DETECTION THE OCCURRENCE OF *HELICOBACTER PYLORI* AMONG DYSPEPTIC PATIENT AND THEIR SUSCEPTIBILITY TO ANTIBIOTIC AGENTS

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Abstract

Fifty dyspeptic patient include 31 female and 19 male were subjected to esophageal gastroduodenoscopy and gastric biopsy and gastric biopsy specimens were taken from antrum and body were applied for microbiological analysis which include : urease test bacterial culture using selective and non selective media and histological examination for detecting *H. pylori*, and studying their susceptibility to various to antimicrobial agents.

Results showed that occurrence of *H.pyloria* was (100%) among gastric ulcer patient,71% among duodenal ulcer and these with normal endoscopic appearance, as well as, (70%) of gastric patient were found to be infected with *H.pylori*.

Biopsy urease test were proved to be most sensitive method for detecting *H.pylori* infection when 76% gave positive result for presence of *H.pyloria* using it, followed by histological examination when 70% of gastric tissues showed the existence of *H.pylori* organisms.

Results also showed that using selective media was batter than not selective media for primary isolation of bacteria, with a recovery rate 61% using brain heart infusion agar and 39% was recovery rate for non selective blood agar.

When isolates of *H.pylori* were subjected to the sensitivity test against (8) antibiotic groups, result, showed that ciprofloxacin was the most effective antibiotic against the isolates when the sensitivity percentage 90%pencilin G,tetracycline, amoxicillin and metronidazal on other hand, were the last

Effective antibiotic antibiotics when bacterial sensitivity range between 30% and 90%.

Introduction

H. pylori is a spiral, Gram negative bacterium which inhibits the stomach of more than (50%) of human, although the new organism was only cultured in 1982.

1. Its manifestations have been reported in the scientific literature for over 100 years *H.pylori* is a typical curved rod-like or short spiral (up to three turns) motile, even in the highly viscous mucous layer in which they live.

The organism has up to 7 sheathed flagella attached to one pole which allow for motility through propelling itself with a rapid corkscrew like movement that allows it to penetrate the mucosal gel layer overlaying the gastric epithelium curved, gently spiral bacteria or S-shaped. While, on cultivation true spiral forms may be few or absent and the organism appears mostly as bacilli or slightly curved.

2. *H.pylori* has repeatedly been shown to be associated with chronic superficial gastritis (CSG) which involves the antrum and the fundus of the stomach.
3. Combinations of bismuth, tetracycline, metronidazole were acquired for adequate eradication of the organism.
4. There for a breakthrough came when Unge and colleagues, 1989, in Sweden observed that the action of amoxicillin was greatly enhanced when gastric acid was suppressed with a proton pump inhibitor, notably omeprazole. Thus, since 1996, *H.pylori* has been able to be treated relatively easily with a 7 days therapy of omperazole (to render the gastric pH neutral) in combination with two antibiotics, usually amoxicillin and clarithromycin. Omeprazole, clarthromycin.

Primary treatment failure occurs in (15%) of patients treated with antibiotics combined with antisecrectory drug. Poor compliance with antibiotic regimens and antibiotic resistance in *H.pylori*.

5. Contribute to treatment failures. Recently, it has rapidly acquired resistance to some antibiotics and this event might also account for clinical failure. Also misuse of antibiotics that would increase selection of antibiotics resistant *H.pylori* and negatively affect the ecology of gastric microflora.
6. The aim of this Study is to isolate and characterization of *H.pylori* organism from dyspeptic patients. Histological examination of *H. pylori* from gastric tissue sections and studying their susceptibility to antimicrobial agents.

Materials and Methods

Samples Collection and treatment

Patient Groups:

50 patients with various gastrointestinal symptoms representing different age groups from both sexes were under want samples were obtained from Endoscopy Department of Al-Kadhmia Hospital in Baghdad. Informed written consent was obtained in advance from each patient.

Collection of Gastric Biopsy Specimens

Patients were advised to fast for overnight before endoscopy. Endoscopies performed under local anesthesia (xylocaine) 7. The endoscopy was disinfected with (2%) glutaraldehyde (cidex) before and after each procedure 8, 9. Biopsy forceps were washed with water and disinfected with glutaraldehyde (cidex) for (10min), then washed with distilled water before each procedure. During upper gastrointestinal endoscopy, four gastric biopsy specimens were taken (3-4)cm, two from each of corpus (body) and antrum region of the stomach 10. Standard pinch biopsy forceps were used. One biopsy from each region was fixed in 10% formal buffer saline for histological investigation and the other was used for bacteriological investigation. Biopsy specimens were transported to the laboratory in 0.5ml Brain - heart infusion broth with ice and kept at 4°C for no longer than 4hr before processing.

Laboratory Treatment:

The biopsy samples were minced and homogenized between the frosted ends of sterile microscope sides in a sterile petridishes near benzene burner, then subjected for the following tests:

1-Biopsy Urease Test:

The first minced biopsy sample was inoculated on urea agar slant containing phenol red (as an indicator) and incubated at (37°C). Slants were examined for color change from yellow to pink before and after (1hr) and after (24hr). The test was not finally declared as negative till 24hr 11, 12.

2-Biopsy Culturing:

The second minced biopsy was inoculated in Brain-heart infusion broth and on each of selective media (Columbia, Brain - heart infusion) agar media plates and non selective media (Blood) agar media plates that was used for primary isolation of *H.pylori*. The cultures were incubated at 37°C under microaerophilic conditions in an anaerobic jar with a gas generating kit. Plates were examined for positive growth for intervals of 3-5 days for the selective media and 7 days for the nonselective media before discarding as negative. For positive growth, the colonies must be tiny, glistering, translucent or gray and covered with entire edges.

Gram Staining:

Dry heat fixed smears were taken from colonies and placed on microscopic glass slide to examine the morphology of bacteria.

3-Biochemical Tests Of *H.pylori*:

A- Urease Test Of Colonies:

Grown colonies were picked from the agar plate with a sterile loop and then inoculated on urea agar slant. Positive result was detected by changing color from yellow to pink within few minutes.

B-Catalase Test :

One drop of hydrogen peroxide 3% was added to part of the grown isolated colonies which was picked up from the agar with a woody stick on the surface of a sterile slide. Production of gas bubbles within 20-30 seconds from *H.pylori* growth on the slide indicated a positive reaction.

C- Oxidase Test:

A few drops of freshly made oxidase reagent 1% were added on a strip of filter paper and then an isolated colony was rubbed by using a strike woody sticks. A positive

reaction is indicated by an intense deep purple color appearing within 5-10 seconds.

D- Growth at 25 °C and 45 °C:

Plates of Brain - heart infusion agar were inoculated with *H.pylori*, then incubated at 25°Cand 45°C for 24-48 hr. Positive results were obtained by the appearance of *H.pylori* growth.

E- Susceptibility Test for Nalidixic Acid and Cephalothin:

Isolates were inoculated in sterile brain-heart infusion broth then incubated at 37°C for 24-48 hr then, 0.1ml of this inoculum was spreaded on Muller-Hinton agar supplemented with 5% horse blood and then with a sterile forceps, cephalothin and nalidixic acid disks were placed on the surface of inoculated plate and incubated at 37°C for 24hr under microaerophilic conditions.

4- Histological Examinations:

Histological examination was diagnosed under supervision of the histopathologist. The biopsy specimen which was fixed in 10% formalin solution was washed by tap water for few minutes and left in ethanol 50% for 30min while 70% ethanol was used to keep the specimen for a long time. The specimen was transferred to 2.5% absolute ethanol + 75% butanol and left for 2hr . Paraffin wax sectioned in 4μm thickness to be easier to use, then specimen was stained with Giemsa and hematoxyline - eosin stain.

Hematoxylin - Eosin Method (13):

Histological sections were placed in the following solutions and reagents as follows:

- Xylol for 5 min.
- Absolute alcohol for 1 min.
- A graded series of ethanol (80%, 70%, 50% and 35%) to be dried then rinsed in distilled water for 1 min.
- Iodine for 1 min.
- Sodium-hydrosulphate to erase the iodine and turn the color of tissue to white and rinsed in tap water for few min.
- Hematoxyline for (1-5) min then rinsed in tap water to get rid of the excess dye.
- Acid alcohol, till the color turned to pink then rinsed in tap water.
- Na- bicarbonate till the color turned to blue, then rinsed in tap water.
- Eosin for 5min, then rinsed in tap water.
- Crystal violet dye for 2 min.
- Lugol - iodine dye for 3 min and left to dry for few min.
- Xylol for 3 min, then mounted in Canada balsam.

5- Antibiotic Susceptibility Test (14):

Five milliters of brain heart infusion broth supplemented with 5% horse or human sera were inoculated by *H.pylori* isolates, then incubated at 37°C for 24 hr. A liquid of 0.1ml of the inoculated broth was transferred and spreader by sterile cotton swab on Muller-Hinton agar plates supplemented with 5% horse blood in three different planes (by rotating the plate approximately 60° each time to obtain an even distribution of the inoculums). The inoculated plates were then placed at room temperature for (10-15) min to allow absorption of excess moisture. With a sterile forceps, the selected antibiotic disks were placed on the inoculated plates and incubated at 37°C under microaerophilic conditions for 48 hr. After incubation, the diameters of inhibition zones were measured by a ruler (mm). Results were determined and compared according to the National Committee for Clinical Laboratory Standards 15.

Results and Discussion

1-Paitionts Study Design

Fifty patients with dyspepsia included 31 female and 19 male, aging between 19-70 years and mean age 45. They were underwent for diagnostic upper gastrointestinal endoscopy at endoscopy department in Al-Kadhmya hospital in Baghdad, Iraq .Several gastric biopsy specimens were taken from antrum and body. Urase production, histological &cultural examination tests were used to detect the presence of *H. pylori* among gastric biopsy samples.

2- Detection the Occurrence of *H. pylori* Among Dyspeptic Patient

As shown in Table (1), gastric ulcer shows highest percentage of infection 100% when all dyspeptic patients were found to be infected with *H. pylori*, followed by duodenal ulcer and those with normal endoscopic appearance 71% when 5 out of 7 patient showed existence of H. pylori. As well as 70% of gastritis patients were found to be infected when 7 out of 10 patient gave positive result to it, and in 62% of gastric erosion also 66% of duodenal patients had *H. pylori* in 5 out of 8 patient and in 6 out of 9 patients, respectively.
While non of gastric cancer patient showed the existence of *H.pylori.*

Table (1)
Occurrence of H. p*ylori* among dyspeptic patients.

Endoscopic Diagnosis	No. of Cases	Infection cases no.	%
Duodenitis	9	6	66
Gastric erosion	8	5	62
Duodenal ulcer	7	5	71
Gastritis	10	7	70
Gastric cancer	2	----	---
Peptic ulcer	4	2	50
Gastric ulcer	3	3	100
Healthy	7	5	71
Total	50	33	

3-Laboratory Treatment of Gastric Biopsy Specimens

1- Biopsy Urease test

Preliminary studies were conducted in 50 patients to evaluate the relationship between biopsy urease activity and the presence of *H. pylori* as shown in Table (2), there was a very good correlation between the presence of *H. pylori* as detected in antrum and body sites of stomach and positive gastric urease test

In this test out of 50 dyspeptic patients, 38 (76%) gave positive.

Result for presence of *H. pylori* using biopsy urease test during upper gastrointestinal endoscopy, four gastric biopsy specimens (two for each of antrum and body) were applied to diagnose the presence of *H. pylori* infection using biopsy urease test.

Table (2)
Biopsy urease test result at different periods.

Gastric biopsy	Location site	Within 30 min	After 1 hr	After 24 hrs	Total
Infected	Antrum	6	5	15	26
No infected		2	1	4	7
Infected	Body	3	2	2	12
No infected		-	3	2	5

In the antrum site as shown in Table (2) 6 of gastric biopsy specimens gave positive result, for presence of *H. pylori* within 30 min, 5 showed a color change for urease production after 1 hr, and 15 of cases were positive after 24 hr. While in the body site, 3 of specimens showed positive result after 30 min. 2 of specimens were able to produce color change after 1 hr and 7 of gastric biopsy specimens were positive after 24 hr it has been shown that doubling the amount of tissue in the urease hasten the positive result by approximately 1.5 to 2 hrs 11.

2- Histological Examination of Biopsy Specimen

The advantage of histological diagnostic is through confirmation of active infection as well as evaluation of mucous for presence of potential associated pathogenic state acute, chronic gastritis, atrophy, metaplasia, dyspepsia, gastric lymphoma and malignancy (16).

In this test out of (50) dyspeptic patients, 30 (70%) gave positive result for presence of *H. pylori* using hematoxylin–eosin stain of gastric tissues, as shown in Fig. (1).

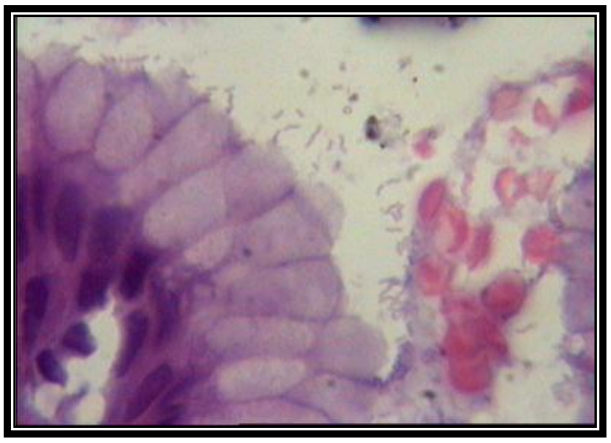

Fig.(1) : *Helicobacter pylori* of Gastritis Antral Biobsy Speciemen Stained by H and E Stains(100xs).

Table (3) shows chronic gastritis and chronic atrophy gastritis were found in 36% (13) and in 24% (12) of the dyspeptic patient followed by gastric lymphoma and gastric Aden-carcinoma with 10% (5) and 4% (2) ,respectively *H. pylori* was detected in 12 of 13 cases with normal tissue section appearance 92% , in 15 out of 18 cases with chronic gastritis 83% , 8 out of 12 with chronic atrophy gastritis (66%) and in 1 out of 5 cases with gastric lymphoma 20%.

Table (3)
Histological diagnosis of dyspeptic patient in association with *H. pylori* infection.

Histological Diagnosis	Occurrence		Infection with H.pylori	
	No.	%	No.	%
Chronic gastric	18	36	15	38
Chronic atrophy gastric	12	24	8	66
Gastric lymphoma	5	10	1	20
Gastric adenocarcinoma	2	4	----	---
Healthy	13	26	12	92
Total	50		35	

Normal gastric biopsy could be associated with *H. pylori* infection. In this study 12 (92%) normal gastric biopsies out of 13 cases revealed the presence of *H. pylori* it was found that 6.3% of normal biopsy were infected with *H. pylori* organisms 17, and found its presence in 14% of normal biopsy 18 .

These observations could be also attributed to recent infection, or patchy involvement, pathogenicity might be present in gastric region other that invaded by bacteria.

Culturing of *H. pylori*:

Although various methods have been developed for detecting *H.pylori* infection, bacterial culture remains extremely important. Isolation of *H.pylori* enables susceptibility testing, which predicts the like hood of eradication. *H.pylori* is fastidious organism so various factors, including bacterial density, transport conditions, culture medium and microaerophilic condition, directly influence the yield of culture19.A variety of media, selective and nonselective, or a combination of both has been proposed for use in the primary isolation of *H. pylori*. A total of 200 gastric mucosal biopsy specimens were obtained (two pieces) from each of antrum and body of 50 dyspeptic patients undergoing endoscope, by using selective and nonselective media. *H.pylori* organisms were detected in 31patient, yielding as isolation rate of 62%. Brain-heart infusion agar was the most sensitive culture media for isolation of *H.pylori* among dyspeptic patients with (recovery rate = 81%) when 25 of them were positive to it followed by Columbia agar 64.5% when 20 of out 31 *H.pylori* isolates recovered from it. While blood agar gave lowest rate when 12 of isolates 39% were recovered using.

Practical contamination is difficult to avoid and, therefore, a suitable selective media with antibiotics are usually essential to detect *H.pylori* 20.

It was recorded that nonselective media yielded the lowest rate because of the abundant growth of contaminants (especially *Proteus* spp., *Pseudomonas aerugenosa*, *Strptococcus* spp. and *Candida* spp.). That obscured the growth of *H.pylori*. So growth of *H.pylori* could not be detected on the plate in presence of a high number of contaminants 21.

Identification of *H. pylori* Isolates:

Culture examination of *H.pylori* appeared colonies after (3-5) days of plating on selective columbia and Brain heart infusion agar and

between (7-10) days on non selective media blood agar used under microaerophilic conditions at 37°C. The colonies were tiny glistering, translucent convex with entire edges as shown in Fig. (2).

Fig. (2) : Colonies of *H. pylori* on Brain - Heart Infusion Agar.

When part of a suspected colony grown on Brain-heart infusion agar was smeared on a microscopical slide and stained by Gram staining technique, cells appeared as slightly curved or straight rods to curved bacilli with Gram - negative reaction. Extended cultivation to (10-15) days changed morphology of the bacteria from a helical form to a coccoid one.

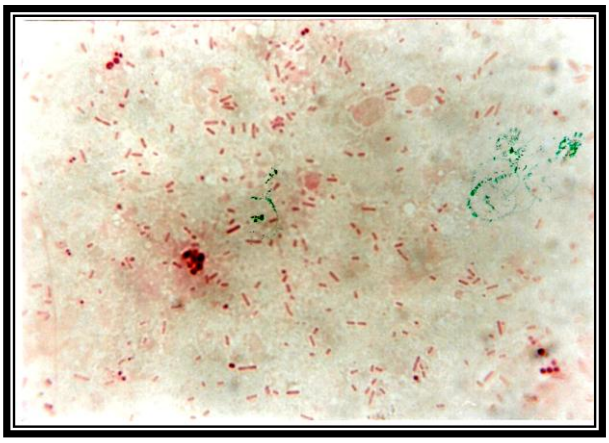

Fig.(3) : Gram Stain of *H. pylori* after 5 Days Culturing on Brain - Heart Infusion Agar.

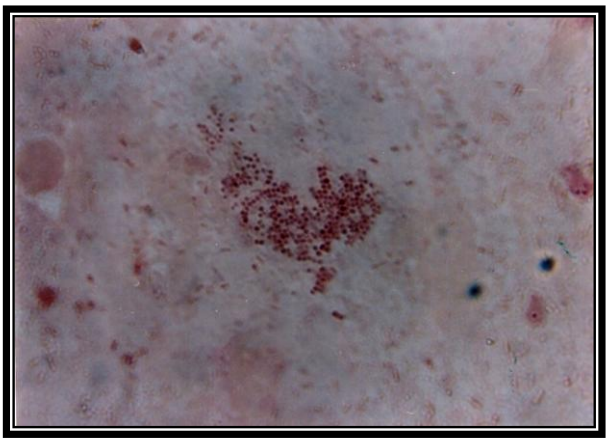

Fig. (4): Gram Stain of *H. pylori* after 15 Days Culturing on Brain - Heart Infusion Agar (100xs).

Furthermore, morphology of *H.pylori* which is observed in gastric biopsies may differ markedly from that observed in a Gram stained preparation of organisms. It usually appeared as a slightly curved or rods straight whereas stained tissue biopsy specimens usually reveal a helical or more curved appearance (22).

Isolates of *H.pylori* were also catalase positive through air bubbles production and oxidase positive through color changing from violet to dark violet on the filter paper of suspected isolates. Moreover, good growth was obtained at 37°C incubation while no growth for *H.pylori* isolates was observed, at 25°C and 45°C incubation temperatures.

Results of susceptibility test to nalidixic acid and cephalothin indicated that approximately all isolates of *H.pylori* showed resistance to nalidixic acid but susceptible to cephalothin.

Antibiotic Sensitivity of *H. pylori*:

Standard disk diffusion method was used to determine the antibiotic resistance pattern of rapid urease producing *H.pylori* isolates against 8 different antibiotics, as shown in Table (4). Generally, a vast of resistance was detected among *H.pylori* isolates against the antibiotic used. Among them, no single antibiotic was resisted by all the isolates of *H.pylori* or sensitive to them.

It was found that the more effective antibiotic against *H.pylori* isolates was ciprofloxacin (from quinolons groups) when 7 isolates were sensitive to it, while only 3 isolates were resistant.

Table (4)
Antibiotic susceptibility of *H.pylori* rapid urease producer isolated determine diameter of inhibition zone (mm).

Isolate	AMX 20 meg	CRO 30 meg	Crr 15 meg	Mt 5 meg	PG 10 meg	TE 30 meg	E 30 meg	Cip 0 meg
HP1	R	R	S	S	R	R	S	S
HP2	S	R	R	S	R	R	R	S
HP3	R	R	S	R	R	R	S	R
HP4	S	R	R	R	R	S	S	S
HP5	R	R	R	R	R	R	S	R
HP6	R	R	S	R	S	R	R	S
HP7	S	S	S	S	R	S	S	S
HP8	S	R	S	R	R	S	S	S
HP9	R	S	R	S	R	R	R	R
HP10	R	R	S	R	R	R	R	S

1-AmX=Amoxicillin. 2-CRO=Cefrixime.
3-Crr=Clarithromycin.
4- Mt=Metronidazol. 5-PG=Pencilin G.
6-TE=Tetracycline.
7-E=Eryethromycin. 8-Cip=Ciprofloxacin.

Followed by erythromycin and clarithromycin (of macrolide groups) when 4 isolates were resistant and the other 6 isolates were sensitive to them. Adversely, the less effective antibiotics were pencillin G (from beta lactam group) when all (except one) isolates were resistant to them. Followed by cefrixome with only 2 isolates sensitive, while the other remaining 8 isolates were resistant, and tetracycline when only 3 isolates were sensitive to it while others were resistant. Followed by metronidazole (nitrometadazole group) and amoxicillin when only four isolates were sensitive to them while the remaining 6 isolates were resistan.

Development of antibiotic resistance may be explained by different mechanisms, it may either involve a modification in DNA gyrase, the target enzyme of quinolones, or due to a modification in the bacterial outer membrane proteins, rendering the drug unable to penetrate inside the bacteria.

It was found that in patients heavily infected with *H.pylori* ($\geq 10^9$ colonies at some sites of infection), spontaneous mutation may be selected for few resistant mutants which would subsequently replace the susceptible population of bacteria. This selection of resistant organism may be further facilitated by low concentration of drug in some areas of the colonized stomach reduced antibacterial activity in the presence of a low pH 23.

It was discovered that differences in susceptibility were found in isolates obtained from both corpus and antrum patients, the discrepancies may be due to co - infection in an individual with two different strains of distinct linage 24.

Frequency of resistance *H.pylori* isolates was also estimated in this study as shown in Fig.(5).

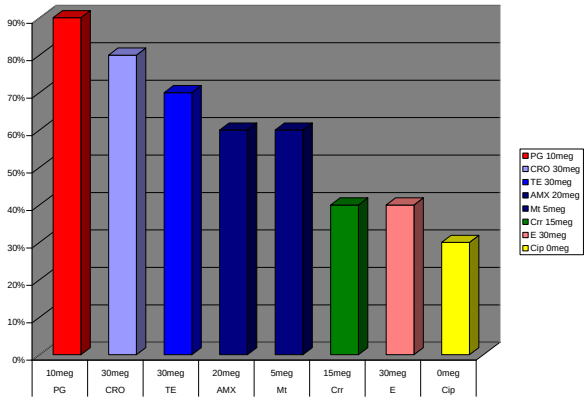

Fig. (5) : The frequency of resistance *H.pylori* isolates.

Ciprofloxacin has the lowest percentage (30%). Followed by erythromycin and clarithromycin 40% On the other hand, *H.pylori* isolates showed high percentage of resistant to ß-lactam antibiotics used 90% of penicillin G by, followed Cefrixome when 80% of them were resistant to it, and 70% of isolates were resistant to Tetracyclin. Additionally 60% of the isolates were resistant to metronidazol and Amoxicallin this could be explain by increasing colonization of the stomach with a mouth or intestinal flora that led to transfer antibiotic resistance- encoding plasmids to *H.pylori* from other bacteria 25. Also resistance of ß-lactam antibiotics used may be due to possessing of ß-lactamase by the isolate which may be encoded by transferable plasmids and found in various *Enterobacteraceae* members such as *E.coli*, *Proteus mirabilis, Klebsiella pneumonia* and *Salmonella typhimurium* 19 and 26. Or could be due to apparent of tolerant strains which have been identified by 27 who found that amoxicillin tolerance was detected among *H.pylori* strains after rescuer of amoxicillin resistance using gradient p.

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الخلاصة

50 شخصا يعانون من عسر الهضم من ضمنهم 31 أمراة و 19 ذكر اذ اخضع جميعهم الى التنظير المعدي العفجي وتم أستئصال الخزعات النسيجية من غار وجسم المعدة لأجراء الاختبارات المايكروبايولوجية عليها والتي شملت اختبار انتاج انزيم اليوريز، الزرع البكتيري بأستخدامأوساط زرعيه مختلفة استخدام الوسط الانتقائي وغير الانتقائي، والاختبار النسيجي للكشف عن بكتريا*Helicobacter pylori* بأستخدام صبغتي الهيماتوكسلين والايوسين و كمزوإقيست مدى مقومتها لعدد من المضادات الحيوية.

أظهرت النتائج ان نسبة تواجد بكتريا *H.pylori* في المرضى (100%) من الذين يعانون من عسر الهضم ، وكانت هناك علاقة بين الخمج ببكتريا *H. pylori* تقرح المعدة (83%) ومع قرحة الاثنى عشر (81%) علما أنه التنظير المعدي العفجيكان النتيجة طبيعية.

أظهرت النتائج ايضا ان استخدام الوسط الانتقائي المحوركان افضل من الوسط الغير الأنتقائي للعزل الاولي للبكتريا اذ بلغت نسبة الكشف بأستخدام أغار نقيع الدماغ القلب الانتقائي(61%) و(31%) عند أستخدام أغار الدم غير الانتقائي.

عند اخضاع بكتريا *H.pylori* لإختبار الحساسية اتجاه (8) مضاداً حياتياً، أظهرت النتائج ان السبروفلوكساين كان اكثر المضادات تأثيراًبينما كانت مقاومة للبنسلين (90%)، ايرثرومايسين، كالروثرومانسين ، ميترانازول والاموكسلين تيتراسيكلين كلوكسلين.من (40%)الى (80%) على التوالي.