylori* in gastric tissue biopsy samples (pÂ0/01). It appeared that PCR with 1.24 mean is better than RUT with 1.36 mean and LAMP with 1.13 is better than PCR with 1.24 and RUT with 1.36. In this study, 9 cases with negative result in PCR and 2 cases with negative result in LAMP had positive false results in RUT, which may be due to pollution of biopsy sample with blood, stomach acid or bile reflux or due to existence of other positive Urease microorganisms such as *Proteus* or stomach *Lactobacillus*. Further, in this study 21 cases with positive PCR results and 25 cases with positive LAMP results, had negative RUT which may due to existence of few numbers of active bacteria (At least 10000 bacteria) and stomach environment conditions as stimuli of gene Urease expression. Also the bacteria change in conditions such as pH changes, oxygen improvement and its forms changes under the effect of antibiotics such as Amoxicillin to coccoid form. Coccoid form of *H. pylori* caused to decrease Urease activity, in addition antibiotic treatment of patient leads to removal of active form of bacteria from stomach and decreasing the activity of Urease enzyme. It should be mentioned that proton pump inhibitors leads to Urease enzyme activity and sensitivity[37-40]. Furthermore, 11 cases which detected as positive by LAMP reported negative by PCR which 7 cases reported positive by RUT, indicating higher accuracy and sensitivity of LAMP than PCR.

As negative results of RUT do not definitely indicating infection with *H. pylori* and positive results do not mean the infection with bacterium that may be due to infection with other positive Urease bacteria. PCR test is based on using thermal profile, expensive thermo cycler, and time consuming and hard detection and manifestation of product, therefore a more rapid and simple technique such as LAMP which is one stage amplification technique could be used with more absolute positive results than PCR , RUT and more sensitivity and accuracy.

ACKNOWLEDGEMENTS

The authors would like to appreciate the science based Iraian gene fanavar (IGF) institute

and its respectful personnel (Sam Masahi, Atefeh Shahhosseiny, Elham Moslemi), also Microbiology laboratory of Baqiyatallah University and hospital and its digestion center and medical science research center of liver and digestive diseases of Shahid Beheshti university to their great help and generosity.

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