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Prevalence of *Helicobacter pylori* infection in patients with peptic ulcer disease and non-ulcer dyspepsia and comparison among six diagnostic methods

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Abstract: *The aim of this study is to determine the prevalence of Helicobacter pylori (H. pylori) infection among patients presenting with peptic ulcer disease (PUD) andnon- ulcer dyspepsia (NUD) in Aleppo city, Syria and to compare the sensitivity and specificity of six diagnostic tests using real-time polymerase chain reaction (RT-PCR) as gold standard.The study included 50patients with PUD and 25 patients withNUD who underwent upper gastrointestinal endoscopy at AL-RAZIhospital and ALEPPO UNIVERSITY hospital between May2015 and February 2017.Seven diagnostic tests for H.pylori were used and compared including: RT-PCR, Culture, Rapid Urease Test (RUT), Stool Antigen Test (SAT), Urea Breath Test (UBT), Serology IgG, test. The overall prevalence rate of H. pyloriinfectionamong50 patients with PUD was 70% by RT-PCR method,on the other hand,the prevalence was 52%among NUD patients,we noticed that Age and gender were not statistically significant in H.pylori infection, while smoking was an important risk factor. Serology IgG test had the highest sensitivity which was 100% followed by SAT (93.7%). The specificity of all diagnostic tests was 100% except serology IgG test (92.5%). We concluded that the frequency of H. pylori among the studied population is remarkably high. Stool antigen test showed high specificity and acceptable sensitivity compared to RT-PCR, therefore it i sconsidered reliable diagnostic methods when RT-PCR is unavailable.*

Keywords: *Helicobacter pylori, diagnostic methods, RT-PCR, culture, RUT, UBT, SAT, Serology IgG.*

Introduction

Helicobacter pylori (*H. pylori*) remains one of the most common worldwide human infections and is associated with a number of important upper gastrointestinal (GI) conditions including chronic gastritis, peptic ulcer disease, and gastric malignancy. The prevalence of *H. pylori* is closely tied to socioeconomic conditions and accordingly, this infection is more common in developing countries than in developed Countries [1], The mode of transmission and other aspects of the epidemiology of *H. pylori* infection still remain unclear [2], Several virulence factors that aid *H. pylori* in colonization of the host and contribute to disease development have been identified bya variety of methods. One of these factors is the urease enzyme that *H. pylori* needs to survive in the low-pH gastric lumen as it makes its way to the gastric mucosa, which has a more neutral pH [3]. Molecular methods like Polymerase Chain Reaction(PCR) have been used extensively for the diagnosis of *H.pylori*from gastric biopsy specimens, saliva, faeces and archival specimens, as well as for detecting clarithromycin resistance[4]. The targets of these PCR methods include urease A (*ure*A) gene, *cag* A gene, phosphosaminemutase (*glm*M) gene and 16S rRNA gene(Lu, 1999). Real time PCR (RT-PCR) is more accurate than PCR. Furthermore, it permits a concomitant easy and reliable determination of expression level of genes[5].

Invasive and non-invasive techniques are used to diagnose *H. pylori* infection. Invasive methods such as histology, rapid urease test (RUT), microbiological culture and polymerase chain reaction (PCR), require endoscopy and are also known as biopsy-based tests. Non-invasive tests include stool antigen test (SAT), serology and urea breath test (UBT). Some factors which influence the choice of a given testing strategy include sensitivity, specificity, the clinical circumstances and the cost-effectiveness of the test [6]. Notably, all these techniques have their own limitations [7].

The aim of this study is to detect the prevalence of helicobacter pylori infection among peptic ulcer disease (NUD) patients and compare it with that among non-ulcer dyspepsia(NUD) patients, and to compare among the sensitivity and specificity of six diagnostic tests using real-time PCR as gold standard in order to detect the best alternative diagnostic method for RT-PCR which is an expensive one.

Materials and Methods

1- Patients: A total of 75 patients attending two local hospitals (AL-RAZI hospital and ALEPPO UNIVERSITY hospital) were enrolled in this study. The patients were divided into two groups. Group A is peptic ulcer disease patients (PUD), this group included 50 patients suffering from dyspepsia and epigastric pain, and the endoscopic findings showed existing gastric or/and duodenal ulcer. Group B is non-ulcerdyspepsia patients (NUD), it included 25 patients suffering from dyspepsia and epigastric pain but, the endoscopic findings were normal. Patients were excluded if they had recent use (within 4 weeks) of antibiotics, proton pump inhibitors (PPIs), or non-steroidal anti-inflammatory drugs (NSAIDs). All the patients provided informed consent to participate in this study. Clinical features of patients are clarified in (Tables 1,2,3,4,5,6)

Table 1: Distribution of PUD patients according to the age

Age range	**Number**	**Percentage**
18-20	2	4%
21-30	8	16%
31-40	8	16%
50-41	9	18%
60-51	9	18%
61-70	7	14%
70<	7	14%
Total	50	100%

Table 2: Distribution of PUD patients according to the gender

Gender	**Number**	**Percentage**
Male	36	72%
Female	14	28%
Total	50	100%

Table 3: Distribution of PUD patients according to the smoking habit

Smoking	**Number**	**Percentage**
Smoker	24	48%
Non- smoker	26	52%
Total	50	100%

Table 4: Distribution of PUD patients according to type of ulcer

Type of ulcer	**Number**	**Percentage**
Gastric ulcer	29	58%
Duodenal ulcer	14	28%
Combined gastric and duodenal ulcers	7	14%
Total	50	100%

Table 5: Distribution of NUD patients according to the age

Age range	**Number**	**Percentage**
18-20	4	16%

21-30	9	36%
31-40	3	12%
50-41	3	12%
60-51	3	12%
61-70	2	8%
70<	1	4%
Total	25	100%

Table 6: Distribution of NUD patients according to the gender

Gender	Number	Percentage
Male	13	52%
Female	12	42%
Total	25	100%

Table 7: distribution of NUD patients according to the smoking habit

Smoking	Number	Percentage
Smoker	7	28%
Non smoker	18	72%
Total	25	100%

2- Collection of Samples

2.1Biopsies samples

Three biopsies from the gastric antrum were taken using sterile biopsy forceps, two of them were inserted into special transport medium (portagerm pylori, Biomeriux, French), and the third biopsy was used immediately for doing rapid urease test.

2.2 Blood samples

Blood samples were collected through veins puncture into tube containing lithium heparin. The blood was then centrifuged at 5000RPM for 3 minutes at room temperature to obtain plasma. Specimens were labeled and stored at -20°C.

2.3 Stool samples

Stool samples were collected then kept in sterile container and stored at -20°C.

2.4 Exhaled breath samples

Samples of exhaled breath were collected within special commercial bags (kibion, Sweden).

3- Methods

3.1Real time PCR method (RT- PCR)

RT- PCR method was used to detect *H.Pylori* specific ureAgene, this method wasconsidered the gold standard for determination the sensitivity and specificity for the other diagnostic tests. Extraction of DNA was done on QIAamp Mini spin columns by using commercial DNA extraction kit (QIAamp DNA Mini Kit, Qiagen).TaqMan PCR was used to amplify fragments of the ureA gene. Real-time PCR was carried out by using Light Cycler apparatus (Roche) at Faculty of medicine, Aleppo University with the following thermal cycling conditions (Table 8).

Table 8: The points used to program LightCyclerapparatus

	Cycles	TT	IT	TTR	STT	SS	SD	AM
Enzyme activation	1	95°C	10 min	0.00	0	0.0	0	None
Denaturation	50	95°C	10sec	0.00	0	0.0	0	Single
Amplification	50	60°C	60sec	0.00	0	0.0	0	

TT= Target Temperature (°C), IT= Incubation Time (hrs:min:sec),TTR=Temperature Transition Rate (°C/s), STT= Secondary Temperature Time (°C), SS= Step Size (°C),SD= Step Delay (Cycles), AM= Acquisition Mode.

The regents used in RT-PCR reaction are:

- pylori specific primer/probe mix labeled with the fluorescent dyes 6-carboxyfluorescein (6-FAM) on the 5′end (PrimerDesign^{TM}genesig)
- pylori positive control ((PrimerDesign^{TM}genesig)
- 2xPrecision Mastermix kit (PrimerDesign^{TM}genesig) consists of (2x reaction buffer ‹0.025 U/µl Taq Polymerase‹5 Mm MgCl2 ‹Mix dNTP ((200µM each dNTP)
- RNAse/DNAse free water (Roche).

All regents including positive and negative controls were prepared according to the manufacturer's instructions. RT-PCR reaction mixture was prepared as described in (Table 9).

Table 9: The components of Pylori detection mixture

Component	Volume
2x PrecisionTMMasterMix	10 µl
Pylori Primer/Probe mix	1 µl
RNAse/DNAse free water	4 µl
Final Volume	15 µl

For each sample, 15µl from pylori detection mixture was added to 5µl DNA extract, thenall the processes were done as described in manufacturer's manual.

3.2 Microbiological culture

The standard microbiological culture technique was used to isolate and identify *H.pylori*. The biopsies were cut into small pieces byusing sterile scalpel and forceps and placed in sterile saline. The suspension was then inoculated on selective pylori agar medium (Biomeriux, French). The plates were incubated using commercial bags (Biomeriux, French) to providemicroaerophilicatmosphere,at37°C for7 days. The bacteria were identified as *H. pylori* by Gram staining, colony morphology, positive oxidase, catalase and urease reactions.

- **Urease Test**

The Pure colonies were inoculated on the commercial indol-urea medium (Biomeriux, Fernch) the tubes were inoculated at 37°C in the incubator. The formation of purple color was examined after 24 h.

- **Oxidase Test**

The isolates were tested for oxidase activity by using oxidase test strip.

- **Catalase test**

The isolates were tested for catalase activity by using slide (drop) method in which the sterile wire loop is stacked on the surface of the pure colony and transferred to a microscope slide and one or

two drop of 3% H2O2 were added onto the organism on the slide and observed for immediate oxygen bubble formation.

3.3 Rapid Urease Test (RUT)

The test was performed immediately after taking biopsy sample, In the rapid urease test, a gastric biopsy was placed on agar gel containing urea and a pH indicator such as phenol red (Pronto Dry, Medical Instruments Corporation, France). The urease produced by *H. pylori* degrades the urea in the gel, producing ammonium and bicarbonate ions which raise the pH of the media. The resultant alkalinity is reflected in a color change (from yellow to red) by the phenol red. Results were interpreted within an hour.

3.4 Serology

Plasma samples were tested for the presence of anti H. pylori IgG. Commercial kits used were DIA.PRO Helicobacter pylori IgG and IgA ELISA kits (Enzyme-Linked Immunosorbent Assay Test (ELISA) manufactured in Italy. The procedure was followed as described in the manual of the manufacturer.

3.5 Stool Antigen Test (SAT)

Commercial kits were used to extract *H. pylori* antigen from stool samples and to analyze it by using ELISA method (DIA.PRO, Italy).

3.6 Urea Breath Test (UBT)

Urea breath test was done using a commercially available kit (Kibion, Sweden) that consists of 50 tablets containing C-14labelledurea and 50 special bags to collect air samples . The C-14 urea in the presence of the enzyme urease is hydrolyzed, liberating CO2-14, which is detected by Gamma-counter apparatus. Result is expressed as counts/minute, the result is positive when it is more or equal to 50 counts/ minute.

Statistical analysis

The data was collected, summarized, tabulated and analyzed using the statistical package for social sciences (SPSS) V.13 software. Differences in proportions were assessed by a chi-square test and mean comparisons by the t-test, p-value < 0.05 was considered statistically significant. Sensitivity, specificity, and negative and positive predictive values were calculated according to the following equations.

Sensitivity=100 xTP/(TP+FN)

Specificity=100 xTN/(TN+FP)

TP:Number of true positive patients, FN:Number of false negative patients, FP:Number of false positive patients, TN:Number of true negative patients

Results

1- Prevalence and risk factors

the prevalence of *H. pylori* among PUD patients was 70% by RT-PCR method, while it was 52% among NUD patients (Table 10).Analysis of risk factors showed that the age and gender of PUD patients were not statistically important ($p > 0.05$). Smoking as risk factor was statistically significant ($p < 0.05$). The highest *H. pylori* prevalence was among combined duodenal and gastric ulcer patients (85.7%) followed by duodenal ulcer patients (78.6%) then gastric ulcer patients(62.1%). However there was no statistical relationship between the type of ulcer and *H. pylori* infection ($P > 0.05$) (Table 11). No statistically significant relationship was observed between*H. pylori* prevalence and the age, gender, and smoking habit of NUD patients ($p > 0.05$) (Table 12).

2- Statistical analysis of diagnostic methods

Considering 50 PUD patients and 25 NUD patients as one group consisted of 75 patients; sensitivity and specificityof different diagnostic tests were calculated. RT- PCR was taken as gold standard. IgG serology had highest sensitivity which was 100%, followed by SAT (93.7%). The specificity of all diagnostic tests was 100% except serology IgG (92.5%). (Table 13,14)

Table 10:The prevalence of *H. pylori* infection among PUD and NUD patients using RT-PCR

Patients	PUD N=50	NUD N=25
Positive	35	13
negative	15	12
Prevalence %	70	52

Table 11: The relationship between *H.pylori* infection and potentially risk factors among PUD patients

Factor		H. pylori positive		H. pylori negative		P value
		Number	Percentage	Number	Percentage	
Age	18-20	1	50%	1	50%	0.615
	21-30	6	75%	2	25%	
	31-40	6	75%	2	25%	
	41-50	7	77.8%	2	22.2%	
	51-60	4	44.4%	5	55.6%	
	61-70	6	85.7%	1	14.3%	
	>70	5	71.4%	2	28.6%	
Gender	Male	25	69.4%	11	30.6%	0.891
	Female	10	71.4%	4	28.6%	
Smoking	Smoker	22	91.6%	2	8.4%	0.001
	Non-smoker	13	50%	13	50%	
Type of ulcer	Gastric	18	62.1%	11	37.9%	0.336
	Duodenal	11	78.6%	3	21.4%	
	Gastric and duodenal	6	85.7%	1	14.3%	

Table 12: The relationship between *H.pylori* infection and potentially risk factors among NUD patients

Factor		H. pylori positive		H. pylori negative		P value
		Number	Percentage	Number	Percentage	
Age	18-20	2	50%	2	50%	0.195%
	21-30	3	33.3%	6	66.7%	
	31-40	3	100%	0	0%	
	41-50	2	66.7%	1	33.3%	
	51-60	1	33.3%	2	66.7%	
	61-70	1	50%	1	50%	

	>70	1	100%	0	0%	
Gender	Male	7	53.8%	6	46.2%	0.848%
	Female	6	50%	6	50%	
Smoking	Smoker	5	71.4%	2	28.6%	0.225%
	Non-smoker	8	44.4%	10	55.6%	

Table 13: The results of diagnostic tests for the total patients

Methods	**RT-PCR**	**RUT**	**Culture**	**SAT**	**IgA serology**	**IgG serology**	**UBT**
Positive	48	37	32	45	47	50	38
Negative	27	38	43	30	28	25	37
Total	75	75	75	75	75	75	75

Table 14: Diagnostic values of the different tests employed for *H. pylori* infection

Methods	**Sensitivity**	**Specificity**
Culture	66.6%	100%
RUT	77%	100%
UBT	79.1%	100%
SAT	93.7%	100%
Serology IgA	97.9%	100%
Serology IgG	100%	92.5%

Discussion

This study was carried out during the period between May 2015 and July 2016. The total patients was 75 patients, 50 PUD patients and 25 NUD patients . The importance of this study come from being the first study that detect the prevalence of *H.pylori* in Aleppo city and compare it between two groups of patients (PUD and NUD) by using sex different diagnostic methods including RT-PCR which is the most accurate method, and the comparison of the sensitivity and specificity among these methods. The prevalence of *H.pylori* among PUD patients was found 70% by using RT-PCR method. This prevalence is approximately the same as in many country such as Saudi Arabia (63%), Iran (68.4%), Pakistan (65.7%) [8,9], however, it is higher than this in Malaysia (49.4%)[10] and lower than this in Libya (96.8%)[11]. We can explain this wide range of prevalence as The prevalence of *H. pylori* differs both between and within countries, with high rates of infection being associated with low socioeconomic status and high densities of living [12,13]

Our study showed that smoking was a risk factor in *H.pylori* infection in PUD patients with statistically important relationship ($p<0.05$), this can be interpreted as the destructive effect of smoking on the immunity of gastric mucosa and lining layers and hence it causes increase in its susceptibility to infection by *H. pylori*[14]. This finding is in agreement with a study conducted in USA that reported

there was a relationship between *H. pylori* infection and smoking [15]. However, another study in USA reported no significant relationship [16].

The study also shed the light on the relationship between the type of ulcer and *H.pylori* infection, the most prevalence was among the patients suffering from combined gastric and duodenal ulcer (85.7%). The prevalence among duodenal ulcer patients (78.6%) was more than that among gastric ulcer patients (62.1%). Other studies carried out in Thailand, India, and Iran showed the same result [17,18,19]. However,the Pakistani study showed that *H.pylori* prevalence among gastric patients is higher than that among duodenal ulcer patients [9].

Our study observed that there was no statistically important relationship between *H.pylori* prevalence and the age of patients ($p>0.05$). On the other hand, some studies referred that the percentage of infected people increases with age, since 50% of infected people were those over the age of 60 compared with around 10% between 18 and 30 years[20]. In a large French cross-sectional study, a significantly lower prevalence of *H. pylori* infection was observed in females as compared with males[21]. However, in our study the gender was not statistically significant. Age, gender, and smoking were not statistically important in *H.pylori* infection among NUD patients.

In the clinical setting, a rapid and cost-effective detection method for diagnosis of *H. pylori* infection is desirable. We calculated the sensitivity and specificity of six diagnostic tests using RT-PCR as gold standard. We found that the specificity of all diagnostic tests was quite high (100%) except serology IgG (92.5%). The sensitivity of the diagnostic tests was 100% in serology IgG, 93.7% in stool antigen test, 77% in RUT, 79.1% in UBT, 66.6% in culture. The comparison between the sensitivity and specificity found in our study and the results found in the global studies was not accessible because of the difference between the commercial kits used and the method considered as gold standard.

The lowest specificity of serology IgG in comparison with other tests is due to the inability to differentiate between current and past infection. So that, previously treated patients who showed positive serology test might reflect past infection or false positive test result [22]. The second reason for the lowest specificity of serologyIgG test observed in our study may be due to the increased carriage of campylobacter jejuni by people in developing countries and presence of spiral bacteria like Gastrospirillium which interfere with the results[23].The lowest sensitivity of culture may be because of the fastidious nature of *H. pylori*, the patchy distribution of the organism in the stomach, and the presence of pharyngeal flora [24]. To increase the sensitivity, care should be taken regarding the transport of biopsy specimens and storage, media plate, and microaerophilicconditions. The transport medium is also essential for the successful detection of the bacteria.

To prevent the possible contamination by flora such as Gram-positive cocci from buccal or intestinal flora, we recommend using selective transport media which contain antimicrobial compounds to inhibit the growth of flora [25]. The interpretation for the highest sensitivity of serological tests may be because they are the only tests which are not likely to give false negative results in patients who have taken antibiotics, bismuth compounds or PPIs in the recent past[26]. Minor differences in sensitivity between RUT and UBT was observed in our study. As the urea breath test (UBT) and rapid urease test (RUT) rely on robust urease activity by *H. pylori*, their sensitivities may decrease by medications that reduce organism density in the biopsy or urease activity, including bismuth containing compounds, antibiotics and proton pump inhibitors (PPIs) [27]. Thus, patients should stop taking their medications two weeks before the diagnosis to prevent false negative results [28]. The presence of

blood in the stomach may also reduce the sensitivity of RUT as it can promote false-negative results, probably because of the buffer effect of serum albumin that interferes with the chemical reaction [29]. Since IgGantibody can remain high for months after elimination of infection [30], Serology IgG tests are not reliable to check eradication of *H. pylori*[31]. Since *H.pylori* is fastidious and difficult to grow in the laboratory, the role of culture in diagnosis of the infection is limited mostly to research and epidemiologic considerations. RUT permits inexpensive and rapid detection of *H.pylori* with high specificity (100%) and acceptable sensitivity (77.1%). However, it is invasive methods. UBT is non-invasive test but it requires special equipment, which may not be available in routine clinical laboratories. In our study,SATis considered as good choice for post-treatment follow-up of *H. pylori* infection [32].

Based on the results provided by this study, Stool antigen testare a good alternative for RT-PCR method which is expensive one and sometimes it is unavailable in all diagnostic center.

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