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Evaluation of *glmM* Gene in Diagnosis of *Helicobacter pylori* with Another Invasive Methods

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Abstract: *Helicobacter pylori* (*H. pylori*) is a colonizer in the gastric mucosa of more than 50% of the human population. Complications due to this bacterium include chronic gastritis, gastric mucosa-associated lymphoid tissue lymphoma, peptic ulcer and gastric adenocarcinoma. This study aimed to compare molecular detection method of *H. pylori* by using *glmM* gene with another invasive detection tests as Rapid urease test (RUT) and histopathological examination. Invasive tests has been performed for detection *H. pylori* and five gastric biopsies has been taken from each individual, one biopsy from antrum for detection of *glmM* gene by polymerase chain reaction (PCR) as a molecular method, another antral biopsy for rapid urease test, whereas 3 biopsies from different parts of stomach used in histopathological examination. A total of 123 (58.5 %) from 210 patients were positive at least in two invasive tests for *H. pylori* infection. *H. pylori* were detected in 50% (105/210) by RUT, 54.7% (115/210) by histology and 85/210 (40.4%) for *glmM* detection by PCR as a molecular method.
In Conclusions the *glmM* gene it's not suitable for *H. pylori* detection in compare to another tests.

Keywords: *Helicobacter pylori* , *glmM* Gene, RUT**,** Molecular Detection.

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Introduction

The discovery of Helicobacter pylori (*H. pylori*) offered the etiologic agent of the initiating event of the inflammatory cascade. It colonizes half of the human population worldwide and is known as the causative agent of gastric ulcers and adenocarcinoma, chronic gastritis, gastric mucosa-associated lymphoid tissue lymphoma (MALT) (1, 2, 3).

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 prevention of gastric cancer (4). In addition, *H. pylori* treatment can potentially prevent gastric cancer by reducing the progress of precancerous lesion 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 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 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 a rigorous treatment to prevent an increase in bacterial resistance (5).

A variety of non-invasive and invasive tests are available for the diagnosis of the infection. Noninvasive testing includes serologic tests, urea breath test (UBT), and stool antigen test (6, 7). The invasive diagnosis using endoscopy includes rapid urease tests (RUT), histology, or culture. RUT is generally the first choice test, which depends on clinical conditions, accuracy of tests, costs, and availability (6)

The rapid urease test is an indirect test for the presence of *H. pylori* in or on the gastric mucosa. In the presence of *H. pylori*, urea is hydrolyzed by this enzyme to produce ammonia and bicarbonate, leading to a pH increase in the gastric mucosa, which is indicated by a change in the color of phenol red from yellow to pink or red. A positive RUT requires approximately 10^5 *H. pylori* in the biopsy sample to change color. The positivity of test depends on the concentration of bacteria and the temperature. Most will turn positive within 120 to 180 minutes, but it is best to hold those that appear negative for 24 hours. After 24 hours, the test may turn positive from the presence non-*H. pylori* urease-containing organisms. Positive results after 24 hours are most often false positive and should not be used for treatment decisions (8, 9).

There are many histochemical stains used for the histological detection of *H. pylori* in gastric biopsies and resections and the routine stain in this case is hematoxylin and eosin (H&E) stain. However, several studies have shown that immunohistochemical (IHC) staining with specific polyclonal *H. pylori* antibodies has the highest sensitivity and specificity and better interobserver agreement compared to histochemical stains (10).

Molecular methods are used with expansion in the diagnosis of infections caused by *H. pylori* along with virulence and drug resistance analysis, due to the high sensitivity and specificity (11). The housekeeping *glmM* gene encodes a phosphoglucosamine mutase, an enzyme catalyzing the interconversion of glucosamine-6-phosphate into glucosamine-1-phosphate, which subsequently transformed into N-acetylglucosamine. This monosaccharide is one of the main cytoplasmic precursors of bacterial cell wall murein and outer membrane lipopolysaccharides. Consequently, the *glmM* gene is essential for bacterial cell growth and assists directly with cell wall synthesis (12), also called ureC and used in many studies for *H. pylori* detection along with *16S rRNA* (13, 14).

The present study aimed to evaluate of polymerase chain reaction (PCR) *glmM* gene detection within *H. pylori*, and compare the result with another invasive tests as RUT and histopathological examination.

Methods

In this study, which was directed during (April 2018 - March 2019). A total of 210 patients biopsy samples were collected by gastroenterologists from each patient who underwent upper gastroduodenal endoscopy in the gastroenterology and hepatology tertiary center, Baghdad - Iraq. In total, 210 patients (126 males and 84 females) aged 12 to 85 year, complaining from clinical manifestations of dyspepsia or burning, vomiting, bloating, weight loss, loss of appetite, dysphagia and melena, were enrolled in the study (Table1).

Table (1): Description of the studied patients.

Patients characters (n=210)	Number (%)
Sex	
Males	128 (60.9)
Females	82 (39)
Age (years)	12-85 (Mean 47)
Signs and symptoms	
Dyspepsia	110 (52.3)
Vomiting	20 (9.5)
Bloating	15 (7.1)
Weight loss	13 (6.1)
Loss of appetite	9 (4.2)
Dysphagia	19 (9)
Melena	24 (11.4)

Five gastric biopsy were taken from each patient , three of them were kept in10% formal saline for histopathological examination, other one biopsy for RUT , and the last one for molecular test.

Rapid Urease Test

The test done by using AMA RUT EXPERT device (AMA Co. Ltd. – Russia) , through taking fresh antral biopsy and apply it on sample well of test slide then reseal test slide and insert it on device, and waiting for 3 different periods (1, 2 and 3 mins) because this test consider as a semi-quantitative and depend on urease quantity released from samples. The results will represented as (+ or ++ or +++ or -) depend on urease production according to the instructions of manufacturer company. This device measures the wavelength of urease through spectrophotometer principle.

Histopathological Examination

The biopsy tissues in 10% formal saline were processed for histopathology using an automated tissue processor (ATP). Formalin Fixed paraffin Embedded (FFPE) tissue blocks were sectioned using a microtome, cut into thin sections of 4 micrometre thickness, dewaxed and used for histopathological staining. Dewaxed and cleared slide sections were stained in Haematoxylene for 5 minutes and rinsed in 1% acid alcohol for a few seconds. The sections were blued in tap water and counterstained in Eosin solution for 5 minutes and washed, then sections were dehydrated in ethanol (70%, 95%, and 100%) for 3 minutes each and mounted in Dextrene plasticizer xylene (DPX) to increase refractive index (15).

Severity of gastritis was recorded based on the updated Sydney system (16). This work has been done by histopathological laboratory technicians under supervision of histopathologists.

DNA Extraction and Quantification

The DNA was extracted from biopsies using QIAamp DNA Mini Kit cat.no (51304) (Qiagen – Germany) according to 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 and that depend mainly on conjugated dye attached only to dsDNA.

Detection of *H. pylori glmM* gene

Virtual PCR were done for checking the exact primer binding site and also the expected product site, this procedure obtained after downloading the FASTA file for complete genome of *H. pylori* J99 strain from GenBank database and primer binding site have been checked by Geneious Prime software while *in Silico* PCR done by UGENE software.

Stock primers were dissolved in free nuclease water and then stepped down to 10 pmol as a working primer. PCR optimization were done for counting the optimal annealing temperature for *glmM* gene.

The PCR reaction (25 µl) composed from 12.5 µl OneTaq master mix (NEB©), 1.5 µl from each primer, 3 µl from DNA template with average concentration (69 ng/ µl) and volume completed to 25 µl by adding 6.5 µl from PCR grade water.

The optimum amplification conditions for *glmM* gene were achieved consisting an initial denaturation of target DNA at 95 °C for 3 min (stage 1), followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at (57)°C for 1 min and extension at 72 °C for 30 s (stage 2). The final stage included only one cycle for extension 8 min at 72 °C.

Eight microliter from PCR products were subjected to electrophoresis on 2% (wt/vol) agarose gel with 80 voltage for 90 min using horizontal electrophoresis apparatus and 1X TAE as a running buffer. The gel were stained with RedSafe DNA staining dye (INTRON© – Korea) and PCR bands were visualized gel documentation instrument under ultraviolet light.

Results

The endoscopic findings have been obtained from gastroenterologists' report in OGD endoscopy related to 210 patients suffered from dyspepsia, unexpected weight loss, vomiting, and abdominal discomfort. The data reveals that 126 (60%) were males and 84 (40%) were females. The gastritis (pan gastritis and mild gastritis) observed in high number of patients reach to 95 (45.2%) as that shown in (Figure 1). There are 25 (11.9%) cases were suspected to be gastric cancer (GC).

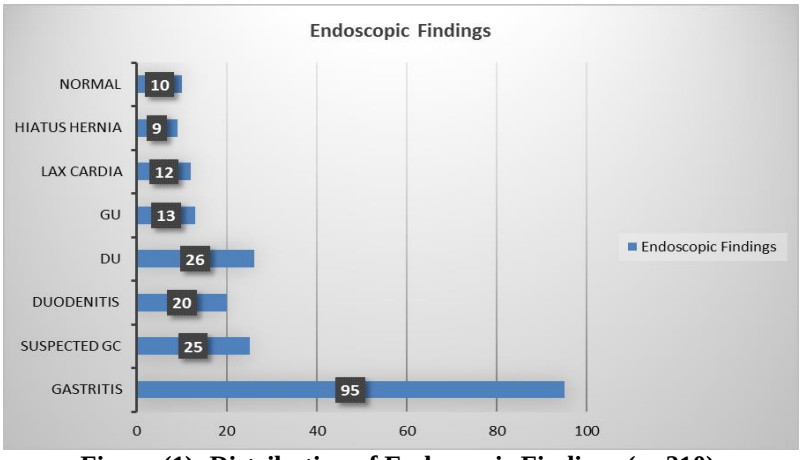

Figure (1): Distribution of Endoscopic Findings (n=210).
DU: duodenal ulcer, GU: gastric ulcer, GC: gastric cancer

Detection of *H. pylori* by Rapid Urease Test (RUT)

There are 105(50%) samples were positive to RUT out of 210 samples, it was found that highest number of RUT positive samples obtained from patient with active DU and gastritis, 20/21 (95.2 %), 46/52 (88.4 %) respectively, but the lowest number with intestinal metaplasia 13/18 (72.2%) and gastric cancer 9/17 (52.9%) (Figure 2).

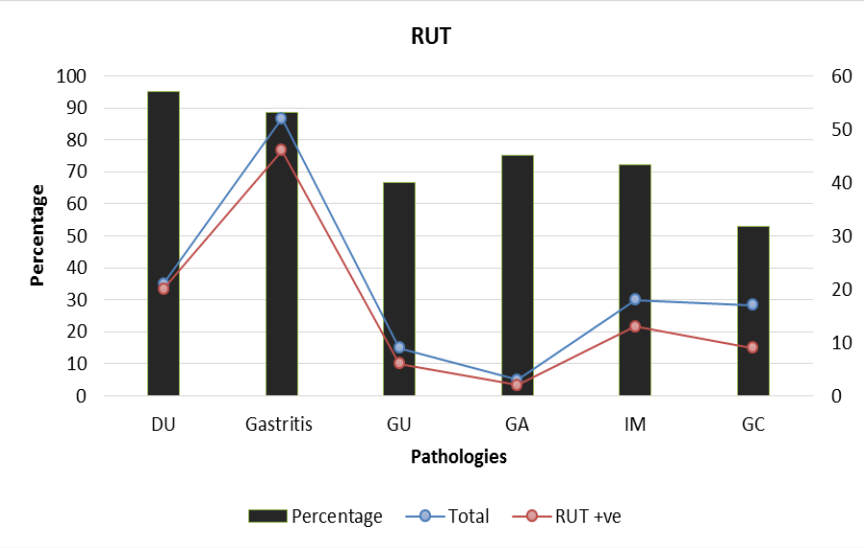

Figure (2): Detection of *H. pylori* infection using RUT in patients with various disease. DU: duodenal ulcer, GU: gastric ulcer, AG: atrophy gastritis, IM: intestinal metaplasia, GC: gastric cancer

Histopathological identification of *Helicobacter pylori*

Multiple biopsies were obtained from each individual (210) underwent to OGD endoscopy department whom suffered from different complains to determine the inflammation grade of tissue and detection of *H. pylori* by hematoxylin-eosin (H&E) staining method. Samples are diagnosed with different grades and kind of inflammation according to histopathologist's report. Histology plays a pivotal role in detecting *H. pylori* and it also provided more information about the degree of inflammation and associated pathology, such as, atrophic gastritis (AG), intestinal metaplasia (IM), and gastric cancer.

There are 115/210 (54.7%) samples were positive to *H. pylori* and distributed by different percentage according to histopathological findings. Highest number of positive *H. pylori* samples observed with active chronic gastritis followed by adenocarcinoma and superficial gastritis as 57 (62.6%), 11 (50%) and 31 (44.2%) respectively, while the lowest observation noticed with atrophy gastritis were only one *H. pylori*-positive out of 5 cases (Figure3).

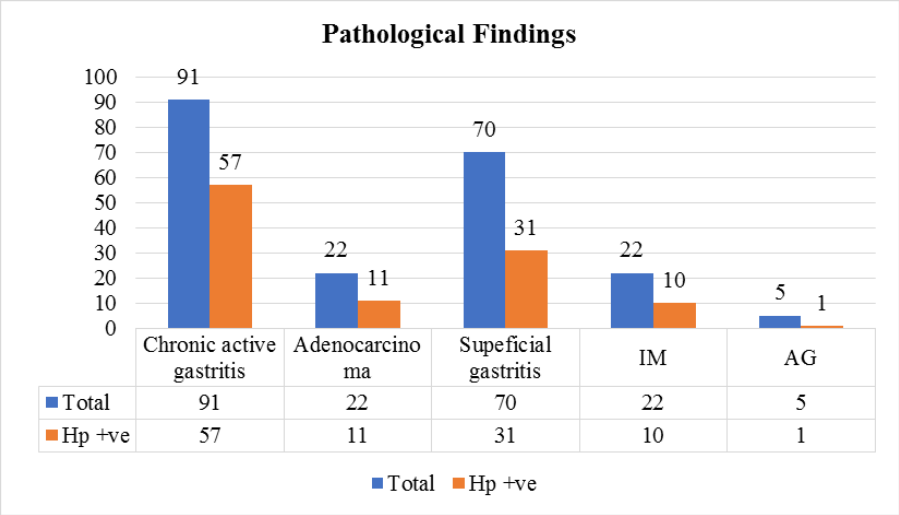

Figure (3): *Helicobacter pylori* and histopathological findings in patients with dyspepsia.

Identification of *H. pylori* Through Detection of *glmM* Gene

The molecular analysis was conducted to compare this result with another two previous methods (RUT and Histopathological examination) and also checking the possibility of using this test with *glmM* gene as a confirmatory test for *H. pylori* infection. PCR technique used in this part of study as a more powerful and accurate method for diagnosis, that allow to detect *H. pylori* in small samples even with low quantity.

There are 85/210 (40.4%) samples are positive to *glmM* gene and this result considered to be lowest sensitivity and specificity in comparison with another two methods that have been used Figure (4).

The same PCR reaction have been applied on DNA template extracted from *H. pylori* NCTC 11916 as a positive control and also gave negative result, (Figure 5), this step has been performed to confirm the result and especially the *glmM*-negative result which obtained from positive isolates and also to validate *glmM* gene.

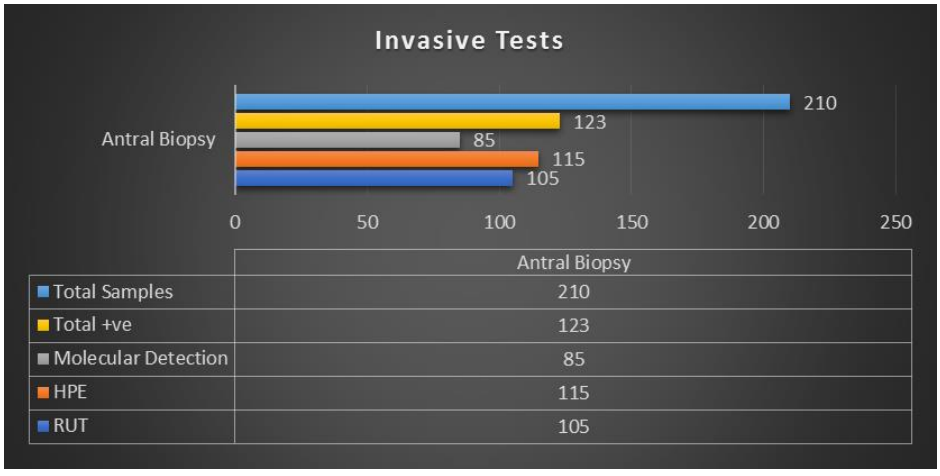

Figure (4): Invasive tests for detection of *H. pylori*
RUT: Rapid Urease Test , HPE; Histopathological Examination

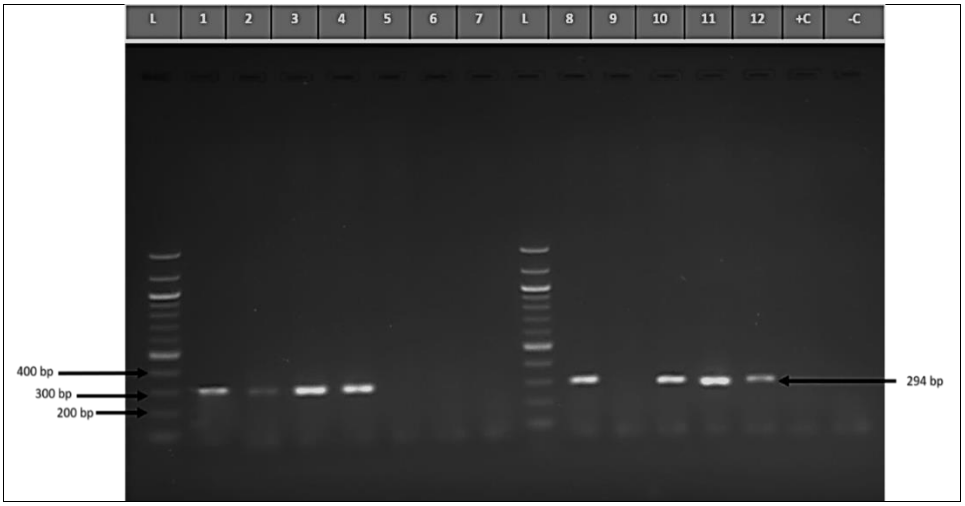

Figure (5): Agarose gel (1.8%) stained with RedSafe dye with 75V electrophoresis for detection of *glmM* gene as a PCR product. Lane 1,2,3,4,8,10,11 and 12 shows PCR product of with an expected size of 294 bp . Lane 5,6,7,9 and +C shows no PCR product
L: DNA ladder , +C : Positive control , -C : Negative control

Discussion

In this study the results showing higher prevalence of *H. pylori* infection compared to previous investigations. Ali *et al*.(2017) (17) found in their study high *H. pylori* infection was detected using three invasive methods (RUT, histopathological examination)

Detection of *H. pylori* by histopathological examination showing the highest sensitivity in comparison to another invasive methods. In the present study, this test identified *H. pylori* infection in 115 (93.4%) samples out of 123 as a total *H. pylori*-positive samples.

The possible explanations for inability of detection all positive samples by histopathological examination is that because intestinal metaplasia is often focal process, and *H. Pylori* may stay alive on the gastric epithelium in the surroundings of intestinal metaplasia (18).

There are many previous studies done in this field with various pictures of disease as Ayoola *et al*.(19) in Saudi Arabia who reported that 60% of people infected with *H. pylori* suffered from chronic gastritis, whereas Zhang and his team observed *H. pylori* in 52.4% of patients with gastric cancer in Japan (2). In this study the *H. pylori* detected in 10/22 (45.4%) diagnosed with intestinal metaplasia and this result similar to previous study which conducted by Grgov *et al*.(2002) (20).

Rapid urease test also showing good sensitivity just after histopathological examination results, and the sensitivity of this test reached to 85.3%, there are 18 samples gave false negative results in this test. This test mainly depends on the copy number of bacteria present in samples and more than one biopsy sample may give a highly rapid test result. The size of biopsy itself may implicate the number of bacteria within these biopsies due to the patchy distribution of the bacteria within the sample (21).

However, if the patient has atrophic gastritis and large areas of intestinal metaplasia, which contain few *H. pylori*

organisms, the results will often be disappointing (22).

The lowest sensitivity (69.1 %) occupied for molecular detection of *glmM* gene and this result could be due to sequence polymorphisms in the *glmM* loci and the variation between strains. This outcome have highly agreement with different other studies were they also noticed there is a high polymorphisms within this gene, Kalaf *et al.*(2013) (23) in Iraq, detected different genes were *glmM* gene is included also for *H. pylori* samples by using molecular PCR kit from (Maxim Biotech.Inc. USA) in their study , another Iraqi study conducted by Haider *et al.*(2018) who identified *glmM* gene in 19 samples out of 49 as a total positive (38.8%). Sadeghi *et al.*(2018)(1) in Iran have been obtained the results of *glmM* positive samples with very low sensitivity (26%). In contrast there is another previous study from Iran also but PCR detection method through *glmM* gene have been scored the quit higher sensitivity (24). Also, the researcher from India have been detected *glmM* gene in most of positive samples with good sensitivity (95.9%) (25). After cross matching the results of all three diagnostic methods it was found there are 123/210 (58.5 %) samples are positive to at least two of involving tests. This gene (*glmM*) have been used in this study for validation and checking if this gene suitable for detection of *H. pylori* from Iraqi isolates, because many researchers around the world are using it in molecular method, but here in Iraq seems this gene is not favorable for this mission in compare to another invasive methods like histopathological examination which is routinely used in hospitals, also the same samples have been tested with *16S rRNA* genes and the sensitivity its much better and the research paper for this gene under publish.

The accurate estimation and detection of *H. pylori* is very important because that lead to correct treatment and eradication of this pathogen.

In conclusion and according to recent results, histopathological examination consider as a golden standard method for *H. pylori* detection and *glmM* gene should be performed with another housekeeping gene for example *16S rRNA* or *ureA* genes in a multiplex reaction. (have been used on the same isolates) (under publish)

Other tests might be unable to detect infection if there is a low-level of *H. pylori*. Furthermore, 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.

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