Comparative study of *Helicobacter pylori* best diagnosis methods and the Effect of some Risk Factors associated with infection.

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الخلاصة

هذا الاستعراض هو محاولة لإيجاد أفضل طريقة لتشخيص الإصابة بجرثومة المعدة الحلزونية في المرضى الذين يعانون من امرض القرحة الهضمية، وكذلك، تحديد تأثير بعض عوامل الخطورة المرتبطة بهذه الإصابة. تضمنت هذه الحالات عزل عشر عزلات من بكتريا ***H. pylori*** من110 نموذج خزعة نسيجية استؤصلت بواسطة جهاز الناظور من معدة مجموعة المرضى فقط الذين كانوا يعانون من أعراض عسر الهضم والوافدين إلى وحدة تنظير الجهاز الهضمي في مستشفى اليرموك التعليمي. مع الفئة العمرية 15-85 سنة من كلا الجنسين. أخضعت هذه الخزع للاختبارات المايكروبايولوجية والتي تتضمنت: اختبار الزراعة البكتيرية للكشف عن وجود أجناس بكتيريا ***H. pylori***. واختبار خزعة أخر هو فحص اليوريز الذي أجري بطريقتين (طريقة وسط أكار اليوريا المائل وطريقة عدة اليوريا السريعة). وشارك 30 فرد آخر من المتطوعين غير مصابين ببكتيريا ***H. pylori*** كمجموعة سيطرة من الفئة العمرية 16-75 سنة، ولا يعانون سابقا من أمرض القرح الهضمية. بالإضافة إلى ذلك، جمعت 140 عينة دم وريدي من مجموعة المرضى والسيطرة لغرض أجراء اختبار مقايسة الممتز المناعي المرتبط بالإنزيم. وأيضا اختبار فصيلة مجاميع الدم ABO. النتائج أظهرت فرق معنوي لانتشار الإصابة ببكتريا ***H. pylori*** بلغت 60 (54.55٪).

استنتجت الدراسة أن فحص عدة اليوريا السريعة اعتبر بمثابة الاختبار التشخيصي الأكثر كفاءة بين الأشخاص المصابين بالبكتيريا ***H. pylori*** مع حساسية (93٪) ونوعية (80٪). وكشفت الدراسة أن هناك تأثيرا معنويا كبيرا لكل من عامل العمر, الجنس و النمط الظاهري لفصيلتي الدم B و O على انتشار بكتريا ***H. pylori*** و زيادة اكتساب الإصابة بإمراض القرح الهضمية باحتمالية ($P<0.0001$).

ABSTRRACT

The present review is an attempt to find the best diagnosis method of ***H. pylori*** infection in patients with Peptic Ulcer Disease (PUD), as well as, determined the effect of some risk factors associated with this infection.

These prospective cesses included, Ten ***H. pylori*** isolates were isolated out of 110 tissue gastric biopsies specimens existed from the patient's stomach that were suffering from dyspeptic symptoms, whom attended to Gastroenterology Centre in AL-Yarmook Teaching Hospital. With an age range 15-85 years from both genders. These biopsies subjected to, bacterial Cutler test for investigate the presence of ***H. pylori*** *spp*. and other biopsy test is Urease test that was performed by Two methods (slant urea agar base and Rapid Urease Test Kit). A further 30 healthy volunteers individual, uninfected with ***H. pylori*** were involved as a control group. Besides that, 140 venous blood specimens were collected from patients and control group for Enzyme Linked Immuno Sorbent Assay, also for ABO blood group test. And the results obtained clearly showed increase the frequency prevalence of ***H. pylori*** distribution to reach 60 (54.55%) patients.

Key words: PUD; Skirrows Selective Medium; RUT; Anti-***H. pylori*** IgG antibodies.

Introduction

The infection with ***Helicobacter pylori*** (***H. pylori***) as opportunistic pathogen should be paying more attention since, it formed a Gastroduodenal problem. And because only few studies on these bacteria in Iraq, so the present review is amid to determining comparison among ***H. pylori*** effective method for identifying suspected ***H. pylori*** infected individuals.

This bacterium chronically infect the stomach mucus layer over half the world's human population with clinical signs of infection only manifesting in less than (20%) of these individuals causing gastritis [(1)]. ***H. pylori*** had been recognized as the etiological agent of different Peptic Ulcer Diseases (PUD) and the infection with this bacterium highly links to PU as well as, increases the risk of Gastric Cancer (GC) in humans [(2)]. Epidemiological studies have confirmed the distribution of the ***H. pylori*** infection in a worldwide and the prevalence rate range approximately (30%) in developed countries to more than (90%) of the population in developing areas, and the infection rate increases at a rate of approximately (1%) per year [(4)]. The infection is usually acquired in early childhood and persists for decades if left untreated [4]. The way of infection spread throughout the world suggests the possibility that there were multiple pathways of transmission [(5).] ***H. pylori***, has been designated a ″Class -I- human carcinogen″ by the World Health Organization International Agency for Research on Cancer [(6).] It is worth mentioning of this bacterium was fastidious and, it may not grow despite the availability of all the growth requirements and often were called uncultured bacteria, while, sometimes called fickle germ [(7)] There are variety in diagnostic tests are now available for ***H. pylori*** infection; include Endoscopic and non-Endoscopic tests [(8, 9)].

Materials and Methods

Culture media: Culture media used in this study: Blood Agar Base, Columbia Blood Agar Base, Skirrows Selective medium and Urea Agar Base.

Kits: Kits used in this study: Blood groups kit, Gas Generation Kit, Gram stains set Kit, ***H. pylori*** IgG- ELISA Kit and RUT kit.

Samples Collection: According on these criteria One hundred ten (110) gastric biopsies specimens were taken from patient's stomach. During period January/2015 to March/2016. The patients include 67 females and 43 males with age range from 15-85 years. The investigations results have been compared with 30 healthy volunteers were performed individuals conceder as healthy control group; they were used in Serological test and ABO blood group test.

Specimens: specimens were collected from the patients before antibiotic therapy and PPI taking, in which at least Two multiple gastric biopsies specimens were taken from patient's stomach (suffering from PU symptoms) guided Upper Oesophageal Gastroduodenal Endoscopy (OGD) under consultant supervision endoscopist to evaluate the presence of PUD. One biopsy specimen was placed quickly on RUT kit device while, the endoscopist has examined the stomach, and the second biopsy was immediately

introduced into an appropriate transport media subjected and processed for Culture test as soon as possible, ideally within Six hours for ***H. pylori*** isolation and identification [10]. The drown venous blood specimen of Three mL were collected from both patients and control group divided into Two parts: One mL put in heparinized tubs for Haematological test to detect ABO blood group test, and Tow mL put in sterilized plain tube, and allowed to coagulate at 37°C for 30min. then serum were separated by centrifugation for 10min. for Serological test to estimating the levels of IgG Anti-***H. pylori*** antibodies by using Enzyme Linked Immuno Sorbent Assay (ELASA).

Laboratory diagnosis:

- **RUT test:** RUT kit was performed strictly following the instructions of the manufacturer Kolkata Company/India. One biopsy specimen from patients place was taken and sealed on plastic slide of the kit, subjected during Endoscopy, and then few drops of distal water was added to the well then kept undisturbed for 10minute. The test was judged to be positive if a color changed from yellow to pink indicative of ***H. pylori*** infection in order to the ability of bacteria to secrete Urease enzyme that hydrolysis urea to ammonia and raises the pH of the medium which cause change in color within 5-10minute [11].
- **Serological test:** This test has ability to detection IgG antibodies against ***H. pylori*** bacterial antigen in human serum or plasma based on ELISA depending sandwich technique [12]. In which all patients' serum were ran to the ***H. pylori*** IgG-ELISA test according to the instructions of Novalisa Company/Germany.
- **Bacteriological test:** the Bacterial identification assays included gastric biopsy specimen was first suspended in One mL of sterile normal saline, gently ground in manual homogenizer. However, the suspension was streaked onto Skirrows Modified Selective Medium and Colombia Agar plates, the plates were regularly observed for 4-5days at 37°C under microaerophilic atmosphere using GasPaks generation kit supply (5%O2, 10%CO2, 85%N2 and 7.1%H2) placed in an aerobic jars [13]. Several small round colonies from each patient's plate were selected and sub-cultured them 1or 2 times to obtain a pure culture. According to the diagnostic procedures recommended by Brown *et al* [14]. The isolation and identification of ***H. pylori*** associated with patients were performed as follows:-
  - Colonial morphology and microscopic examination: ***H. pylori*** identification depended on the morphology properties (colony size, shape, color, edge), and microscopically colonies appeared 1-2mm like pin point, smooth and transparent, as well as, Smear from bacterial growth was stained by Gram stain to observe reaction for different colonies under light microscopy; ***H. pylori*** were seen as curved, rod, Gram–negative bacteria [15].
  - Biochemical tests: for further, identification of ***H. pylori*** from other isolations, after culture was done, the following biochemical tests were performed including: Urease test, Catalase test, Oxidase test [13].
- Susceptibility test to Naldixic acid (NA) and Cephalothin (CEF): All ***H. pylori*** isolates were tested to antibiotic susceptibility test by inoculating few

colonies on the surface of Colombia Agar plate, incubated at 37°C in microaerophilic conditions for 48hrours [16]. The results showed ***H. pylori*** NA sensitive and CEF resistant according to Amsterdam [17] recommendations.

➢ **Blood groups test:**

The ABO blood groups phenotypes were carried out by assay standard Hemagglutination ABO blood group solutions, according the instruction kit of Randox-company/USA [19].

Results and discussion

A total of 110 patients with gstroduodenal symptoms under this review, subjected to various investigations for diagnosis the prevalence of ***H. pylori*** infection including (Cutler, RUT, and Serology) tests. Patients were considered infected by ***H. pylori***, if at least Two biopsy-based tests were positive results [20].Thus, used of multiple tests to detect the presence of ***H. pylori*** is recommended, This is come a approved with other results who suggested uses multiple tests for ***H. pylori*** diagnosis [21]. Our data investigated that ***H. pylori*** infection was detected in 60 (54.55%) out of 110 patient based on the pre-defined case definition. Whereas, the patients without ***H. pylori*** were 50 (45.45%) as seen in (Table1).With low significant difference (P = 0.003). This study clearly demonstrates the high rate of ***H. pylori*** detection in this work indicates that this bacterium type play an important role in the development of PUD among the infected patients.

Table 1: The prevalence of *H. pylori* infection in patients group with P.U.D.

***H. pylori* status**	**Gastroduodenal disease No. 110**
***H. pylori*- positive**	**60 (54.55%)
***H. pylori*- negative**	50 (45.45%)
Statistical Analysis	
Cell: *H. pylori*- positive; Effect : row 1 on row 2 Significance level: 0.05	P = 0.003

***** Patients were considered infected with ***H. pylori***, if at least both Two tests were positive results either by Culture and RUT or by ELASA and RUT; ****** There is low significance difference (P = 0.003).

The result obtained in our work was little bit higher than that recorded by other data who found the prevalence of ***H. pylori*** were presented in the 79 from 90 (87.7%) patients suffering from this infectious [22]. While, the results completely differ with that results, who revealed that 94 (74.01%) out of 127 patients were showed positive evidence of ***H. pylori*** [23].

- **Isolation and identification *H. pylori* from Gastroduodenal patients:**

Out of 110 biopsy specimen collected, Ten isolates were primary identified as ***Helicobacter pylori*** out of 60 infected patients. This bacterium can quickly identify as ***H. pylori*** on the Colombia Blood Agar and Medium Modified Skirrows Selective Medium (Figure1)

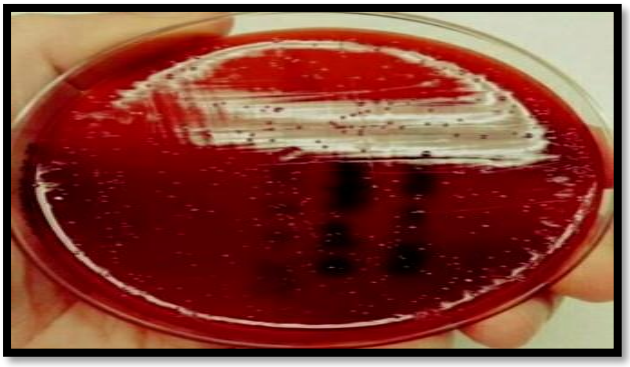

Figure 1: Morphology of *H. pylori* colonies was inoculated onto a Modified Skirrows Selective Medium after 4-5 days previously incubation period under optimal condition.

The identification and characterization were done based on morphological properties of the colonies which, are circular, convex, 1-2mm in diameter, smooth translucent to grey colour and distinct edges.

Furthermore, microscopic identification conformed by typical appearance on direct smear to observe reaction for different colonies under light microscopy at a final magnification of 1000x. (Figure 2) ***H. pylori*** were appeared curved or spiral Gram–negative bacteria and non spore forming, this corresponded to characterization mentioned in [24].

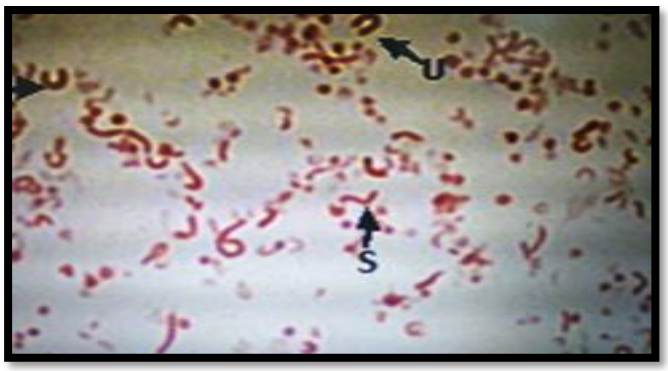

Figure 2: Typical appearance morphological forms of *H. pylori* (gull-wing, curved rod and spiral) shapes on direct smear stained by Gram stain can be clearly seen using a power light microscope enlarging on magnification 1000x objective oil).

Further, Ten ***H. pylori*** isolates were subjected to Biochemical tests, to give Catalase, Oxidase and Urease positive results, within 15-20 min. (Figure 3) after incubated at 37ºC under microaerophilic conditions. And this agrees with other result who found that some isolates gave positive results within 15 min [25]. But, disagrees with the result that found positive results given in 5min [26]

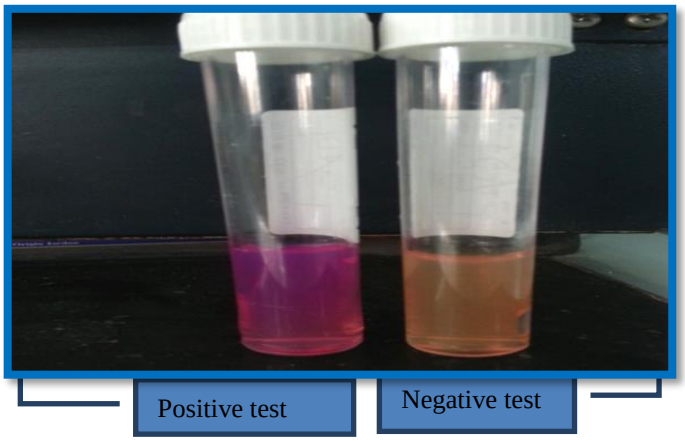

Figure 3: Production of Urease enzyme test on Urea agar base slant.

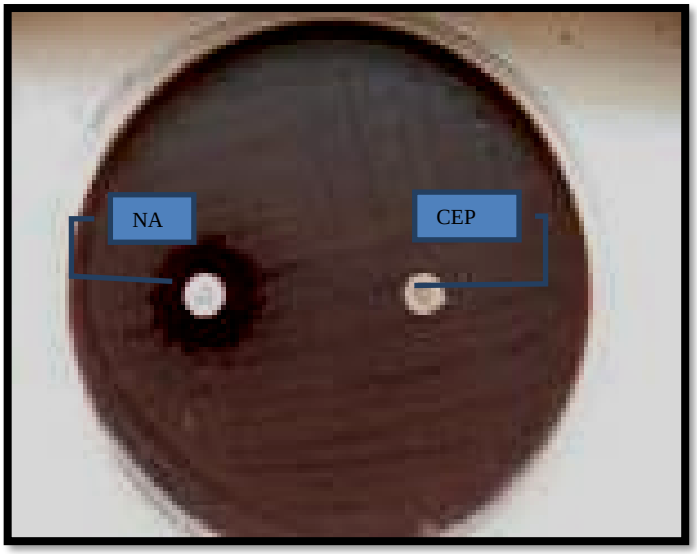

Figure 4: *H. pylori* resistant to Cephalothin (CEP) and sensitive to Naldixic acid (NA).

Moreover, all isolates tested for antibiotics sensitivity to Cephalothin and Naldixic acid on Colombia Blood Agar Medium and the result were identical for all of them , which is resistant to Cephalothin and sensitive to Naldixic acid (Figure 4). Our data were much similar to the result of [27].

- **Rapid Urease Test (RUT):**

This test is important rapid invasive test to detect ***H. pylori*** infection through period does not exceed 10 min or less than this period [30], due to the ability of ***H. pylori*** to secreted Urease enzyme that hydrolysis the urea to ammonia and raises the pH of the medium and cause change color into pink which indicates the positive results (Figure 5) depending basically on the number of bacteria in the biopsy as well as, the number of excision biopsies by using RUT of ***H. pylori*** kit device. Our findings revealed that 60 biopsy samples from patients group subjected to RUT kit test.

In comparison with previously work, the remaining 50 infected patients, failure to Culture due to the fastidious nature of ***H. pylori*** depends on the amount and viability of the bacteria since, ***H. pylori*** were sensitive to oxygen and temperature also the efficacy of ***H. pylori*** culture depends on the time between sampling biopsy and the start Culture test under microaerophilic conditions [28]. That led the bacteria loss of viability of the specimen during transportation [29]. In the Iraq, due to methodological difficulties in isolating this bacterium, detection of the organism by Culture methods has not been popular.

in a positive test results because it has a large number of bacterial cells [31].
While, Ten false positive result with –ve ***H. pylori*** patients attributed to the possibility of contamination biopsies or the forceps with other bacterial species that produced Urease enzyme, like ***Pseudomonas*** *ssp.* [32].

A-Positive test B- Negative test

Fig 5: Detects the Urease enzyme in *H. pylori* test device Kit

- **Serological test:**

This test was provided useful information for the primary certain ***H. pylori*** infection states [33]. Its evaluated the Anti ***H. pylori*** IgG antibody in serum using ELISA, was performed on 140 person to examined ***H. pylori*** IgG antibody, (Figure 6) showed the microtitration well plate that associated with ELISA kit, the patients whom infected with ***H. pylori*** give 52 true positive result and Moreover, the observations found that there are Eight false positive results appeared from 30 control group, the probable cause of the cross-reactivity of the test with other patient's disease such as hepatitis B and C virus, Syphilis, low immune disease and some infection caused by Enterococci.

The results give 56 out of 60 biopsy had true positive results for +ve ***H. pylori*** infected patients and 40 out of 50 had true negative results for –ve ***H. pylori*** patients with low significance difference ($P<0.001$) as tabled in (Table 2). The probable cause of a biopsy gives Four false negative results with +ve ***H. pylori*** infected patients could be ascribed to the small numbers of bacteria to the size of biopsy can affected Eight false negative result with high significance difference ($P\leq 0.0001$), this attributed to the poor immune response or the duration of infection and age. While, the test for patients uninfected with ***H. pylori*** give 43 true negative result and Seven false positive results with significance difference ($P\leq 0.0001$), Because the level of antibody remain for a months after recovery from a previous infection as well as, the hemolytic blood give false positive result [34].

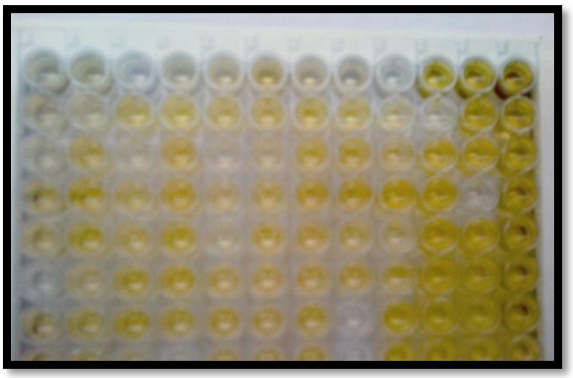

Figure 6: ELISA Microtitration well plate 96 that associated with ELISA kit of IgG in serum patients contains positive and negative cases.

The results of this date are strongly disagreeing with other results that investigated (44%) of control group give false positive [27]. And agree with the data explain the appearance of positive results in control group due to these person were already infected with ***H. pylori***, but asymptomatic in which it's not

necessarily to turn every infection to the disease [35].

Comparison of positive *H. pylori* test (Culture, RUT and ELISA) in different groups:

The sensitivity and specificity for each test depending on true positive, true Negative, false positive, false negative values as compared to theoretical golden standard methodof diagnostic tests [36]. When bringing the sensitivity and specificity of the three tests Culture, RUT and ELISA the results will be (17%), (100%), (93%), (80%) and (87%), (86%) respectively according to data recorded in (Table 2). In this study, using several tests together represented as golden standard method. Our results suggest that collection multi-group tests were found the best way to determine the ***H. pylori*** infection. Identical results were found by other data that revealed the uses of multiple diagnostic methods are recommended to accurately diagnose ***H. pylori*** infection [25].

Table2: Comparison among *H. pylori* diagnostic tests (Culture, RUT and ELISA) in different groups.

No.	Test		Control 30	Patients group 110		Sensitivity	Specificity
				50 (-ve) *H.pylori*	60 (+ve) *H.pylori*		
1	Culture 110		-	50	*10	17 %	100 %
2	RUT 110		-	40	**56	93 %	80 %
3	ELISA 140	(+ve) ELISA	8	43	52	87%	86%
		(-ve) ELISA	22	7	8		
Statistical Analysis							
Cell: Culture Effect: column 4 on column 3 Significance level: 0.05			$P < 0.0001$				
Cell: RUT Effect: column 4 on column 3 Significance level: 0.05			$P < 0.001$				

***** There is highly significance difference ($P< 0.0001$) between (-ve) and (+ve) ***H.pylori*** patients subjected to Culture test.

****** There is low significance difference ($P < 0.001$) between (-ve) and (+ve) ***H.pylori*** patients subjected to RUT.

On the basis of these findings, the results obtained in this study recorded, the low sensitivity (17%), and high specificity (100%) rate for Cutler test and from these rates we can clarify that gastric tissue Culture is not highly sensitive, making it un useful test in the detection of ***H. pylori*** infection. Therefore, should be done jointly with another diagnostic dependable test such as Histology or RUT. Its low sensitivity is acceptable since this method is not recommended as a screening test, but, high specificity related to increase the incubation time. These percentages are relatively lower than the reported by [25] in Philippines proposed that ***H. pylori*** Culture showed a sensitivity of (45%) and specificity of (98%) respectively. while, the other results showed that Cutler test gives (70%), (80%) sensitivity and specificity respectively [10]. Based on our previous observations about

RUT the results have been suggested a great efficiency in diagnosis ***H. pylori*** infection and is a more reliable as well as, better than other reported tests with highly sensitivity (93%) and specificity (80%) that qualified it's as a good detection test among suspected ***H. pylori*** infected individuals. Increased sensitivity rates of the RUT from antral biopsy, may be related to that other tests show heterogeneity in their sensitivity that ranged in (17%) for Culture and (87%) for ELISA, whereas, its specificity is little bat than ELASA (86%), but far out of Culture test in its specificity (100%). So this review supported the other study, who proved that RUT test whether in a gel manually preparation or on rapid kit device test is considered as a strongly precise tool than others [37]. In contrast, the other researchers, argued that, because the sensitivity and specificity of RUT are (66%) and (91%), respectively, and explain their data when reading the results longer than 30min. leading to minimised the accuracy of the test, suggesting that decreasing the incubation time may improve the sensitivity of RUT but, decreases its specificity [38]. While, a perfect performance of RUT has been demonstrated with the high sensitivity of (98%), specificity (100%) registered by Mojgan *et al* [39] whom affirming that RUT is widely used in diagnosis of ***H. pylori*** infection around the world due to a number of accompanying advantages, including less expense and more rapid results compared to Histology or Culture tests. Moreover, One of the great advantages of the RUT in the diagnosis of ***H. pylori*** is that the result can be obtained before the patient leaves the Endoscopy room this will facilitated to perform the test without need to hold the samples to the laboratory which required other equipment making clinical management being easier and faster with cost effective. Also the uses small size kit device leading the possibility of storage for a long time without drying or contamination. Our data agree with other data who noted the ability of RUT to determine the infection with ***H. pylori*** in dyspeptic patient and it is considerable the essential test in diagnosis this bacterium to gives more accuracy result than others methods that giving less accuracy with many advantages [26]. From other hand, the ELISA results revealed the ability of this Serology test in monitoring treatment and successful eradication of infection through detecting the fall in level of IgG antibodies in serum after Three months of treatment, as well as, could be depending this test to become a good alternative method in primary diagnosis ***H. pylori*** infection comparing with tests need to be examined Endoscopically, and it is recommended for situations when Endoscopy is not necessary. Nevertheless, an endoscopy test is always necessary because a gastric biopsy is required to perform the Histology, RUT and Culture tests [40]. The sensitivity and specificity of ELISA are (87%) and (86%). Our current work comes in concordance to the study of [41]. With sensitivities (90%) and specificity ranging from (80%-85%), while, the other data recorded the sensitivity and specificity of ELASA test by many workers varies from (70-80%) and (90-95%) respectively ([42]. The high sensitivity (87%) resulting from the large capacity of the test to investigation of ***H. pylori***, whereas, the specificity depend on the nature of cure antigen that used which is raised in case of used recombinant antigen [35]. Furthermore, the low specificity (86%) related to the cross reactivity with other bacteria such as ***Campylobacter*** *spp.* [43]. These findings are in agreement to what was found by Al-Rawy [21],

who referred to the possibility indication of using ELISA test as hopefully diagnostic assay to diagnosis ***H. pylori*** Serologically, while, other results consider this test not specific enough for diagnosis of ***H. pylori*** infection because this method is not influenced by the consumption of PPI drug [44]. This was currently a common treatment among patients seeking a specialized consultation diagnosis [45]. And the results were completely different with other literature that they proposed that positive finding of IgA antibody to ***H. pylori*** in patients who were symptomatic may be significant clinical value especially if IgG was negative and this making ELIZA test not dependable in diagnosis [46]. Notably, in our previous work, the choice of Endoscopic or non-Endoscopic tests depended to a large extent on availability and cost, as well as, includes a distinction between tests used to establish a diagnosis of the infection and confirm bacterial eradication, other important factors such as: clinical situation, population prevalence of infection, pre test probability of infection, differences in test performance, and factors that may influence the test results, like the uses of antibiotics [9]. Our finding also, indicated that the effect of patient's age, sex, blood group phenotypes, Endoscopic clinical findings and patient's symptoms types were studied as risk factors associated with the distribution ***H. pylori*** infected patients, in which the statistical analysis of the results investigated highly significance differences were found between blood group B and O phenotypes factor affecting distribution ***H. pylori*** acquisition, which led to prolonged increase infection with PUD caused by this bacterium in probability (P<0.0001) But, Endoscopic clinical findings were found to be not significantly recorded in distribution ***H. pylori*** infection with (P<0.075). While, patient's age and sex are showed to be highly significance differences with (P<0.0001), and patient's symptoms types showed no such effects were exist with (P< 0.37).

Conclusion

The study concluded that Rapid Urease Test considers as a more efficient diagnosis test among suspected ***H. pylori*** patients with sensitivity (93%) and specificity is (80%). As well as the data revealed that patient's age, sex and blood group B and O phenotypes factor affecting on distribution ***H. pylori*** acquisition in probability (P<0.0001).

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1

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