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A Fast Multiplex PCR Method to Detect *Helicobacter pylori* Directly from Gastric Biopsies in Patients from Erbil Province

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ABSTRACT

H. pylori are the causative bacteria of gastric ulcerations and carcinomas among hundreds of millions of people worldwide and is converted viable but non-cultivable bacteria (VBNC) outside our body. The diagnosis of *H. pylori* still done by traditional methods like rapid urease test (RUT) although it is not very accurate, therefore applying a fast, cost effective and accurate method to detect the pathogen directly from patients is crucial for the treatment and prevention of chronic ulceration or worse consequences of the pathogen. The aim of the present study was to assess the accuracy of RUT and specificity of detecting *H. pylori* directly from biopsy samples by specific flagellar and *16SrRNA* genes of the bacterium as a new fast approach. For this purpose, seventy biopsy samples isolated from patients in Erbil city who suspected to be infected with *H. pylori*. Samples were tested using RUT and molecular methods (PCR) through two sets of specific primers targeting the *16SrRNA* and Flagellar gene. Obtained results were (52.9%) positive RUT vs. (74.3%) for both *16SrRNA* and flagellar gene test, RUT showed 15 false negative samples (21.4%) detected as positive by the flagellar gene test. No false positive RUT detected, while results of *16SrRNA* and flagellar gene test showed (100%) compatibility. It can be concluded that RUT is not very accurate comparing to molecular methods and using flagellar gene in combination with the *16SrRNA* gene to detect *H. pylori* showed (100%) accuracy that can be depended.

Keywords: *H. pylori*, Molecular Diagnosis, Multiplex PCR, *16SrRNA* gene, flagellar protein gene.

1. INTRODUCTION

H. pylori bacteria grow in the digestive tract with a very high prevalence infection that may be present in more than half of the world's population (1, 2). *H. pylori* cause many consequences when infected human, such as chronic inflammation, increases the risk of developing duodenal ulcer, gastric ulcer disease, and gastric cancer (3, 4).
There are many diagnostic methods for detection of *H. pylori* infection, but only those methods considered as useful and recommendable which have high specificity and sensitivity, According to a traditional classification, those methods are classified into invasive and noninvasive methods (5, 6). Invasive methods include tests such as PCR polymerase chain reaction (PCR), rapid urease test (RUT), culture, and histology which require endoscopic surgery, and biopsy specimens, while noninvasive methods include tests such as *H. pylori* antigen in stool specimen, urea breath test (UBT), and serology, each method has certain advantages and disadvantages (7).

The rapid urease test (RUT) which is among invasive methods, has some advantages that made it popular diagnostic test, it is a rapid, cheap, and simple test that detects the presence of urease from gastric mucosa. Its sensitivity ranges between 80 to 100% and specificity between 97% to 99% (8). Despite these advantages, RUT has some disadvantages that limit its accuracy like false negative or positive which could result from several factors (7, 9).
Molecular test, PCR also belongs to invasive methods that used to detect *H. pylori* infections with a very high accuracy. With some modifications, its sensitivity and specificity could be increased up to 100%. Unlike other tests, a positive result from a specific PCR approach is enough to decide a sample as *H. pylori* positive, there is no need to combine the positive result of it to the culture positive result like RUT. Also, PCR can detect samples with limited

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amount of bacteria when other tests fail and give negative due to low bacterial density (10-12). Molecular methods detect the presence of housekeeping (conserved) genes for detection, the popular conserved genes used in *H. pylori* detection include: *ureA*, *ureC*, *16SrRNA*, *23SrRNA* and *hsp60* (12).

Recently, direct detection of *H. pylori* from biopsy sample as an approach to overcome the limitations of bacterial growth on media as *H. pylori* can switch to a viable but not cultivable state and lose the ability to grow in culture media (13, 14). Current study aimed to assess the accuracy of rapid urease test versus a new developed molecular method based on PCR detection of *H. pylori* by targeting the flagellar gene and the *16SrRNA* genes of the bacterium in different age ranges and in both sexes.

2. MATERIALS AND METHODS

2.1. Specimen collection

Seventy biopsy samples were collected from patients, 31male (%51) and 39 female (49%) aging from 18 year and above. The specimens were collected at Hawler Gastroenterology and Hepatology Center at Rizgary. Samples collected between January 2020 and March 2020.
Biopsy Samples were taken through endoscopy apparatus of the upper gastrointestinal endoscopy. All patients were undergo overnight of fasting before the endoscopy and asked not take any anti-inflammatory, antiacidic and even antibiotic drugs before the endoscopy to ensure that the results not affected. Two biopsy samples each about 0.5 cm of collected from each patient, one for rapid urease test and the other one preserved in 1.5 ml microcentrifuge tube that contain Dulbecco's phosphate-buffered saline (DPBS) (1X) for molecular test. The samples were preserved at -20 °C in laboratory of Hawler Cardiac Center until time of processing DNA extraction (15).

2.2. Genomic DNA Extraction

Genomic DNA for molecular studies have been isolated from the stored 1.5 ml microcentrifuge tubes containing the biopsy samples from *H. Pylori* patients, using PureColumn DNA Extraction Kit (BIORON, Germany) according to manufactures instructions. Extracted genomics was stored at –20 °C before running PCR (11).

2.3. Assessing the isolated gDNA

The extracted DNA samples were tested for their concentrations and purities using nanodrop assay (NanoDrop, ThermoScientific. UK), following instrument calibration using 1ul elusion buffer, 1ul of each DNA sample. Extracted gDNA yielding from 1.7 – 2.0 as 260/280 ratio were considered pure and used in our study (16).

2.4. Primer Design

Two sets of primers were used in the present study targeting the *16SrRNA* and the flagellar protein gene, the first primer used to ensure the presence of H. pylori genome, while the other one is used to detect the presence of flagella protein gene. Primers were designed according to NCBI software program (https://www.ncbi.nlm.nih.gov/tools/primer). Sequence and base pairs of the primers are shown in (Table 1).

Table 1: Primers used for detection of *H. pylori,* their base pairs and annealing temp.

Target Gene name	Primer Name	Primer Sequence 5'-3' sequence	Ampico size (bps.)	Annealing temp. (ºC)	Accession number
16s rRNA	16SR-F	ACTCCCTACGGGGGAAAGAT	141	59 ºC	NR_044761.1
16s rRNA	16SR-F	GGACCGTGTCTCAGTTCCAG			
Flagellar protein	Flag-F	CGCAAAACAAGGACTACGCC	373	59 ºC	NZ_AP017632.1
Flagellar protein	Flag-R	TCTATCGCTTTTTGGATCGCTG			

2.5. Polymerase chain reaction

Amplification of Flagellar Protein and *16SrRNA* gene

Selected genes were amplified using DNA amplification with some optimization, cycling was carried out by (Gradient thermal cycler Alpha Cycler PCRmax series). The primers used for PCR were specific for the detection of flagellar protein and *16SrRNA* (Table 1). PCR condition for amplification was as the following: initial denaturation step for 5 min. at 95 ºC, followed by 35 cycles of denaturation for 40 sec. at 95 ºC, annealing for 45 sec. at 59 ºC and extension for 40 sec. at 72 ºC. A final extension step for 5 min. at 72 ºC was performed. Multiplex PCR carried out

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using total volume of 50 µl by adding 25 µl of the master mix, for each primer 1 µl of the forward primer and 1 µl of the reverse primer, 8 µl of the extracted genome, and complete the volume to 50 µl by adding 15 µl free nuclease water to the 0.5 ml microcentrifuge tube.

2.6. Agarose Gel Electrophoresis

PCR amplicon yielded from both *16SrRNA* and the Flagellar gene were run on a 1.0% of safe stained agarose gel. Five microliters of each PCR amplicon was mixed carefully with 1µl of loading dye, well mixed then poured carefully into its determined gel well. The loaded PCR products run at 85V for approximately 45 minutes. The separated bands were visualized by ultraviolet (UV) transilluminator (17).

2.7. Statistical analysis

Data analysis was performed using GraphPad Prism software version 8.0.2. Statistical significance was assessed via the One-Way ANOVA (Turkeys Multiple Comparisons). Logistic regression analysis was used for multivariate analysis. Variables that achieved a probability of (P-value<0.05) was considered statistically significant.

3. RESULTS

The rapid urease test was first performed for all the 70 biopsy samples resulting in 37 (52.9%) positive samples (Figure 3). No significant differences were observed between male and female, nor between ages of patients (P<0.073) with positive results of infection by *H. pylori* for both RUT and PCR tests.

Multiplex PCR for detection of *16SrRNA* and Flagellar genes of *H. pylori* conducted over all positive and negative RUT samples indicating 52 (74.3%) positive cases for both target genes respectively. Selection of proper oligonucleotide primers, cycling parameters, target genes, and PCR reaction brought about the simultaneous amplification of these genes in a single PCR reaction tubes with high specificity and sensitivity of identification. When comparing the PCR products with our 100 bps interval ladder, our PCR products yielded 141 bps and 373 bps amplicon sizes for both *16SrRNA* and Flagellar gene primer targets respectively, as shown in Figures (1) and (2).

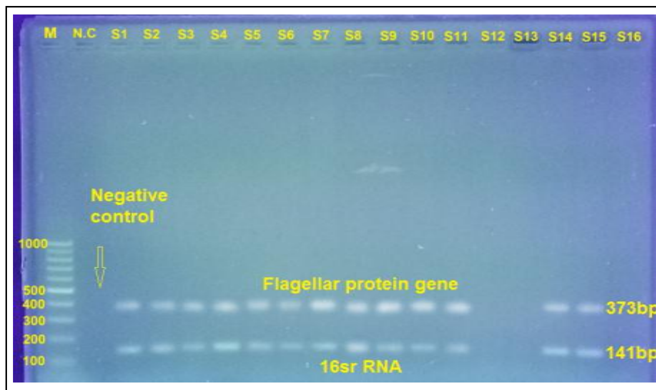

Figure (1): Agarose gel electrophoresis analysis 1% (stained with safe stain) of PCR products presenting gene amplified with specific primers that showed the expected size 141 bp For 16SrRNA and 373 bp for Flagella protein. Lane M: is 100 bp DNA Marker; Lane 2 negative control: Lanes 3-18: gene product amplified from *H. pylori* genome run on 85V for 45min including samples from 1 to 16. Samples number 1 to 11, 14 and 15 showed positive results for both Flag. Protein and 16s rRNA, other samples, 12, 13 and 16 were negative for both Flag and 16SrRNA.

Figure (2): Agarose gel electrophoresis analysis 1% (stained with safe stain) of PCR products presenting gene amplified with specific primers that showed the expected size 141 bp For 16SrRNA and 373 bp for Flagella protein. Lane M: is 100 bp DNA Marker; Lane 2 negative control: Lanes 3-11: gene product amplified from *H. pylori* genome run on 85V for 45min including samples from 62 to 70. Samples number 62, 63, 65, 66 and 69 showed positive results for both Flag. Protein and 16SrRNA, other samples, 64, 67, 68 and 70 were negative for both Flag and 16SrRNA.

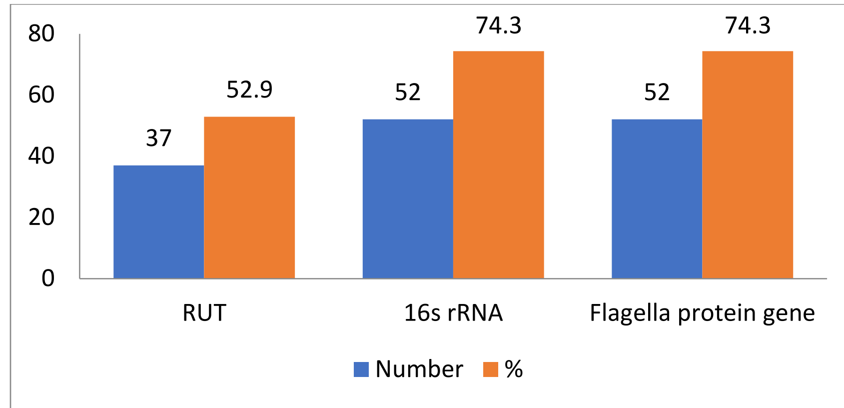

Figure (3): The positive results and frequencies of RUT, *16SrRNA* and Flagellar protein gene in the present study.

All positive and negative RUT samples were subject to the primer specific PCR detection of *H. pylori* using *16SrRNA* and Flagellar gene targets. All RUT positive specimens resulted in positive PCR amplification for both target genes indicating 100% specificity and sensitivity for both *16SrRNA* and Flagellar gene targets. On the other hand, the specific PCR amplifications resulted in 52 (74.3%) positive cases with a difference of 15 cases (28.8%) more than RUT positive results indicating a significant false negative results ($P < 0.0001$) for the later test versus the PCR based tests (Figure 3, Table 2). No significant differences were recorded between the specific *16SrRNA* and the Flagellar gene based PCR results, indicating the possibility of using both targets alone or together for detecting the pathogen regardless the RUT test (Table 2).

Table 2: The specificity, sensitivity, negative predictive value, and positive predictive value of RUT versus each of *16SrRNA*, and flagellar protein.

Tests	Sensitivity (%)	Specificity (%)	+PV (%)	−PV (%)	Accuracy (%)
RUT	71.15	100	100	54.55	78.57
16S rRNA	100	100	100	100	100
Flag protein	100	100	100	100	100

Results of statistical analysis showed non-significant differences regarding to gender and age ($P<0.073$), while showed high significant differences between RUT, 16SrRNA and Flagellar protein with ($P<0.0001$), and no significant differences between 16SrRNA and Flagellar protein ($P<0.1429$), as shown in (Table 3):

Table 3: Results of statistical analysis of RUT versus each of *16SrRNA*, and flagellar protein.

One Way ANOVA Tukey's test	Significant	Summary	Adjusted P- Value
RUT vs. 16S rRNA	Yes	****	<0.0001
RUT vs. Flag protein	Yes	****	=0.0001
16S rRNA vs. Flag protein	No	ns	=0.1429

4. DISCUSSION

The multiplex PCR yields of *16SrRNA* and flagella gene amplification showed 52 (74.3%) positive and 18 (25.7%) negative for *H. pylori* respectively. According to these results, flagella protein genes showed 100% accuracy in detecting *H. pylori,* all samples that were positive for *16SrRNA* showed also positive results using Flagellar protein gene which is normal and logic considering that flagella are a main structure for *H. pylori* and any normal and active *H. pylori* must have flagella (18). Such a result makes flagella gene valuable in detecting this bacterium rather than *16SrRNA* gene even with high sensitivity as this gene shared by many bacteria (19). Moreover, the results of the

RUT test indicated only 37 positive samples (52.9%) out of total 70 samples within first three hours. While, the rest 33 samples (47.1%) were negative, indicating a difference of 15 RUT false negative results which is considered a significant differences for the later test versus the PCR based tests (Figure 3, Table 2).

According to these statistical analyses, there were highly significant differences between results of RUT and PCR ($P<0.0001$). Results indicated that detection rate of *H. pylori* using PCR is higher than RUT, such a result approved by a research carried out by Okullu and his colleagues in 2017 (20). Another research carried out by Nevoa and his colleagues, they did a comparison between molecular technique and RUT for detection of *H. pylori*, according to their results, PCR detected higher number of positive samples than RUT (11). No significant differences were recorded between the specific *16SrRNA* and the Flagellar gene based PCR results ($P<0.1429$), indicating the possibility of using both targets alone or together for detecting the pathogen regardless the RUT test (Table 2).

It should be considered that RUT could give false negative or false positive results. Several factors affect the accuracy of RUT including size of the biopsy sample, location of the biopsy, bleeding patients, and taking medications (21). Some medications could reduce the density and/or urease activity of *H. pylori*, consequently, the test sensitivity could decrease to 25%. Also, Formalin contamination of biopsy forceps may also generate false negative, several florae such as *Proteus mirabilis*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Staphylococcus aureus*, isolated from the oral cavity and/or stomach, also present urease activity and can be potential false positive when using RUT (8, 9).

Another explanation for such differences and detecting higher positive results by PCR comparing to RUT could be resulted from using treatments as RUT become less accurate after treatment or after PPI use and could give false negative (22). While PCR is sensitive enough to detect the presence of *H. pylori* despite being on PPI treatment (23). Accuracy of PCR comparing to RUT in detection of *H. pylori* in the stomach tissue has been approved by other researches which confirmed that PCR showed higher sensitivity and specificity (11). Also, Fadilah *et al.* (2016) recommended adding multiplex PCR method in routine diagnosis of H. pylori infection. They showed that this method increases the detection of *H. pylori* in samples with non-cultivable *H. pylori* organisms and mild inflammation where it is undetectable by other methods. Furthermore, Nevoa *et al.* in 2017 evaluated the use of PCR in *H. pylori* detection and compared it with the rapid urease test, the authors found that the rate in the detection of H. pylori by the molecular method was significantly higher when compared to the rapid urease test (11).

5. CONCLUSIONS

PCR is a useful method in the laboratorial routine to detect the presence of *H. pylori* in the stomach tissue, due to high sensitivity and specificity, but it requires a more careful analysis and standardization. Detection of *H. pylori* using the Multiplex PCR method is more accurate and reliable than the conventional RUT method, where the last one is not accurate comparing to the primer-specific-PCR method. The Multiplex PCR assay indicates the possibility of using both primer sets alone or together for detecting the *H. pylori* regardless the RUT test.

Authors Contribution

Ahmed N. Hassan, performed sample collection, molecular test, and writing the research.
Dr. Ari Q. Nabi, designing the primers, statistical analysis, participated in practical work, and supervised the whole research.

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