Primary isolation and detection of *Helicobacter pylori* from dyspeptic patients: a simple, rapid method

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الاستفراد البدئي وكشف جرثومة الـمَلويَّة البوابية *Helicobacter pylori* في مرضى القرحة المعدية: طريقة سريعة وبسيطة

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الخلاصة: استهدفت هذه الدراسة استنباط طريقة سريعة وبسيطة للاستفراد البدئي وكشف جرثومة الـمَلويَّة البوابية *Helicobacter pylori* في مرضى القرحة المعدية. وقد أخذت خزعتان، بالمنظار، من الغشاء المخاطي للمنطقة البوابية للمعدة من 136 مريضاً شخصت إصابتهم على أنها قرحة هضمية، وذلك في مستشفى البصرة العام، في العراق. ومن خلال الفحص الهيستوباثولوجي للخزعات، تم التعرف على الـمَلويَّة البوابية في 81 مريضاً بالقرحة المعدية (59.6٪). أما بالنسبة للزرع الجرثومي، فقد استُنبِتت الخزعات في مُسْتَنْبِتَين متوازيين: الأول غير انتقائي استخدم فيه وسط كولومبيا التقليدي، وكان الآخر انتقائياً استخدم فيه أغاريوريا كولومبيا المحوَّر. الذي أظهر معدلاً أعلى للاستفراد الناتج من استخدام أغار كولومبيا التقليدي (67.6٪ مقابل 44.1٪ من المرضى)، كما أن النتائج ظهرت بصورة أسرع (24 ساعة مقابل خمسة إلى سبعة أيام) مع صورة أوضح لا تترك مجالاً لأي شك.

ABSTRACT The study aimed to develop a rapid and simple method for the primary isolation and detection of *Helicobacter pylori* from dyspeptic patients. Mucosal antral biopsy specimens were obtained from 136 consecutive dyspeptic patients diagnosed with peptic ulcer by endoscopy at Basra General Hospital, Iraq. From histopathological examination of biopsies, *H. pylori* was detected in 81 (59.6%) peptic ulcer patients. For bacterial culture, specimens were cultured in parallel on 2 media: the non-selective classic Columbia agar and the selective modified Columbia urea agar (MCUA). MCUA showed a higher isolation rate than classic Columbia agar (67.6% versus 44.1% of patients), and the results were obtained faster (24 hours versus 5–7 days) with more clear-cut identification.

Méthode rapide et simple d'isolement primaire et de détection de *Helicobacter pylori* chez des patients dyspeptiques

RÉSUMÉ L'objectif de cette étude était de mettre au point une méthode rapide et simple permettant d'isoler et de détecter *Helicobacter pylori* chez des patients dyspeptiques. Des échantillons de muqueuse ont été prélevés grâce à une biopsie antrale sur 136 patients dyspeptiques consécutifs pour lesquels un diagnostic d'ulcère gastro-duodénal avait été établi par endoscopie à l'hôpital général de Bassora (Basra) en Iraq. L'examen histopathologique des biopsies a permis de détecter *H. pylori* chez 81 (59,6 %) patients atteints d'un ulcère gastro-duodénal. Concernant la culture bactérienne, les échantillons ont été mis en culture en parallèle dans deux milieux : la gélose Columbia classique non sélective et la gélose Columbia sélective modifiée à l'urée. Cette dernière a présenté un taux d'isolement plus élevé que la gélose Columbia classique (67,6 % des patients contre 44,1 %) et les résultats ont été obtenus plus rapidement (24 heures contre 5 à 7 jours), avec une identification plus nette.

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Introduction

Peptic ulcer disease is a common problem encountered by physicians in everyday practice. Since the isolation of *Helicobacter pylori* by Marshall and Warren in 1983 [*1*], tremendous progress in the understanding of the etiology, pathogenesis and management of this disease has occurred.

It is now widely agreed that *H. pylori* is the cause of most peptic ulcer disease [*2,3*] and that this microorganism is endemic in some developing countries, affecting as much as half of the population [*4*]. The strongest evidence for the pathogenic role of *H. pylori* in peptic ulcer disease comes from treatment trials. Eradication of the organism results in ulcer healing and reduces the risk of ulcer recurrence and complications [*5,6*].

Infection by *H. pylori* has been diagnosed by a variety of invasive and non-invasive tests [*7*]. However, the "gold standard" for *H. pylori* detection, as suggested by the Maastricht consensus report [*8*] is positive culture or both a positive histologic examination and a positive rapid urease test. The sensitivity of histology is generally high; however, because *H. pylori* colonization is focal, negative biopsy results do not exclude the possibility of infection in areas not sampled [*9*].

A variety of culture media have been used for isolation of *H. pylori*. Both selective and non-selective media have been used [*10,11*] . The most commonly used are Columbia, Brucella, brain–heart infusion, trypticase soy or blood agar media, each supplemented with 5%–10% blood. Supplementation of culture media with serum, albumin or activated charcoal instead of blood has been used to support the growth of *H. pylori* [*12*]. These materials are assumed to play a role of detoxifying the toxic substances in the media [*13*]. Bacte-rial growth usually appears as translucent, small, pinpoint colonies [*1,14*].

We aimed to develop a rapid and simple method for the primary isolation and detection of *H. pylori* from dyspeptic patients.

Methods

Sample

Between October 2002 and August 2004, 136 patients with symptoms suggestive of peptic ulcer were diagnosed as having peptic ulcer (gastric and duodenal) using endoscopic examination at the endoscopic unit, Basra General Hospital, Iraq, by a specialized surgeon. There were 72 males and 64 females. The ages of the patients ranged from 18 years to 69 years. Cases with negative endoscopic results for peptic ulcer were excluded from the study.

Data collection

Two gastric tissue specimens were taken from the antral region of the stomach of each patient during the endoscopic examination. Presence of the bacterium *H. pylori* in the tissue specimens was detected using 2 methods: histopathological examination and bacterial culture growth.

Laboratory methods

For histopathological detection, surgical specimens were fixed in paraffin wax and stained with haematoxylin and eosin (H&E) and Giemsa stains. The sections were examined for the presence of *H. pylori* and any inflammatory changes that might be present in the gastric antral sections using the ordinary light microscope. The Sydney system was used to assess the histological changes. In this system the topography of the gastritis as seen on endoscopy is included (antral, body, pangastritis), together with the macroscopic appearances (oedema,

haemorrhagic, flat, raised, etc.). In addition, some of the histological and microscopic features may be graded in terms of severity as mild, moderate or severe.

For bacterial culture detection, antral biopsy specimens were transported to the microbiology department within 1 hour in 5 mL tryptic soy broth as a transport medium. In the laboratory, specimens were first ground in a sterile mortar with the aid of a sterile fine glass rod until the formation of a homogenate. Two kinds of culture media—the classic Columbia agar and the modified Columbia urea agar (MCUA)—were used. MCUA contains per litre: 41 g Columbia agar base, 10 mL of haemin solution, 20 g of urea, 0.0012 mg of phenol red and 0.04 mg of vancomycin. Slants of this medium were prepared in 14 ×16 mm test tubes. Each slant tube was made up to contain 5 mL of MCUA.

Haemin solution was prepared by dissolving 50 mg of haemin in 1 mL of 1.0 mol NaOH. Distilled water was then added to make 100 mL solution which was then sterilized by autoclave at 121 °C for 15 minutes. The solution was stored at 4 °C and used as stock solution.

From the homogenate, 1 mL was taken and placed at the bottom of the MCUA slant tube so that the transport medium itself is the liquid phase. The tube was tilted a few times to allow the added broth homogenate to moisten the upper slanted portion of the tube before its settlement into the bottom of the slant. The resulting system is a simple monophasic–diphasic culture setup (MDCS); a diphasic solid liquid environment at the bottom of the test tube and a monophasic solid one above it [15]. At the same time some part of the homogenized biopsy specimen from each patient was spread and plated on standard Columbia agar medium.

With MCUA medium, the inoculated tubes were incubated microaerophilically at 37 °C for 24 hours, after which the colour changes from orange to pink in the solid phase, indicating urease activity, and the appearance of isolated *H. pylori* colonies was observed. With classic Columbia agar medium, the incubation time was extended from 5 to 7 days. After that time *H. pylori* growth was observed as a few, tiny transparent colonies. The isolated *H. pylori* colonies were then subcultured on plates of the same MCUA medium for purification, identification, and performing antibiotic susceptibility tests.

Identification of isolated *H. pylori* was confirmed by a negative reaction to Gram staining and by positive results of each of the following biochemical tests: oxidase test, catalase test and urease test, as indicated by colour change of the medium from orange to pink [16].

Results

Of the total 136 patients with positive endoscopic diagnosis of peptic ulcer, 92 patients (67.6%) showed positive evidence of *H. pylori* infection using bacterial culture and 81 patients (59.6%) histopathologically. They were referred to as *H. pylori* ulcer patients. In the remaining 44 patients (32.4%), called non-*H. pylori* ulcer patients, *H. pylori* were not detected.

Patient characteristics

Among *H. pylori* ulcer patients, the highest detection rates of the bacterium (79.2%) was recorded in the age group 41–50 years, while no single case was recorded in the age group ≤ 20 years (Table 1). These findings were statistically nonsignificant ($\chi^2 = 0.733$, $P > 0.05$, df = 4).

Of the 72 males with peptic ulcer, 49 (68.1%) showed positive tests for *H. pylori*, while 43 (67.2%) out of 64 females in this study showed positive tests for *H. pylori* (Table 1).

The highest occurrence of *H. pylori* was recorded in patients of low educational status. The bacterium was detected in 74.1% (40 out of 54) of these peptic ulcer patients with low educational level compared with 58.3% (21 out of 36) with high educational level but this was not statistically significant ($\chi^2 = 2.447$, $P > 0.05$, df = 2).

A higher rate of *H. pylori* was recorded in patients from rural areas. In 68.5% (50 out of 73) of patients living in rural areas *H. pylori* was detected compared with 66.7% (42 of 63 ulcer patients) in urban areas; again these results were statistically not significant ($\chi^2 = 0.04$, $P > 0.05$, df = 1).

The habit of cigarette smoking was reported by 55.1% of all ulcer cases. Among cigarette smokers 55 out of 75 (73.3%) had *H. pylori* infection compared with only 60.7% (37 out of 61) of nonsmoker peptic ulcer patients, a statistically non-significant finding.

Histopathology findings

For histopathological detection of *H. pylori*, the distribution of *H. pylori* was examined in both H&E-stained and Giemsa-stained sections. The changes in background mucosa included inflammation, atrophy and intestinal metaplasia. The degree of inflammatory changes in the gastric biopsy specimens were evaluated in every *H. pylori* ulcer patient. Table 2 shows that the most frequent pattern of inflammatory changes of antral biopsy specimens from patients with *H. pylori* ulcers was severe gastritis, recorded in 41 patients (44.6%), followed by mild gastritis in 31 patients (33.7%) and chronic gastritis in 20 patients (21.7%).

Culture findings

The total isolation rate of *H. pylori* was about 44.1% by the classic Columbia agar, as it identified 60 positive infections out of 136 patients tested. The growth of *H. pylori* on this medium was scanty, the colonies were few in number, transparent and tiny in size. Bacterial contamination of the medium was frequent. The contaminant bacteria were *Pseudomonas* spp., *Proteus* spp. and *Klebsiella* spp. The growth rate of *H. pylori* on classic Columbia agar was slow; in most cases 5 to 7 days were needed for the colonies to appear.

The MCUA using MDCS showed a greater isolation rate of *H. pylori*. Posi-

Table 1 **Distribution of patients with *Helicobacter pylori* and non-*H. pylori* ulcer by age group and sex**

Variable	No. of patients	*H. pylori* ulcer		Non-*H. pylori* ulcer	
		No.	%	No.	%
Age (years)					
≤ 20	2	0	0	2	4.5
21–30	27	17	63.0	10	37.0
31–40	31	23	74.2	8	18.2
41–50	24	19	79.2	5	20.8
51–60	27	18	66.7	9	33.3
61–69	25	15	60.0	10	40.0
Sex					
Male	72	49	68.1	23	31.9
Female	64	43	67.2	21	32.8
Total	136	92	67.6	44	32.4

Table 2 **Histopathological grading of inflammatory changes in *Helicobacter pylori* patients**

Degree of inflammation	*H. pylori* ulcer	
	No.	%
Mild gastritis	31	33.7
Severe gastritis	41	44.6
Chronic gastritis	20	21.7
Total	92	100.0

tive results were found in 92 out of 136 ulcer patients (67.6%). The colonies of the isolated bacteria were abundant in number, creamy in colour and larger in size, about the size of a pinhead, when compared with the colonies on the classic Columbia agar. There was no contamination at all. The isolation was very rapid; only 24 hours were needed for the growth to be identified. The change in colour of the slanted medium from orange to pink caused by urease action occurred at the same time, giving additional evidence for the presence of *H .pylori* in the tissue specimens.

All *H. pylori* isolates were subjected to Gram staining. The characteristics of this bacterium were observed as Gram-negative, spiral shaped rods.

The 3 additional biochemical tests (oxidase, catalase and urease) performed to confirm the identity of *H. pylori* were positive for all 92 isolates.

Discussion

The discovery of *H. pylori* by Warren and Marshall in 1983 has changed the conventional concept of gastroduodenal ulcer disease [*1*]. Studies over the years suggest a high correlation between *H. pylori* infection and peptic ulceration [*17*]. The prevalence of *H. pylori* infection shows a wide geographical variation [*18,19*]. It is reported that 60% to 70% of patients with gastric ulcer, and 90% to 95% of patients with duodenal ulcer, have marked gastric colonization of *H. pylori* [20].

Although it cannot be said that *H. pylori* causes ulceration, as half of the healthy population also harbours this organism, it has been shown that infection certainly makes the occurrence of ulcers more likely [*20,21*]. Also, eliminating this bacterium reduces the rate of ulcer recurrence to less than 25% [*5*].

In Iraq, several studies have been conducted to evaluate the prevalence of *H. pylori* infection in peptic ulcer disease indicating a range of 60%–70 % [*22–24*].

The major anatomical focus of this Gram-negative microaerophilic bacterium is the gastric antrum, the narrower lower part of the stomach, and also the duodenum when the normal type of duodenal epithelium is replaced with antral type mucosa. *Helicobacter* will infect this antral type of mucosa [*20,25*]. The results of our study also support this concept, since all positive cases for *H. pylori* were identified in antral biopsy specimens.

Inflammatory changes in antral biopsy specimens were found in all *H. pylori* ulcer patients in this study. These changes were mild gastritis in 33.7%, severe gastritis in 44.6%, and chronic gastritis in 21.7% of *H. pylori* ulcer patients. Identification of *H. pylori* as Gram-negative spiral-shaped organisms in stained antral biopsy specimens was possible in only 81 out of 92 *H. pylori* ulcer patients. The possible explanation for the remaining 11 cases (false-negative results) may be due to the patchy distribution of the organism in the antral part of stomach in peptic ulcer disease [*9*].

The gold standard for the presence of most infectious disease is successful culture of the microorganism [*8*]. At present, culture of *H. pylori* from gastric antral biopsy specimens is a reference technique in bacteriology and is essential for drug susceptibility testing and analysis of putative virulence factors [*12,26*]. Although it is usually considered a tedious, time-consuming and expensive procedure, culturing on solid medium is the standard technique used in most laboratories for the isolation of *H. pylori* from gastric biopsy specimens [*11*].

Primary isolation of *H. pylori* from a biopsy specimen is a difficult process, the typical success rates in culturing the organ-

ism are reported to be in the range of 70% to 80% with 90% to 95% sensitivity and 100% specificity [20]. Several factors, which are difficult to control, cause difficulty with the culturing of the organism: patchy distribution of the organism on the gastric mucosa, contamination of biopsy forceps, presence of oropharangyeal flora, loss of viability of the organism during transportation, etc. All these may be responsible for a poor negative predictive value associated with culture of *H. pylori* [4,27].

In the present study the isolation rate of *H. pylori* by bacterial culture method in 136 peptic ulcer patients was 67.6%. This rate is comparable to the above mentioned international results.

A variety of media, selective and non-selective, or a combination of both, have been proposed for use in the primary isolation of *H. pylori*, but the optimal method of recovery still remains to be established [28]. Columbia blood agar is a non-selective medium used for many years alone or in combination with other non-selective and selective media for culturing *H. pylori* from antral biopsy specimens taken from peptic ulcer patients during upper gastrointestinal endoscopy [11,29]. The isolation rate of *H. pylori* using this medium alone is very variable. Results as low as only 28.5% total isolation rate were reported by some authors [30]. The isolation rate of *H. pylori* using Columbia blood agar in the present study was 44.1%; the colonies were few in number and tiny in size. Bacterial contamination of the medium was frequent. The contaminant bacteria were *Pseudomonas* spp., *Proteus* spp. and *Klebsiella* spp ., the source of which could be contaminated biopsy forceps and contamination during obtaining, transporting and preparing of the defibrinated sheep blood added to the classic Columbia agar. The growth rate of *H. pylori* on this medium was slow, as 5

to 7 days were needed for the colonies to appear.

Efforts have been invested to improve the reliability of Columbia agar and change it to become selective for *H. pylori*. Westblom et al. in 1991 described egg yolk emulsion agar (EYE) [10]. When this medium was compared with other media, the colony count for EYE agar was significantly higher. However, the results of using EYE agar alone as a selective medium for culturing *H. pylori* in gastric biopsy specimens in peptic ulcer are controversial. While the maximum isolation rate was less than 51% in one study, another study stated that adding egg yolk emulsion to the culture medium was significantly worse than the use of whole blood [31].

Our efforts to establish an optimal method for the recovery of *H. pylori* from antral biopsy specimens in peptic ulcer patients is based on a modification of the Columbia agar medium to make it selective for culturing *H. pylori*. Many reports also mentioned that the use of selective and non-selective media in parallel is superior to the use of one medium alone [10,11,30,31]. By comparing these results of *H. pylori* isolation rates with our results, it is clearly evident that the 67.6% *H. pylori* isolation rate obtained in the present study is reasonably high. Using MCUA is much superior to using a combination of different selective and non-selective media in parallel to culture *H. pylori*. The high *H. pylori* isolation rate with MCUA may be attributed to the use of haemin as a sole iron source, which proved to be superior to other iron sources such as whole blood or serum. Also the clean procedure of preparing and adding haemin solution to the modified medium resulted in a total absence of bacterial contamination, thus removing the antagonistic effect of co-plated contaminants.

Conclusions

The results obtained with this slanted MCUA medium were encouraging as they showed great advantages over many selective media used alone or in combination for isolating *H. pylori*. The advantages of such modifications over Columbia agar medium may be summarized as follows:

1. High isolation rate of *H. pylori*, 67.6 %.

2. Rapid culturing of *H. pylori* as growth of *H. pylori* is very clear within 24 hours of incubation instead of 5–7 days needed with classic Columbia agar. This could be ascribed to the better accessibility of iron from haemin as compared to whole blood or erythrocytes. Also, the lack of contaminant microbes may enhance faster growth by eliminating the effect of competition.

3. Colony count is significantly higher, and colonies are very clear and relatively large in size.

4. Microbial contamination of the medium is totally absent.

5. Incorporation of urease test in the medium provides clear evidence for the presence of *H. pylori* in the tissue specimen. It is indicated by colour change in the medium, from orange to pink, due to the splitting effect of urease enzyme on the urea component of the modified medium.

6. Using MDCS provides several advantages over other culture techniques, exemplified by the fast appearance of results, elimination of transport medium and establishment of simultaneous but separate environments for both the isolation and identification of *H. pylori*. It also provides flexibility in the kinds of media used in the liquid and solid phases. Consequently, the method is inexpensive, and less time-consuming.

We conclude that preparing this single selective medium (MCUA) is clearly simpler, less time-consuming, and much more economic than preparing and working with multiple culture media in parallel.

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Piloting health and climate adaptation: GEF project

WHO is partnering with the United Nations Development Programme (UNDP) in a new project to pilot approaches to protect health under a changing and more variable climate, funded by the Global Environment Facility (GEF). The goal of the project is to implement a range of strategies, policies, and measures that will decrease health vulnerability to current climate variability and future climate change in a range of countries with different health risks. This is the first global project that works directly with developing countries to design and implement practical measures to protect health under a rapidly changing climate.

The project is working in seven countries distributed throughout the world showing a wide variety of health vulnerabilities to climatic conditions. These include Jordan, a water-stressed area, from the Eastern Mediterranean Region. More details on the GEF project can be found at: http://www.who.int/globalchange/climate/gefproject/en/index.html.