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Comparison of Two Rapid Urease Test (RUT) and Culture Diagnostic Methods with PCR Technique to Detect Helical and Coccoid Forms of *Helicobacter pylori* in Clinical and Environmental Samples

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***Helicobacter pylori* (*H. pylori*) infection is a worldwide disease that is the leading cause of digestive and extra digestive diseases. This bacterium exists in two forms, an actively dividing spiral form and a viable but non-culturable coccoid form. Coccoid forms widespread in aquatic environments, so, water as a reservoir of *H. pylori* which infects human. Given the limitations of routine diagnostic methods for detecting of *H. pylori*, Molecular approaches based on DNA amplification by PCR have been developed for accurate detection in both clinical and environmental samples. The objective of this study was to evaluate the performance of PCR compared to rapid urease test (RUT) and culture for the diagnosis of *H. pylori* in two sample groups including 100 gastric biopsy specimens from symptomatic dyspeptic patients and 60 water-induced coccoid samples. Biopsies were subjected to RUT and PCR; water-induced coccoids were subjected to Culture and PCR. By PCR, 85 gastric biopsy samples were confirmed as *H. pylori* positive whereas only 63 were positive using RUT. In water-induced coccoid samples, 22 were detected by PCR but culture method detected *H. pylori* in 4 of them. The results indicate PCR assay is more rapid, sensitive and specific compared to RUT and Culture for identifying spiral and coccoid forms of *H. pylori* in samples.**

Key words: *Helicobacter pylori*, coccoid, RUT, PCR, Culture.

Helicobacter pylori (*H.pylori*) is a prevalent gram-negative microaerophilic bacterium that colonizes the human gastric mucosa, and is known as the major cause of duodenal ulcers, gastric and gastritis, mucosa-associated lymphoid tissue (MALT) lymphoma, gastric adenocarcinoma[1] and a number of non dyspepsia diseases such as brain vessels diseases, heart coroner vessel, hypertension and migraine headaches[2]. *H.pylori* exists in two morphological infective forms: an actively dividing spiral form and a viable but non-culturable coccoid form[3-5]. Rapid and accurate detection is essential for clinical and hygienic management.

Tests for *H.pylori* have been divided into two groups: invasive tests, which require upper gastrointestinal endoscopy and analysis of gastric biopsy specimens, and noninvasive tests[6, 7].One of the rather widely used invasive tests is the Rapid Urease Test (RUT) which is based on the principle that abundant urease enzyme produced by *H.pylori* hydrolyses urea to ammonia[8]. This test is simple and cost effective, but its sensitivity

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depends on the organism density and concentration. Therefore, RUT has low value in duration treatment[6-8]

Culture is a standard method for detecting *H.pylori* among invasive tests, but this bacterium is a fastidious and difficult to grow microorganism and is able to convert from spiral form to coccoid form in water and environmental reservoirs which is due to result from variations in the environment, such as oxygen stress, temperature changes, the presence of antibiotics and other stress-inducing conditions[9-12]. Coccoid *H.pylori* is nonculturable but alive[4] that has been suspected to contribute an important part to the transmission of the bacteria through waterborne or food-borne route from environmental reservoirs, foodstuffs, and water contaminated with human fecal material[13-16]. Morphological and physiological changes through conversion in *H.pylori* cells make them very difficult to recover by culturing method. So non-culturing techniques were examined for the detection of *H.pylori* in water[17,18]

To overcome the limitations of mentioned diagnostic methods, assays based on PCR techniques have been developed to detect the presence of *H.pylori* DNA by using several gene targets directly from the samples. The targets of these PCR methods include the 16S rRNA gene, the random chromosome sequence, the 26-kDa species-specific antigen (SSA) gen, the urease A (*ureA*) gene, and the urease C (*ureC*) gene[15,19,20]. The *ureC* gene has been shown to encode the phosphoglucosamine mutase which was renamed the *glmM* gene[21].

The goal of this research was to compare the diagnostic value of PCR technique with the RUT test and culture for sensitive and specific detection of *H.pylori* from clinical and environmental samples.

MATERIAL 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 37°C incubator[22].

DNA extraction from standard strain

DNA was extracted using DNG (sinaclon) kit and PCR test was optimized on this strain.

PCR test optimization to detect *H. pylori*

PCR primers were designed for *glmM* gene using primer explorer V4 software (http:// primer explorer JP. /e/)(Table1). PCR mixture was prepared as follows: DDW: 14 ¼l, 10X buffer: 2.5 ¼l, MgCl2 (50 mM): 0.75 ¼l, dNTP Mix(10 mM): 0.5 ¼l, (10 ¼M) Forward primer: 1¼l, (10 ¼M) Reverse primer: 1 ¼l, Taq DNA Polymerase enzyme(5u/¼L): 0.3 ¼l. Target DNA (from standard strain): 5 ¼l and total volume is 25 ¼l. Further, thermal profile was optimized as follows: The thermal cycles number were 35, including: Denaturation temperature: 30 sec at 93°C, Annealing temp: 20 sec at 54°C and Extension temp: 20 sec at 72°C and a final extension step of 5 min 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

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 that used for sequencing and also positive control in PCR tests.

Specificity and sensitivity of the primers

To determine the sensitivity, a suspension of fresh *H. pylori* culture was prepared which its concentration was 0.9×109 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 the DNAs of Human, Mouse, *Saccharomyces cerevisiae*, *Escherichia coli*, *Mycoplasma pneumonia*, Herpes Simplex Virus, *Mycobacterium tuberculosis* were extracted and were loaded in lanes accompanied by positive control.

Sample preparation

In this study, two sample groups were prepared including 100 gastric biopsy specimens from symptomatic dyspeptic patients and 30 water-induced coccoid samples.

Tissue biopsy samples

100 patients that referred to Baqiyatallah hospital and Booali Islamic Azad university hospital were studied. These patients had clinical

symptoms of digestive dysfunction with ulcerous, stomach reflux symptoms and ulcer injuries. 19 patients were already treated by antibiotics but recurred after 2 years. 100 biopsy samples of stomach tissues were obtained by Endoscopy surgery for rapid Urease activity and PCR tests.

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 biopsies

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).

Coccoid form induction in water samples

To induce the coccoid forms of *H.pylori*, a freshly prepared suspension of *H.pylori* cells was inoculated into three series of 10-tubes with each tube containing 4000µl of drinking water. The initial concentration of the cells was 10^5 cells/ml. The tubes were incubated at three different temperatures of 4°C, 22°C and 37°C for the durations of 30 and 60 days. At these times, samples were removed aseptically from the tubes, cultured on blood agar plates. DNA was also extracted from them for PCR performance on samples.

DNA extraction from water-induced coccoids

DNA was extracted from 1400 µl of each water sample by boiling method. Briefly, the samples were spinned quickly and were centrifuged for 5 min at 10000 rpm. The supernatants were removed. The precipitates were separately dissolved in 100 µl of sterile water by vortex and were boiled in water for 15 min. Then the tubes were centrifuged for 5 min at 12000 rpm. The supernatants containing DNA were isolated from precipitates for PCR test.

PCR test

PCR was done for entire 100 biopsy and 30 water-induced coccoid samples on the basis of *glmM* gene primers. Test results were studied on 2% Agarose gel and SYBR green and UV light using Transilluminatort.

Table 1. Designed primers for PCR

Sequence (5'…….3')	Primer
5' ACGCCCT TTCTTCTCAA G 3'	Forward primer
5' CGCC TGTTTTAGCG TAAT 3'	Reverse primer

RESULTS

PCR test optimization

Amplicon of *H.pylori* (201 bp) observed in optimized PCR test on Agarose 2% (Fig. 1).

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 10 DNA copies which indicate the high sensitivity of the test (Fig. 2). 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. 3).

Results of RUT and PCR tests of biopsies

A study of 100 biopsy samples showed that 63% were positive using RUT test. DNA of 100 stomach tissue biopsy samples were extracted using DNP and were tested by PCR under optimized conditions, 85% showed positive results with PCR (Fig. 4).

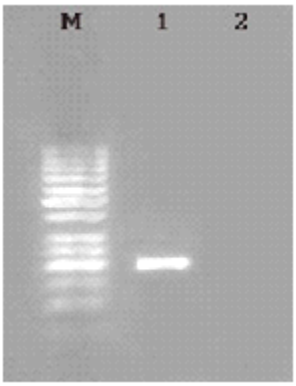

Fig. 1. Optimized PCR test for *glmM* gene of *H.pylori*. M: 50 bp DNA Ladder (Thermo scientific), 1: Amplicon (201 bp) of *H. pylori* (Positive control), 2: negative control

Amplicon sequence was analyzed with forward and reverse primers and confirmed. The result of analysis showed that the size of amplified DNA was about 201 bp.

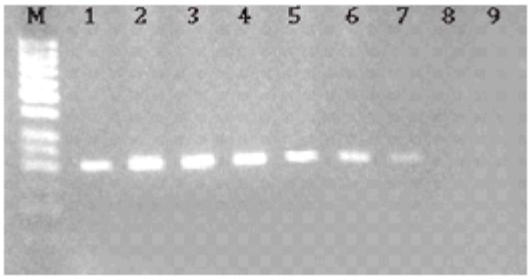

Fig. 2. PCR sensitivity test using serial dilutions of *H.pylori* DNA. M: 50 bp DNA Ladder, 1: positive control, 2: 1000000 DNA per reaction, 3: 100000 DNA, 4: 10000 DNA, 5: 1000 DNA, 6: 100 DNA, 7: 10 DNA, 8: 1 DNA, 9: negative control

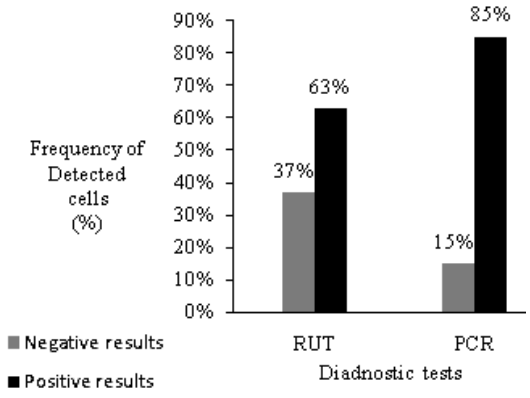

Fig. 4. Results of RUT and PCR for the detection of *H.pylori* in 100 biopsy specimens

Results of Culture and PCR of coccoid samples

A total of 30 water-induced coccoid samples were cultured on blood agar plates and simultaneously were tested with the *glmM* primers.

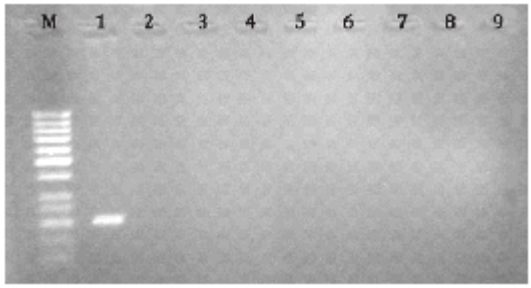

Fig. 3. PCR specificity test. M: 50 bp DNA Ladder, 1: positive control, 2: Human DNA, 3: Mouse DNA, 4: *Saccharomyces cerevisiae* DNA, 5: *Escherichia coli* DNA, 6: *Mycoplasma pneumonia* DNA, 7: *Herpes Simplex Virus* DNA, 8: *Mycobacterium tuberculosis DNA, 9: negative control*

Of these only 4 samples were culture positive. PCR results were positive for 22 samples tasted (Fig. 5)

In the first month of sampling, one sample only were positive culture at 22°C whereas the positive rates of PCR were 30%, 80%, 30% at 4°C, 22°C and 37°C, respectively.

In the second month, the only positive culture result were at 37°C with the rate of 30% and the positive rates of PCR were 20%, 20% , 40% at 4°C, 22°C ,37°C, respectively.

DISCUSSION

In the present study, we have investigated two group specimens including 100

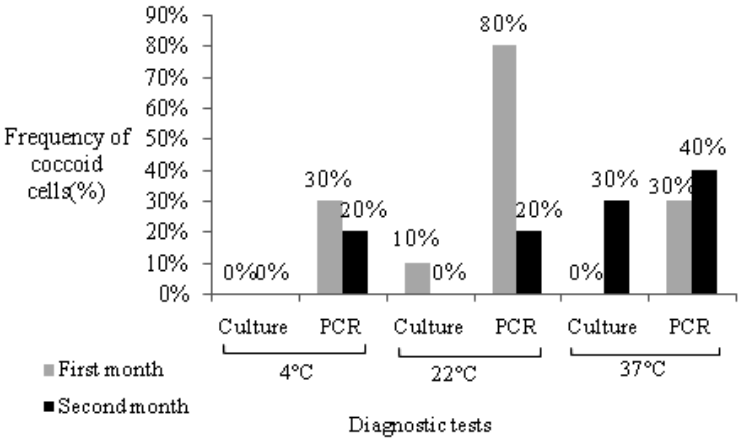

Fig. 5. Results of Culture and PCR methods for the detection of *H.pylori* in 30 water-induced coccoid samples at temperatures of 4°C, 22°C, 37°C for the durations of 1 and 2 months

gastric biopsies and 30 water-induced coccoid samples for *H.pylori* detection. We found that *glmM* (*ureC*) PCR was more sensitive than RUT and culture in both studied groups for detecting *H.pylori* in which PCR test sensitivity was 10 CFU, where least amount of microorganisms in a higher percentage of specimens were detectable using this technique. lu *et al*., (1999) claimed that PCR-based detection of the *glmM* gene, which used in our study, is the most appropriate for detection of *H.pylori*(19). Similar finding were reported by Bunn *et al*., (2002) and smith *et al*., (2004) (20) and shahamat *et al*., (2004) (23).

The RUT is one of the most common *H.pylori* detection tests among invasive techniques that is easy to use and can be performed readily in the endoscopy suite and give a rapid result (8). Donmez-Altuntas (2002) recognized 65.6% of 64 patients with digestive dysfunction symptoms by RUT (24). Additionally, Ottiwet *et al.*, (2010) diagnosed 48% of 130 dyspeptic patients in Thailand using RUT (25). On the other hand, RUT has low sensitivity because of false-negative results which may occur (8, 26). In the present study, Results was studied using chi-Squared and the Student *t* test. The statistically significant difference was suggested by a value of P<0.01. PCR test of biopsy specimens detected *H.pylori* infection in more specimens than did RUT. Significant differences between positive urease(63%) and negative urease(37%), positive PCR(85%) and negative PCR(15%) results show the PCR assay is more reliable than RUT to detect *H.pylori* (p<0/01). In biopsies, 2 cases with negative result in PCR 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, 5 cases with positive PCR 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 which 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 (27-30).

H.pylori fastidious microorganisms require growing on complex media. As regards, viable but nonculturable (VBNC) coccoid forms of *H.pylori* induced by water are capable of colonizing in gastric mucosa and causing gastrititis, culture-independent approaches should be adopted to detect them. Since the coccoids could have provided sufficient *H.pylori* DNA, PCR-based method have been used to detect the cells (23, 31-33). The entrance of *H.pylori* into the VBNC state was first suggested during laboratory studies by Shahamat *et al.*, in which cells were observed to become nonculturable in freshwater microcosms. They have detected *H. pylori* in both helical and coccoid form using PCR with the sensitivity of 0.1 pg of *H.pylori* DNA (23). Further finding were subsequently reported by oliver (2005), suggesting that *H.pylori* is able to enter the VBNC state as cells are exposed to a natural, freshwater environment and that this entry is dependent on the ambient temperature (34). In a study on *H.pylori* detection in drinking water using PCR method conducted by Janzon *et al*., (2009), the *glmM* primers detected all *H.pylori strains* and they showed that *H.pylori* cells are still detectable after 100 days of incubation in tap water microcosms (35). Nevertheless, Sulami *et al*., (2010) claimed the combination of PCR results with culturing of drinking water samples can provide a more accurate picture of *H.pylori* detection (36). Overall, our results are in good agreement with other data for survival of *H.pylori* in water for days, up to weeks at a variety of pH levels and in temperatures (37-40)

In this study, the frequency of *H.pylori*-positive samples detected by PCR was 73.3% (14/30 in the first month and 8/30 in the second month) while only 13.3% (1/30 in the first month and 3/30 in the second month) of these were culture positive. Our data show significant differences between negative and positive results of studied culture and PCR methods to detect the coccoid cells (p<0/01) which show PCR assay is more capable to diagnose the cell. Based on our results, 3 culture negative samples at 37°C in the first month were positive for *H.pylori* DNA using the PCR

method in the second month which could be as a consequence of morphological changes and conversion from the coccoid to Bacillary and culturable forms in optimum growth temperature of the cells. Further the number of cells which detected by PCR in month 2 were more than in the month 1 which indicate the highest rate of growth and viability of the cells at this temperature. The positive PCR rates of the samples at 22°C were 8(80%) and 2(20%) in the first and second month, respectively, that shows more durability of the cells in coccoid form at room temperature for a short period of time. additionally, only one (10%) was positive culture in the first month which suggests the reduction in cells growth and metabolism with decreasing the optimum growth temperature that the cells death phase follow, in which the viable cell population declines. The least positive culture (0%) and PCR (30% and 20% in the first and second month, respectively) results belong to 4°C. This could be a consequence of the stopping the growth of bacteria at chilling temperature that affects metabolic activity and cellular growth of viable but nonculturable cells of *H.pylori*.

CONCLUSION

In Conclusion, as negative results of RUT do not definitely indicate infection with *H.pylori*, positive results also don't mean the infection with bacterium which may due to infection with other positive Urease bacteria. Moreover, given the shortcoming of culture method for the diagnosis of *H.pylori* coccoid cells, we recommend the use of a more rapid and sensitive technique such as PCR detection of DNA from this fastidious microorganism in drinking water and sewage. Since PCR assay was capable of detecting more absolute positive results compared to the rapid urease test and culture method, it seems that PCR is the most efficient to assess *H.pylori* cells than RUT and Culture methods.

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