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Isolation and Molecular Detection of *Helicobacter Pylori* from Biopsy Samples of Gastritis Patients in Iraq

Abeer M. Jabbar
Post Graduate
Department of Biology, College of Science
AL Muthanna University, Samawa, Iraq
email@address.com

Dr Laith A.H. AL Obaidi, PhD
Assistant Professor
Department of Biology, College of Science
AL Muthanna University, Samawa, Iraq
**laith.alobaidi@mu.edu.iq*

Abstract: *Helicobacter pylori have been considered as a primary risk factor for gastric cancer. Attempts were conducted for the diagnosis of H. pylori infection worldwide. The present study was conducted to seek for isolation and diagnoses of H. pylori infection. To achieve the intended aim, biopsy samples were collected from 45 males and females with gastritis in AL Samawa and AL Diwaniya provinces. The period of collection were extended from October 2013 to March 2014. Biopsy samples obtained by esophago- gastro- duodeno- scopy (EGD) were confirmed by rapid urease test (RUT) and qPCR respectively. The results showed that 31 (68.89%) samples were positive for RUT. These samples were highly detected using molecular analysis. Twenty two (71%) of samples were positive for the H. pylori diagnostic gene (ureC). Further confirmation was done using H. pylori virulence genes (ureA, vacA, and cagA). It has been shown that among all gastropathological cases studied; vacA, and cagA, are highly detected (64.28%, and 60.7%, respectively) and 39.28% for ureA with a significant value P 0.01. However, ureA was only detected in oseophagas tumor biopsy, which may have a critical role in stomach cancer.*

Keywords: *Helicobacter pylori, stomach cancer, molecular detection, Quantitative PCR, Biopsy.*

1. INTRODUCTION

Helicobacter pylori was firstly isolated and described from gastritis patient [1]. It was the most common human bacterial pathogen in the world [2]. Over third of million people's deaths each year worldwide may be due to this potentially fatal pathogen [3]. This type of bacteria causes a chronic gastric B-cell lymphoma [3], ulcer diseases [4], and type B gastritis [5]. Moreover, the infection with *H. pylori* was a major risk for developing gastric adenocarcinoma and mucosa associated lymphoid tissue (MALT) lymphoma [6].

Epidemiological studies have been indicated that *H. pylori* have an attributable-risk of 50-60% of gastric cancer [7]. In many developing countries, the majority of people appears to acquire an infection during childhood, especially at preschool age and persists into adult life, whilst primary infection in adulthood is rare [8].

The correlation between *H. pylori* and various human gastrointestinal diseases have been studied extensively [9, 10, 11]. Furthermore, the pathogenesis of this bacterium was studied in mice and rabbit [12]. In this study we aimed for molecular confirmation of *H. pylori* using *vac*A, *cag*A, and *ure*A virulence genes.

2. MATERIALS AND METHODS

2.1. Samples

A total of 45 biopsy samples were collected from the greater curve of the gastric antrum and duodenum [13]. These samples were collected for the period of October 2013 to March 2014 from Al-Hussien Teaching Hospital in the Al-Muthanna and Al-Diwaniya provinces. The samples were prepared for bacteriological and molecular conformation at the Biology department/ College of Science/ AL Muthanna University.

2.2. Gastric Biopsy Specimens

Gastric biopsy specimens (two from each patient) were collected from the stomach antrum or corpus. One of the specimens was used for *H. pylori* isolation, and the other for extraction of total genomic DNA for qPCR purposes.

2.3. Isolation and Identification of H. pylori

Several different solid media were used for culture of *H. pylori* from biopsies. Among the more common ones are Brain Heart Infusion (BHI) agar, Brucella agar, and human blood agar plates. The media are always supplemented with 3 antibiotics, which are Nystatin, Vancomycin, and Nalidixic acid. The culture incubated at 37°C for 3 to 10 days under a 9% CO_2 atmosphere [14].

The bacterial colonies were identified by visual inspection for grey, translucent, and pinpoint colonies using light microscopic. Subsequent biochemical tests including (oxidase, catalase, and urease) were performed from the primary growth; single colonies were propagated in blood agar for an additional 48 h [15, 16].

2.4. Biochemical Tests

The bacterial culture was biochemically identified using catalase, oxidase, and urease analysis [15, 17].

2.5. Molecular Detection of H. pylori

2.5.1. Bacterial Genomic DNA Extraction

Bacterial genomic DNA was extracted from stomach biopsy samples (100mg/ml) using the manufacturing in genomic DNA extraction kit (Geneaid. USA).

2.5.2. Quantitative Real Time PCR

For identification and detection of *H. pylori*, *ureC, ureA, vacA, and cagA* genes were used. The gene primers were designed using two sources; NCBI Gene-Bank data base and [18] (Table 1).

Table1. *The primers used in this study.*

Primer	Sequence		Source
ureC	F	AGCGTTGGCAGTGCTAAAAG	NCBI Gene-Bank data base
	R	TTATAAGCCGCGCCATTAGC	
ureA	F	*TATGGAAGAAGCGAGAGCTGGTA*	[18]
	R	*GAGTGCGCCCTTCTTGCAT*	
vacA	F	*CGCTATCAATCAGCATGATTTTG*	[18]
	R	*CCCGCATCATGGCTATCAAT*	
cagA	F	*TGATGGCGTGATGTTTGTTGA*	[18]
	R	*TCTTGGAGGCGTTGGTGTATT*	

The qPCR master mix was prepared by using (AccuPrep® 2X Greenstar qPCR Master Mix kit, Bioneer. Korea), and done according to company instructions. The master mix including 5 µL DNA sample (80 ng/µL), 2X Greenstar qPCR master (25µL), each of forward and reverse gene primer (2µL) (10pmol) and DEPC water (16µL) for the total of 50µL. The qPCR strips tubes were placed in centrifuge for 3 minutes at 3000 rpm, and then transferred into Miniopticon Real-Time PCR thermocycler. The PCR thermocycler conditions were set as 1 cycle of initial denaturation 95ºc for 3 min; 45 cycle of denaturation 95ºc for 10 sec; annealing 60ºc for 30 sec, 1 cycle melting 65-95ºc for 0.5 sec repeat 1 cycle.

2.6. Statistics Analysis

Statistical analysis was performed by using SPSS computing program for the analysis of the results [19].

3. RESULTS

3.1. Patients Covered Under the Study

Table2. *Gastropathological cases of the patients examined by endoscope.*

Type of disease	Number of patient	Percentage %
Gastritis	10	22.22 %
Gastritis & doudenitis	18	40 %
oseophagas tumer	1	2.22 %
Gastropath ulcer	16	35.56 %
Total	*45*	100%

The study included 45 patients; they are suffering from various symptoms of digestive indigestion patients. All the patient were examined endoscopicaly and clinically. Our results showed the variety of digestive system diseases among the 45 patient (Table 2).

3.2. Isolation and Identification of H. pylori

3.2.1. Bacterial Culture

About 15 bacterial cultures from biopsy samples were isolated and identified as positive culture (33.33 %) (Table 3). All of the isolates were grown on blood agar and incubated for 24-48 h at 37ºC (Figure 1a). Our results showed small colonies, convex, and translucent similar to a drop of water or gray on Brucella agar after 72 h of incubation (Figure 1b). A bacterial swab stained by carbul Fuxin stain and examined under light microscope showed that *H. pylori* are spiral in shape or S like shape (Figure 1c).

Table3. *The number and percentage of positive H. pylori culture isolated from patient biopsy samples.*

Type of disease	No. of patient	No. of positive culture	Percentage %
Gastritis	10	2	20%
Gastritis & doudenitis	18	7	38.89%
oseophagas tumer	1	1	100%
Gastropath ulcer	16	5	31.25%
Total	45	15	33.33%

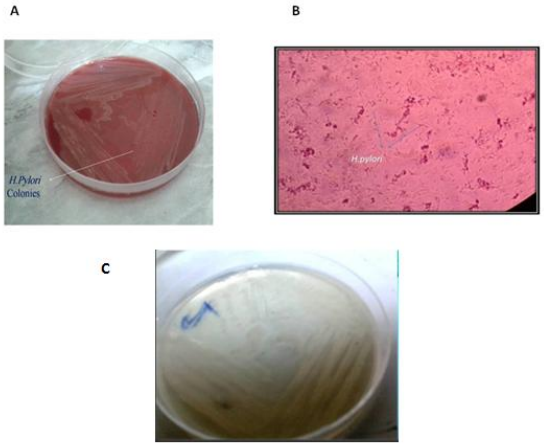

Fig1. *A. The H. pylori bacteria colonies growing on blood agar which are small colonies and gray. B. The shape of H. pylori under light microscope after stained with carbul Fuxin. C. The H. pylori bacteria colonies on Brucella agar after 72 h of incubation.*

3.2.2. Biochemical Tests

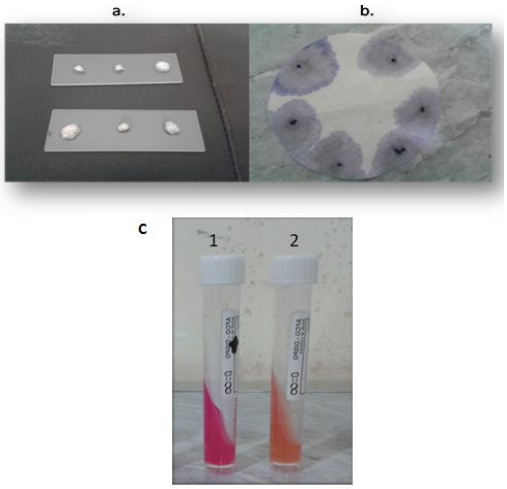

Fig2. *The results of catalase and oxidase test on H.pylori isolates: a. refers to positive result of catalase test. b. refer to positive result of oxidase test. c. The result of urease test on H. pylori isolates: Tubes number 1 and 2 refers to positive and negative result respectively.*

The results showed that 31 biopsy samples were positive (68.89 %) for rapid urease test (Table 4). Moreover, the bacterial culture isolates gave positive results using oxidase, catalase, and urease tests (Figure 2).

Table4. *The number of positive samples tested for urea rapid test from various pathological cases.*

Type of disease	No. of patients	RUT	Percentage %
Gastritis	10	6	60%
Gastritis & doudenitis	18	11	61.11%
oseophagas tumer	1	1	100%
Gastropath ulcer	16	13	81.25%
Total	45	31	68.89%

3.2.3. Molecular detection of H. pylori

Twenty eight RUT positive biopsy samples (300 mg in size) were firstly subjected to qPCR for confirmation of *H. pylori* using *ureC* as a diagnostic gene. It has been showed that 21 (75%) samples were positive for *ureC* (Figure 3). These samples were further tested using *H. pylori* (*ureA*, *vacA* and *cagA*) virulence genes. Our results showed that vacA and cagA were highly detected among 21 different gastropathological cases (64.28% and 60.7% respectively), and at lower rate for ureaA 39% with a significant value of P□ 0.01(Figure 4) (Table 5).

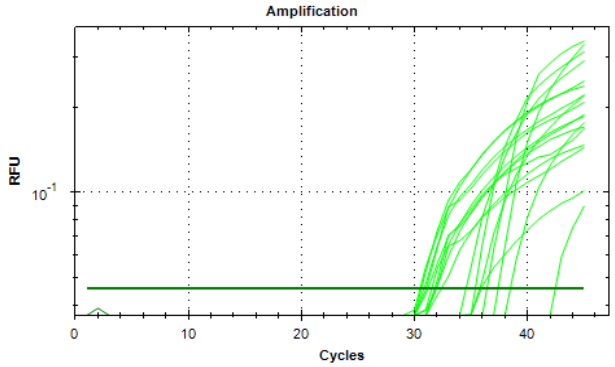

Fig3. *Real-Time PCR amplification log plot of ureC gene in Helicobacter pylori from genomic DNA of human biopsy samples. Where show the positive detection of H. pylori samples.*

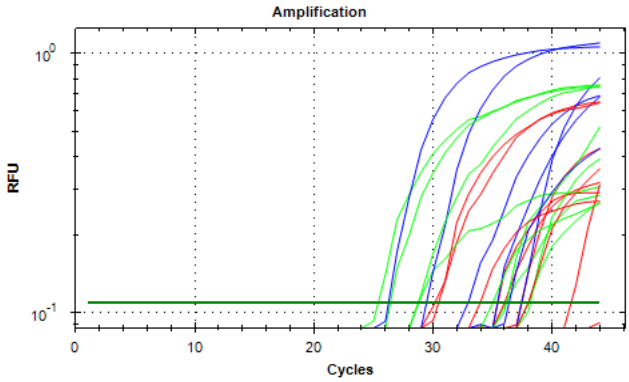

Fig4. *Real-Time PCR amplification log plot of virulence factor genes in H. pylori from genomic DNA of human biopsy samples, where plot green: ureA gene, plot blue: vacA gene, and plot red: cagA gene.*

Table5. *The relationship between H. pylori virulence genes (ureC, ureA, vacA, & cagA) detect by qPCR and Pathogenesis cases.* * *Significant value at P□ 0.01.*

Pathological case		Genes			
		ureC	***ureA***	***vacA***	***cagA***
Gastritis	Total/+ve	4:3 (75%)	*4:3 (75%)	4:2 (50%)	4:2 (50%)
Gastritis & doudenitis	Total/+ve	13:8 (61.53%)	*13:2 (15.38%)	13:9 (69.23%)	13:8 (61.53%)
Oseophagas tumor	Total/+ve	1:1(100%)	1:1(100%)	1:0 (0%)	1:1(100%)
Gastropathy ulcer	Total/+ve	10:9 (90%)	*10:5 (50%)	10:7 (70%)	10:6 (60%)
Total	Total/+ve	28:21 (75%)	*28:11 (39.28%)	28:18 (64.28%)	28:17 (60.71%)

4. DISCUSSION

4.1. Pathological Cases Distribution

The Esophago- gastro- duodeno- scopy (EGD) pointed out high prevalence of duodenal ulcer, gastric ulcer and non-ulcer dyspepsia in the investigated patients followed by other cases (Table 1). Our results were supported by previous study showed that 20% of patients infected with chronic gastritis [20]. Whereas [21, 22, 23, 24] reports higher infection with chronic gastritis among all other gastric pathological cases. However in case of gastric ulcer, gastritis, and doudenitis; our results disagreement with [25], he showed lower infection with above cases. It seems that the distribution of gastric pathological cases among patients was varied and depends on the selection of patients and/ or the host and environmental factors [26].

4.2. Rapid Urease Test

The RUT results was showed 31 sample from 45 were positive (68.89%) (Table 4). Rapid urease test depends essentially upon the number of the bacteria in the biopsies [27]. More than one biopsy may give a highly significant RUT results. The size of the biopsy itself may be implicated the number of the bacteria within these biopsies. It has been suggested that 10^4 colony forming unit (CFU) are required to exhibit positive RUT result [28].

It was suggested that uses of RUT for biopsies in the unit theorizing be sensitive enough for the detection of specific urease formation bacteria, however this test may be less effective for detection if the patient taking antibiotics [29].

4.3. Identification of H. pylori

From our results, it was showed that 15 from 45 samples were positive (33.33%) for *H. pylori* culture (Table 3). Previous studies were also showed difficulty and low percentage for *H. pylori* isolation [20, 30]. Primary isolation of *H. pylori* is a difficult process for routine laboratories. The sensitivity of isolation in specialized laboratories varies widely and it is ranging from 75% to 90%. However, many laboratories have found the primary isolation of *H. pylori* from gastric biopsies is still problematic [31].

Tiny percentage attributed to isolate the bacteria *H. pylori* to the many factors that affect significantly the bacteria, including patient intake of antibiotics or proton pump inhibitors before taking the sample, leading to the small number of bacteria in biopsies [32].

Several factors are implicated in directing the success of *H. pylori* cultivation. They include the method, time, procedure for tissue processing, composition of culture media, patchy distribution of the organism on the gastric mucosa, contamination of biopsy forceps, and the loss of viability of the organisms during transportation, the presence of oropharyngeal flora may be also responsible for a negative predictive value associated with culture of *H. pylori* [33].

The isolated *H. pylori* appeared on petri dish as small colonies convex, transparent, and similar to a drop of water, which is supported by previous studies [34, 35].

4.4. Molecular Detection of H.pylori from Biopsy Samples

The detection rate of *H. pylori* by PCR technique has shown great deal of variation ranging from 0-80% [36]. It depends on the target gene used in the analysis. The first targets recruited were urease operon *ure*A [37] and *glm*M, formerly named *ure*C [38].

Our results of RT PCR demonstrated a wide range of detection rate among the various investigated genes as well as samples underwent the analysis. It has been showed that (75%) of patients were positive for *ureC* gene. It has been shown that the detected ratio of *ureC* was varied between different pathological cases (Table 5). The *ureC* is considered a "housekeeping" gene, which participates directly in cell wall synthesis. It is well-established that the principal ecological niche for *H. pylori* is the gastric mucosa [27]. Other study reported that among patients with gastric ulcer, gastritis, and duodenal ulcer [39].

Meanwhile, results of positive *ure*A gene were (39.28%) also show various ratios in gastritis, oseophagas tumer, and gastropath ulcer (Table 5). The reason of difference gene ratio among pathological cases back to function of this gene which is act on urea hydrolysis and urease may be

excreted from *H. pylori* to the surrounding environment and may protect this pathogen from the humeral immune response. It is found in the cytoplasm and on the membrane of *H. pylori* cells [40].

The study results showed the current ratio of the *vacA* gene demonstrated that (64.28%) of patients have positive findings for *vacA* gene, also show various ratio depended on pathogenetic cases (Table 5). The reason of difference gene ratio among pathological cases back to action of this gene which is act on formation of acidic vacuoles in the cytoplasm of gastric epithelial cells by creation of pores in epithelial cell membranes, allowing release of anions and urea, *vacA* also induces loosening of epithelial tight junctions, potentially allowing nutrients to cross the mucosal barrier to *H. pylori* [41].

Concerning the findings of *cagA* gene (60.7%), the relatively high detection rate of *H. pylori* in the investigated patients also show various ratio depended on pathogenetic cases (Table 5).

The reason of difference gene ratio among pathological cases back to this gene is high antigenicity and its a determinant of *H. pylori* strains of enhanced interaction with gastric tissues [42]. The *cagA* island encodes a functional type IV secretion system, which permits the *cagA* protein to be trans-located into gastric epithelial cells [43]. This gene show high percentage of cagA protein is associated with peptic ulcer disease, gastric cancer, and mucosa-associated lymphoid tissue lymphoma in the stomach [44]. The presence of the *cagA* pathogenicity island has been associated with more severe *H. pylori* disease [45].

5. CONCLUSION

Bacterium *H. pylori* was directly detected from biopsy samples of gastritis patients using qPCR. The study was showed a various relationship between gastropathological cases and *ureC, ureA, vacA, & cagA* genes. Only *vacA* was not detected in tumor biopsy and therefore it should be extensively focusing on the action mechanism of this gene in cancer diseases.

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AUTHOR'S BIOGRAPHY

Dr Laith AL Obaidi is an Assistant Professor in Molecular Biology at College of Sciences, AL Muthanna University, Iraq.