Designing of molecular tool for the detection of *Helicobacter pylori* in Iraqi patients using multiplex PCR technique

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Abstract

*Helicobacter pylori* colonize the gastric mucosa of more than 60% of the world’s human population. This bacterium plays a significant role in the pathogenesis of different diseases of the digestive system, such as chronic gastritis, peptic ulcer, and gastric adenocarcinoma. Accurate diagnosis of *H. pylori* infection is very important in the effective management of many gastroduodenal diseases. There is no gold typical technique that is been well-known for the detection of *H. pylori* infection. A multiplex polymerase chain reaction (mPCR) for glmM and 16S rRNA genes was established in our study for sensitive detection of *H. pylori* from gastric biopsies. Different classical detection techniques have been used lately with mPCR like Rapid Urease Test (RUT), histology and antibody (Serology) test. Detection of housekeeping (HK) genes by monoplex and multiplex PCR with different sets of primers for 16S rRNA due to heterogenicity and high variability in this gene. Our results show that a total of 123 (58.5 %) from 210 patients were positive for *H. pylori* infection. *H. pylori* were detected in 46.6% (98/210) by RUT, 54.7% (115/210) by histology, 85.7% (180/210 false-positive results were included) by *H. pylori* IgG, and 57.1% (120/210) through mPCR. By this molecular technique, *H. pylori* were detected in 100% of biopsies with positive histology and RUT. Our Conclusions prove that the mPCR was able to detect the highest numbers of positive cases although the lowest average scores for inflammation and activity.

Keywords: *Helicobacter pylori*, glmM gene, 16S rRNA Multiplex PCR, Molecular detection.

Introduction

In 1983 Robin Warren and Barry Marshall definitively identified *Helicobacter pylori* by culturing an organism from gastric biopsy specimens that had been visualized for almost a century by pathologists. Among many unique characteristics of *H. pylori*, one of the most remarkable is its capacity to persist for decades in the harsh gastric environment due to an inability of the host to eliminate the infection. Unlike other viruses and bacteria, *H. pylori* have evolved the ability to colonize the highly acidic environment found within the stomach by metabolizing urea to ammonia via urease. Approximately half of the world’s population is infected with *H. pylori*, and the majority of colonized individuals develop coexisting chronic inflammation. In most persons, *H. pylori* colonization does not cause any symptoms.

Accurate detection of the organism is essential for patient management. *H. pylori* eradication results in a marked reduction in the rate of recurrence of peptic ulcer and the prevention of gastric cancer. In addition, *H. pylori* treatment can potentially prevent gastric cancer by reducing the progress of precancerous lesions defined as atrophy, intestinal metaplasia or dysplasia to invasive cancer. Therefore, methods that accurately detect *H. pylori* infection in patients with dyspepsia symptoms are of major importance. The ideal diagnostic method for the detection of *H. pylori* does not exist at this moment, although there are various methodologies presenting advantages and limitations. Thus, clinical indication, costs, and the available resources should be

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considered when choosing type and number of specimens, and also the method to be used. Undoubtedly, patients with gastric disorders require a reliable diagnosis and rigorous treatment to prevent an increase in bacterial resistance (6).

H. pylori have been identified in gastric tissues by a rapid urease test, staining (histological examination), and IgG H. pylori (Serology test). Rapid urease test requires an adequate number of bacteria because urease production by the bacterium is reduced in patients who took proton pump inhibitor (PPI), antibiotics or bismuth compound (7,26,27). H. pylori are fastidious and slow-growing organisms, often difficult and time-consuming to isolate by a culture that requires 3–7 days of incubation (8). Furthermore, H. pylori viability will reduce prior treatment with antibiotics, PPI or during transportation (9, 28). The isolation of H. pylori in culture certainly indicates the presence of the bacteria, however, negative cultures do not prove its absence. Histology is easily affected by factors such as the site of the biopsy taken, number and the size of biopsy, stain used and requires expertise personnel (10).

Molecular tests offer excellent ways for diagnosis of H. pylori infection and do not require the bacteria to be alive when tested and even the organism is present in low numbers. Many polymerase chain reaction (PCR) methods targeting putative H. pylori specific genes have been reported (11, 12). In this study, two genes were targeted for amplification namely a conserved region flanked by genus-specific primer binding sites in Helicobacter 16s rRNA and species-specific sequences and glmM genes. H. pylori were definitively distinguished from Campylobacter and other bacterial genera on the basis of 16s rRNA studies (13). The glmM gene was widely used for identifying H. pylori by PCR (7,14). Therefore, the aim of this study was to develop a commercial molecular tool depend on the multiplex PCR technique for rapid, sensitive and specific detection of H. pylori directly from biopsy samples. The study demonstrated the feasibility of using mPCR to detect H. pylori infection with different cases. Results obtained were also analyzed according to patients’ characteristics.

Methods
In this study, which was directed during April - December 2018, a total of 210 patients biopsy samples were collected by gastroenterologists from each patient, and also blood samples for serology test were collected from patients who underwent upper gastroduodenal endoscopy in the gastroenterology and hepatology tertiary center, Baghdad - Iraq. In total, 210 patients (128 males and 82 females) aged 12 to 85 years, complaining from clinical manifestations of dyspepsia or burning, vomiting, bloating, weight loss, loss of appetite, dysphagia, and melena, were enrolled in the study (Table 1). Those who received Hp eradication therapy protocol, proton pump inhibitors (PPIs), nonsteroidal anti-inflammatory drugs (NSAIDs) within 4 weeks previous to the study were excluded. The remaining participant patient’s only 120 and gastric biopsy, as well as blood from each one, has been collected. Five gastric antral biopsies were taken from each patient, 3 biopsies for histology examination, one biopsy for RUT, and one biopsy for the molecular test.

Table 1. Characters of the patients in this study

<table>
<thead>
<tr>
<th>Patients characters (n=210)</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>128 (60.9)</td>
</tr>
<tr>
<td>Females</td>
<td>82 (39)</td>
</tr>
<tr>
<td><strong>Age (years )</strong></td>
<td>12-85 (Mean 47)</td>
</tr>
<tr>
<td><strong>Signs and symptoms</strong></td>
<td></td>
</tr>
<tr>
<td>Dyspepsia</td>
<td>110 (52.3)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>20 (9.5)</td>
</tr>
<tr>
<td>Bloating</td>
<td>15 (7.1)</td>
</tr>
<tr>
<td>Weight loss</td>
<td>13 (6.1)</td>
</tr>
<tr>
<td>Loss of appetite</td>
<td>9 (4.2)</td>
</tr>
<tr>
<td>Dysphagia</td>
<td>19 (9)</td>
</tr>
<tr>
<td>Melena</td>
<td>24 (11.4)</td>
</tr>
</tbody>
</table>
Rapid Urease Test
The test done by using AMA RUT EXPERT device (AMA Co. Ltd – Russia), through taking fresh antral biopsy and apply it over sample well on test slide then reseal test slide and insert it on device, wait time at 3 levels (1, 2 and 3) mins because this test consider as a semi-quantitative and depend on urease quantity released from samples. The results will show like (+ or ++ or +++ or -) depend on urease production.

Evaluation of \textit{H. pylori} Immunoglobulin G (IgG)
Serum isolated from 210 blood samples through high speed centrifuge 6000 rpm for 5 mins and then we applied 1 drop of serum over specimen well and after 10 minutes we read the results according to the directions of BIOZEK-Netherland.

Histopathological Examination
Gastric biopsies were fixed in 10% formalin and paraffin-embedded section was cut and stained with hematoxylin-eosin. The severity of gastritis was recorded based on the updated Sydney system (15). This work has been done by histopathological laboratory technicians under the supervision of histopathologists.

DNA Extraction and monoplex PCR Optimization
DNA was extracted from biopsies using QIAamp DNA Mini Kit cat.no (51304) (Qiagen – Germany) according to the manufacturer’s instruction. DNA concentration and purity were measured by Nanodrop (Eppendorf – Germany) and then dsDNA concentration was measured by Qubit 4.0 (Thermo-Fisher) this method was done according to Qubit® dsDNA HS Assay Kit. Optimization of PCR for both genes (16S rRNA and glmM) were done separately by using 4 different sets of primer (3 sets are picked from previous studies and 4th set designed in this study) for 16S rRNA gene and one set for glmM gene (Table 2). 1.5µl of each primer (10 pmol), 3µl DNA sample, and 12.5 µl OneTaq master mix (NEB-England) and complete to the final volume 25µl by using free nuclease water. The optimum amplification conditions for 16S rRNA gene were achieved consisting an initial denaturation of target DNA at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at (52,56,56,58)°C for 1 min and extension at 72 °C for 30 s. The final cycle included an extension for 10 min at 72 °C. The PCR conditions for the glmM gene resemble the 16S rRNA gene except for annealing temperature and time are differ as 59°C for 45 s. Eight microliters of monoplex PCR products were subjected to electrophoresis on 2% (wt/vol) agarose gel in 80 voltage for 90 min using horizontal electrophoresis apparatus and 1X TAE as a running buffer. The gels were stained with Red Safe DNA staining dye (INTRON – Korea) and PCR bands were visualized under ultraviolet light.

Multiplex PCR for the detection of 16S rRNA and \textit{glmM} genes
Detection of both genes was done at the same PCR conditions and by using commercial master mix in order to design commercial, a rapid and routine molecular tool for \textit{H. pylori} detection. Multiplex PCR conditions were involved initial denaturation of target DNA at 95 °C for 5 min, followed by 37 cycles of (denaturation at 95 °C for 30 s, annealing at 56°C for 1 min and extension at 72 °C for 45 s). Final extension for 10 min at 72 °C. Eight microliters of monoplex PCR products were subjected to electrophoresis on 2% (wt/vol) agarose gel in 85 voltage for 90 min using horizontal electrophoresis apparatus and 1X TAE as a running buffer.
Table 2. Primers used for monoplex PCR

<table>
<thead>
<tr>
<th>16S rRNA Primers</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set (1)</td>
<td>F ( AGGGGTAAAATCCGTAGAGAT) R ( CGTTTAGGCGTGGAATA)</td>
<td>133</td>
</tr>
<tr>
<td>Set (2)</td>
<td>F-( GCTATGACGGGTATCC) R-( GATTTTACCCCTACACCA)</td>
<td>402</td>
</tr>
<tr>
<td>Set (3)</td>
<td>F-( TGGCAATCAGCGTCAGGTAATG) R-( GCTAAGAGATCGCTATGTC)</td>
<td>522</td>
</tr>
<tr>
<td>Set (4)</td>
<td>F-(TTGGAGGGCTTAGCTCT) R-(AAGATTGGCTCCACCTCGCA)</td>
<td>457</td>
</tr>
</tbody>
</table>

**glmM primers**

<table>
<thead>
<tr>
<th>Primer sequence (5’ to 3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-(AGCTTTTAGGGGTGTTAGGGGTT)(19) R-(AAGCTTACTTTCTAACAACGC)</td>
<td>294</td>
</tr>
</tbody>
</table>

### Results

A total of 210 consecutive patients consisting of 128 male (60.9%) and 82 female (39.1%) aged range from 12 to 85 years, the mean age was 48.5 years with dyspeptic symptoms were involved in this study. The patients were classified into five groups according to endoscopic findings: normal (n = 2), non-ulcer dyspepsia (NUD) (n = 140) [gastitis; n = 110, duodenitis, n = 5, gastritis and duodenitis; n = 25], peptic ulcer disease (PUD) (n = 40) [duodenal ulcer; n = 15, gastric ulcer; n = 25], gastric mucosal abnormalities (GMA) (n = 20) [gastric adenocarcinoma; n=10, atrophic gastritis; n=6, intestinal metaplasia; n=4] and others (n = 8) [hiatus hernia; n = 2, fundal polyps; n = 6] (Figure 1).

![Figure 1. Patients groups according to endoscopic findings](image-url)

### H. pylori detection
H. pylori were detected in 85.7% (180/210), 46.6% (98/210), 54.7% (115/210) and 57.1% (120/210) of biopsy specimens by H. pylori IgG, RUT, histopathology, and mPCR, respectively. The patient was considered to be infected with H. pylori if either RUT or histological examination or mPCR methods gave positive results. As shown in (Figure 2), a total of 120 biopsies gave positive results for at least one of the methods examined except H. pylori IgG test due to false-positive results have been recorded in this study and previous studies for this test. One hundred and ten samples were positive for both (mPCR and histological staining). H. pylori were detected in 98 biopsies by RUT however, two of them were negative by mPCR. A total of 120 samples were positive by mPCR.

Figure 2. Distribution of H. pylori-positive biopsy specimens according to the testing methods.

Monoplex PCR results

Through using different sets of primers for 16S rRNA gene detection obtained from different studies as mentioned in (Table 2), we got unexpected results and not resemble previous studies. Some specimens gave negative results to H. pylori through RUT and histopathological tests while giving positive bands by PCR with primer set 1 (Figure 3) and another two sets of primer (2 and 3) giving nonspecific bands for negative 16S rRNA gene, as shown in (Figure 4 and 5) and these bands are unfavorable and lead to difficulty in distinguishing between positive results and negative one through this technique and also will be unsuitable to design multiplex PCR technique. Whereas using a new set of primers which is modified from a previous study (set 4) we got optimal results and suitable for multiplex PCR in contrast with the glmM gene (Figure 6).

Figure 3. This image show 1.8% agarose gel stained with RedSafe dye as a result of gene amplification. Lane 1-14 shows PCR product of 16SrRNA gene with (set 1) primers with an expected size of 133 bp. L: DNA ladder, C- : Negative control.
Figure 4. This image show 1.5% agarose gel stained with RedSafe dye as a result of gene amplification. Lane 1,2,5,6,7,8,9,10 and 12 shows PCR product of 16S rRNA gene with (set 2) primers with an expected size of 402 bp. Lane 3,4,11 and 13 shows nonspecific bands as a result of negative to 16S rRNA gene. L: DNA ladder, C- : Negative control.
Multiplex PCR for 16S rRNA and glmM genes detection

To determine the presence of *H. pylori* in human gastric biopsy specimens, we assessed the presence status of 16S rRNA and glmM by multiplex-PCR assay. The primers (set 4) have been modified from another study by using Geneious Prime software and making alignment between primers and J99 strain of *H. pylori* which was obtained from the NCBI database, then we have started to substitute 1 guanine with thiamin and 2 cytosines with adenine in primer sequence. The goal behind this modification is to minimize the difference in melting temperature with
game gene primer and detect both genes at the same time through multiplex PCR. We have obtained good results in comparison with other detection tests. The PCR product gave very sharp and clear bands on 1.5% agarose gel (Figure 7).

**Figure 7.** This image show 1.5% agarose gel stained with RedSafe dye as a result of 16S rRNA (set 4) and glmM multiplex PCR with an expected size of 522bp and 294bp, respectively. Lane 1, 2 and 9 positive for 16S rRNA gene only, while Lane 3, 4, 11 and 13 negative for both genes, lane 5, 6, 7, 8, 10 and 12 positive for both genes. L: DNA ladder, C- : Negative control

**Discussion and conclusion**

In our study, we got results showing a higher prevalence of *H. pylori* infection compared to previous investigations. Ali H. *et al.* Found in their study that *H. pylori* infection was detected using conventional methods along with monoplex PCR as a molecular detection method. However, in the present study multiplex PCR (mPCR) was included as one of the testing methods and it proves that mPCR increased the percentage of *H. pylori* detection. Here, we create a specific and unique mPCR assay to detect *H. pylori* in gastric biopsies to detect 16S rRNA and glmM genes at the same time and compared the positivity to other standard routine methods (*H. pylori* IgG, AMA RUT and histology). The ability of PCR to detect *H. pylori* in gastric biopsies has been previously demonstrated (12, 20).

In the present study, mPCR identified *H. pylori* infection in 100% of patients who had positive histology and *H. pylori* IgG. All biopsies except two were positive for AMA RUT gave positive mPCR results. This could be explained by the irregular distribution of *H. pylori* in the gastric mucosa (21). Incidence of non-*H. pylori* urease-producing organisms in the stomach have been recorded (25), and this might result in positive AMA RUT. mPCR shows very specific for *H. pylori* when tested using other bacterial genes (data not shown) the same DNA samples have been tested with another bacterial gene like vacA and cagA. The histopathological features of the biopsies show that there are many patients who had very low average inflammation scores and might not be suspected to have gastritis related to *H. pylori*. However, the mPCR method was able to detect the highest numbers of positive cases although the lowest average score was recorded in the activity, inflammatory, and *H. pylori* colonization, compared to other test methods.

In this study, mPCR was able to detect *H. pylori* in quite a high number of urease test-negative samples. This shows that the urease test -negative biopsies contained *H. pylori* DNA that can be detected by PCR. Urease activity decreased in the coccoid form of *H. pylori* result in a negative urease test (23). The enzyme presence of sufficient numbers of bacterial cells is required to guarantee a large amount of urease production for detection by a rapid urease test. The diagnostic yield of the rapid urease test is said to be increased by over 5% if more than a single biopsy were used (24). *H. pylori* IgG detection methods give very high rate of positive results as shown in (Figure 2), these results are considered to be false-positive results because this antibody which is related to *H.
pylori will remain in the blood circulation for 6 months even if the immune system in the help of medications have been clear of the bacteria.

The great importance of *H. pylori* infection is the association with peptic ulcer disease. Patients with peptic ulcer disease in *H. pylori*-positive patients were higher associated with *H. pylori*-negative patients. Generally, the prevalence rate among the males was higher than the female even though the difference was not statistically significant. Previously noticed that gender-based differences have been detected in delaying the onset of intestinal dysplasia and less development of intestinal inflammation in females infected with *H. pylori*-related to males (25-26).

In conclusion, this study showed that most of the patients had low average inflammation scores and might not be supposed to have *H. pylori* gastritis. However, *H. pylori* infection in these patients can be detected by specific mPCR technique. mPCR detected the highest numbers of positive cases compared to the other test methods and we recommend adding mPCR method in routine diagnosis of *H. pylori* infection. Correct diagnosis is important in view of the high prevalence of *H. pylori* in this region. Other tests might miss a low-level infection of *H. pylori*. Furthermore, the PCR method also can be applied in the detection of highly virulence genes to resolve clinical evaluation. Early diagnosis is very important in helping suitable treatment and management of the patients. *H. pylori* IgG test is not recommended for the detection of active *H. pylori*. This work represents an advance in biomedical science because mPCR increases the diagnosis of *H. pylori* infection in samples with non-culturable *H. pylori* organisms and mild inflammation where it is undetectable by other methods.

**Acknowledgment**

The authors would like to thank gastroenterologists for working in gastroenterology and hepatology tertiary center – Baghdad medical city for their help in the collection of biopsies and members of histopathology laboratory for providing us by the histopathological finding results, also we would like to thank biotechnology laboratory in AL-Razi center for their help and support.

**References**


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