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BACTERIOLOGICALAND MOLECULAR DETECTION OF *H. PYLORI* IN ENDOSCOPIC BIOPSIES FROM DYSPEPTIC PATIENTS RECRUITED TO A GOVERNMENTAL HOSPITAL AT CAIRO, EGYPT

By

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ABSTRACT

Helicobacter pylori (*H. pylori*) has been incriminated as the causative agent of most cases of gastric problems in human. So far, there is no one test agreed upon as a gold standard for the diagnosis of *H. pylori* infection. In the present study, 250 gastric biopsies were collected from male and female dyspeptic patients of different ages recruited to the Gastroenterology Unit at Ahmed Maher Hospital, a big governmental hospital located at the west of Cairo metropolitan, the capital of Egypt, from November 2019 to December 2020. Samples were subjected to bacteriological studies to isolate and identify *H. pylori*. Fifty isolates were identified as *Helicobacter spp.* according to morphological and biochemical bases. The biochemical behavior was variable among different isolates. When a specific semi nested PCR assay was applied on DNA extracted from the helicobacter isolates, only 5 were identified as *H. pylori* while the rest was considered non-*H. Pylori* helicobacter where many of them have been isolated from human gastric samples in previous studies. Therefore, a reliable diagnosis is crucial for better eradication therapy of helicobacter-originated infections in human and animals. Results of more than one test are required to elucidate the real causative agents of human gastritis and to detect animal carriers of helicobacter.

Key words:

Helicobacter pylori, human gastritis, PCR, biochemical tests, bacterial cultivation.

INTRODUCTION

Stomach is unfavorable to most microorganisms owing to the antibacterial effect of gastric acidity. However, a highly specialized group of bacteria has evolved to inhabit the gastric mucosa. Of those bacteria is Helicobacter that exerts many mechanisms by which it can change gastric physiology **(Neiger and Simpson, 2000; Pessione, 2012)**.

Helicobacter, a Gram-negative, spiral and microaerophilic bacterium, is present in the stomach of approximately 50% of the world's human population. Helicobacter is known to play a significant role in chronic gastritis, peptic ulcer, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma pathogenesis **(Blasser and Parsonnet, 1994)**.

Through urease secretion, motility and ability of adhesion to the gastric epithelium, Helicobacter can survive in the gastric tissue **(Sabbagh *et al.*, 2019)**. Urease enzyme breaks down urea to carbon dioxide and ammonia that neutralize the gastric acidity leading to penetration of and colonization in the gastric tissue **(Thakkar *et al.*, 2016)**.

Helicobacter infection represents a major public health issue worldwide so that a systematic review in 2015 indicated that about 4.4 billion individuals worldwide were confirmed to be Helicobacter positive **(Hooi *et al.*, 2017)**.

Different species of Helicobacter have been isolated from human namely, *H. pylori*, *Helicobacter cinaedi*, *Helicobacter fennelliae*, *Helicobacter heilmannii (formerly known as Gastrospirillum hominis), Helicobacter westmeadii, Helicobacter canes, Helicobacter sues, Helicobacter canadensis sp. Nov., Helicobacter pullorum, and Helicobacter rapine (formerly known as Flexispira rappini* **(Tille, 2017)**.

H. pylori has been the most prevalent species blamed for the human gastric disorders. Although the prevalence of *H. pylori* in the western world is decreasing, gastric colonization by *H. pylori* remains widespread in the developing world. Hence, a better understanding of *H. pylori* persistence and pathogenesis is thus mandatory to aid in the development of novel intervention and prevention strategies. Diagnostic methods for detecting helicobacter are classified into invasive (endoscopy) and non-invasive methods such as culture, histopathology, urea breath test, serology, stool antigen detection and molecular techniques **(Hirschl and Makristathis, 2007)**.

Endoscopy has a limitation in accurate diagnosis as the gastric redness; swelling or nodular changes are not specific enough for diagnosis of Helicobacter infection **(Lan *et al.*, 2012)**. Histology is still recommended for the direct detection of Helicobacter infection but its accuracy is affected by several factors such as site, size and number of biopsies and staining methods **(Malfertheiner *et al.*, 2012)**.

Peptide nucleic acid fluorescent in situ hybridization (PNA-FISH) has been found a rapid, accurate, cost-effective method for detection of Helicobacter clarithromycin resistant strains **(Cerqueira *et al.*, 2013)**. However, PNA-FISH technique has disadvantages such as difficult

preparation, fluorescent microscope requirement and special experience to read the slides **(Lan *et al.*, 2012)**.

Urea breath test (UBT) has been used as noninvasive method for diagnosis for almost 30 years. The urease activity of Helicobacter gives this test many advantages such as simplicity, noninvasiveness and safety especially in pediatric infections. However, patient, bacteria and the test itself are factors that may affect the results of the UBT **(Guarner *et al.*, 2010; McColl, 2010)**.Culturing of helicobacter from gastric biopsy specimen is highly specific (~100%) but less sensitive (85%-95%) method due to the delicate and fastidious nature of Helicobacter. The recovery of helicobacter from clinical specimens needs a specific transport and growth media as well as incubation environment **(Park *et al.*, 2011)**. Unfortunately, poor specimen quality, delayed transportation, exposure to aerobic environment and inexperienced microbiologist represent negative impact factors on helicobacter isolation **(Dip *et al.*, 2003)**.

As there is no single accurate sensitive and specific test for Helicobacter detection, it is recommended to combine two or more diagnostic methods of to meet diagnostic criteria for helicobacter **(Rami's *et al.*, 2012)**.

In this study, bacteriological investigation as well as polymerase chain reaction were conducted to detect helicobacter in biopsies collected from suspected human cases.

MATERIAL AND METHODS

Clinical samples:

Gastric biopsies were collected from 250 dyspeptic patients recruited to the Gastroenterology Unit at Ahmed Maher Hospital, Cairo from November 2019 to December 2020. Patients included 98 males and 152 females aging between 21 to 63 years (Table 1).

The gastric biopsies were collected, through endoscopy, into 15 ml falcon sterile tubes containing sterile normal saline and transported to the laboratory within one hour **(Coelho and Zaterka, 2005)**.

Bacterial isolation and identification:

Upon arrival to the laboratory,each sample was gently homogenized for 2 minutes followed bystreakingonto Columbia blood agar base supplemented with 7%sheep blood, campylobacter enrichment supplement and helicobacter selective supplement. The inoculated plates were incubated at 37ºC for 10 days in microaerophilic conditions (5-15% O2 and 10% CO2) using Campy pack and anaerobic jar **(Mégraud and Lehours, 2007)**.

The grown colonies were examined morphologically using Gram's staining method. The suspected colonies were sub-cultured under similar conditions for purification.

Pure isolates were tested biochemically employing oxidase, catalase, nitrate reduction, and urea hydrolysis tests. Also, suspected helicobacter isolates were tested for motility.

The flowchart depicted in Fig. (1) was followed to identify the suspected helicobacter isolates.

Biochemical identification

Biochemical tests methodology and interpretation were carried as described by **Thirunavukkarasu *et al.* (2017)** as follows.

Urease test:

The hydrolysis of urea was detected by streaking Christensen's urea agar slants with fresh bacterial cultures followed by incubation for two days at 37ºC. The color if changed to pink it was considered a positive result.

Catalase test:

A few drops of 3% H_2O_2 were added to a bit of bacterial colonies on a clean glass slide. A positive result was indicated by effervescence of the peroxide solution.

The oxidase test

A loopful of young bacterial growth was streaked on a filter paper impregnated with 1% aqueous tetraethyl-p-phenylene diamine-dihydrochloride (TMPPD). Formation of purple colour within 1 minute indicated oxidase production.

The nitrate reduction test:

Colonies at the log phase of growth were picked up using a loop and inoculated into nitrate broth and incubated at room temperature for 2-3 days. One milliliter of the bacterial broth suspension was transferred to a clean test tube and 3 drops of sulfanilic acid solution followed by 3 drop of α-naphthylamine were added and the tube was left to settle for 5 minutes. Development of red color indicated nitrate reduction test positive.

Motility test:

A loopful of bacterial colonies was transferred to rubella liquid medium and incubated at 37°C for 48 hours with shaking at 200 rpm. A drop of the suspension was tested for motility using the hanging drop method.

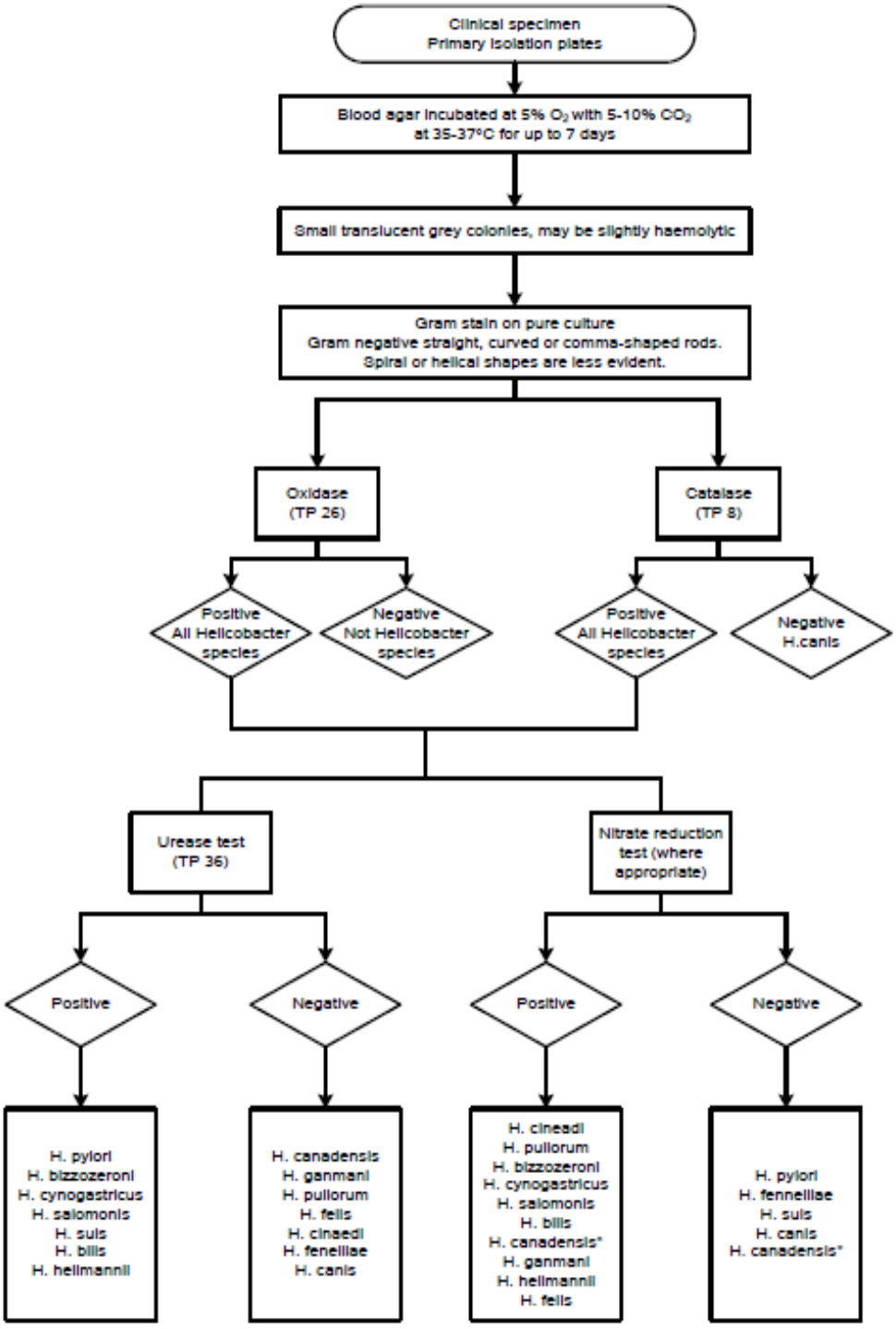

Fig. (1): A flowchart for the phenotypic identification of *Helicobacter species*. Secured from UK Standards for Microbiology Investigations: Identification of *Helicobacter* species. https: // www. gov. uk/uk-standards-for-microbiology-investigations-smi-quality-and-consistency-in-clinical-laboratories.

Seminested PCR for the identification of suspected *H. pylori* isolates:

DNAwasextracted from bacterial colonies of isolates identified biochemically as Helicobacter. Extraction of the bacterial DNA was performed using a commercial DNA extraction kit (Thermo fisher, USA) as described by the kit supplier. DNA concentration and purity were determined spectrophotometrically by measuring the absorbance at 260/280 nm.

Detection of the 16sRNA gene of *H. pylori* was done using an adapted semi nested PCR assay **(Qin *et al.*, 2016)**. For the outer amplification, the forward primer sequence was 5'-CTGGCGGCGTGCCTAATAC-3' and the reverse primer sequence was 5'-CTCACGACACGAGCT GAC-3' which produces 1024 bp amplicons. The inner (Target) amplification utilized the same forward primer of the outer PCR while the reverse primer sequence was 5'-ACCCTCTCAGGCC GGATACC-3'. The inner PCR was targeting a 250 bp product (specific for *H. pylori*). PCR products were visualized by electrophoresis on 1.5% agarose gel containing ethidium bromide. Both agarose preparation and electrophoresis were performed using 1x TAE buffer.

RESULTS AND DISCUSSION

Several methods have been adopted for the detection of *H. pylori* since its discovery in 1983. Each method has been associated with advantages and disadvantages **(Laine *et al.*, 1997)**.

Histology, culture, rapid urease test and polymerase chain reaction (PCR) are the tests of choice on astral biopsies collected by endoscopy. Histology has been proposed to be very sensitive and specific but the question is how diagnostician can claim that, the curved bacterium is exclusively *H. pylori*. Rapid urease test (RUT) has been poor in sensitivity especially with low bacterial density **(Patel *et al.*, 2014)**.

In this study, out of 250 gastric biopsies examined, only 50 specimens (20%) resulted in bacterial growth under the conditions specified in the methodology. All isolates were Gram negative bacilli of which 10 isolates were positive for catalase, oxidase, nitrate reduction and urea hydrolysis (20% of the isolates and 4% of the examined samples).

The suspected helicobacter colonies were translucent and about 1-2 mm in diameter.

On Gram staining, the bacterial cells showed up as Gram negative, non-spore forming, motile, helical, curved, with rounded ends and spiral periodicity. The isolates were found highly motile with the hanging drop method. Cultural and biochemical characteristics of helicobacter isolates were in accordance with those described by **Thirunavukkarasu *et al.* (2017).**

Based on the cultural and biochemical characteristics, the 50 recovered isolates were considered to be helicobacter. Of the fifty isolates, 34 and 16 were recovered from female and male patients, respectively and no correlation was detected between the isolation of helicobacter and either age or sex of the patients (Table 1). From the table, it is noticed that the number of females was more than double of the male number with gastric complaints which may indicate the stressful life under which females are struggling in contrast to men who are more adaptable and resistant to the encountered obstacles at Cairo. Consequently, more isolates were obtained from female-originated samples.

Table (1): Helicobacter isolates from dyspeptic patients according to phenotypic characters in relation to age and gender of the targets

Patient gender	Number of samples	Helicobacter isolates	Age mean in years
Male	**98**	**16**	**37**
Female*	**152**	**34**	**45**
Total	**250**	**50**	**42**

***Females with gastric problems were almost 3 times the number of males subjected to endoscopy at Ahmed Maher Hospital (the place of sample collection).**

Concerning the biochemical behavior, all isolates were catalase positive (100%), 45 were oxidase positive (90%), 30 were able to reduce nitrate (60%) and only 10 resulted in urea hydrolysis (20%). Looking at the results of the four biochemical tests, only 10 isolates were positive for the urea hydrolysis test and only 5 of them were negative for the nitrate reduction test (Table 2).

Table (2): Number of bacterial isolates that showed positive results with the four employed biochemical tests

Patient gender	Catalase	Oxidase	Urea hydrolysis	Nitrate Reduction
Male*	**16**	**15**	**3**	**10**
Female*	**34**	**30**	**7**	**20**
Total	**50**	**45**	**10**	**30**

*** No significant correlation was found between patient sex and the biochemical behavior of the isolates.**

Considering isolation of *H. pylori*, it is required only to characterize the phenotypic criteria of the bacteria such as growth requirements, antibiotic susceptibility, and virulence to develop vaccines. Meanwhile, isolation of *H. pylori* has got several disadvantages such as special transportation and media, incubation conditions and duration which counterpart the speed needed to process the specimens. Moreover, majority of the microbiological laboratories are not well-equipped and trained to isolate such fastidious bacterium **(Mégraud *et al.*, 2016).**

The low isolation rates of *H. pylori* in the current study may be attributed to many factors. Therapeutic approach using proton pump inhibitors (PPIs) can indirectly interfere with *H. pylori* distribution in the stomach. Unfortunately, no data were available whether the patients targeted in this study were administered PPIs or not **(Megraud *et al.*, 1991)**. To avoid false negative results, it was recommended not to consume PPIs two weeks prior to endoscopy as done with the rapid urease. Also, it is advisable to collect multiple biopsy specimens from the same patient**(Megraud andLehours, 2007)**.In previous studies, antibiotic supplements (vancomycin, 10 mg/l, amphotericin B, 10 mg/l and cefsulodin or trimethoprim, 5 mg/l) were recommended for selective media to facilitate primary isolation of *H. pylori*, this was not done in the current study.

As referred to Koch's postulates, culture is still considered to be the most specific way to confirm *H. pylori* infection. However, the sensitivity of isolation of the bacterium has varied greatly among laboratories due to the fastidious nature of the bacteria **(Deltenre *et al.*, 1989; Goodwin and Armstrong, 1990; Loffeld *et al.*, 1993; Feldman and Evans, 1995; van Zwet *et al.*, 1996; Glupczynski, 1998)**. It was also reported that even the experienced diagnosticians can recover the organism in the ranges of 50% to 70% only from actually infected biopsies **(Loffeld *et al.*, 1991; Grove *et al.*, 1998)**. Hence, it is worthy to announce that bacteriological culture is a tedious, time-consuming procedure and should not be the choice for the routine diagnosis of *H. pylori* infection **(Mégraud, 1997)**. Meanwhile, cultures are urgently needed under certain circumstances such as testing of the antibiotic sensitivity of *H. pylori*, better understanding of the pathogen-host interaction, vaccine development as well as phenotypic and genotypic characterization for better understanding of the epidemiologic features of the pathogen **(Cantorna, 1990; Valentine *et al.*, 1991; Lee, 1998)**.

Therefore, PCR methods remained to be the choice to detect *H. pylori* DNA in the clinical specimens. However, there are still speculations for false positivity of the PCR as non-pylori Helicobacter can be misdirected due to genetic sharing. Also, false negativity may be

encountered due to low bacterial counts as well as presence of PCR inhibitors. Fortunately, designing primers extremely specific to *H. pylori* and targeting at least more than one conserved genes may make PCR a gold standard test. Nested PCR can take care of false negatives by countering the inhibitors and low bacterial counts. Therefore, nested PCR based methods, if performed properly, may be proposed as gold standard tests **(Panel *et al.*, 2014).**

The semi nested PCR assay, performed in this study, resulted in the amplification of *H. pylori* specific product (250 bp) from only 5 isolates of the fifty helicobacter isolates identified on cultural and biochemical bases. Of the five PCR positive samples, 4 and 1 were originated from females and males, respectively (Table 3), Fig. (2).

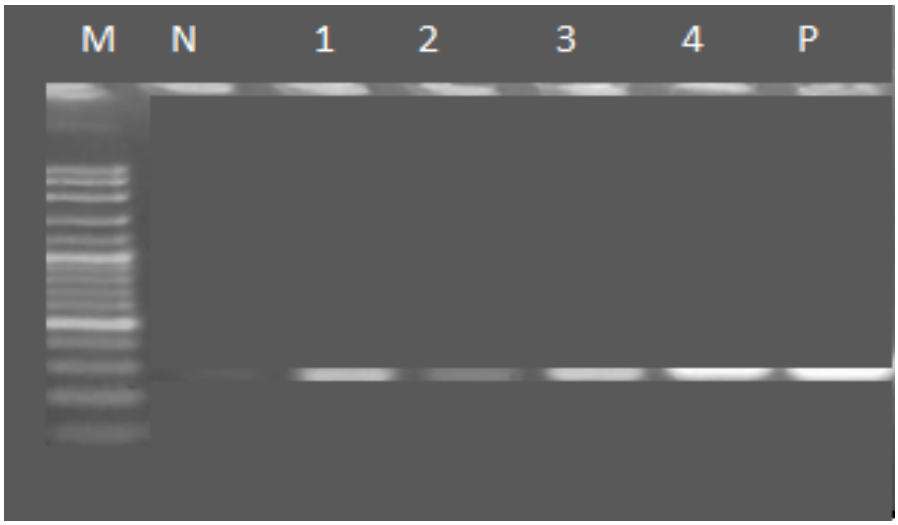

Fig. (2): Agarose gel electrophoresis of the inner program products of the semi nested PCR using *H. pylori* 16s RNA-specific primers. Lanes (M: DNA 100 bp size marker, N: negative control (no template), 1-4: positive *H. pylori* isolates (250 bp product) and P: positive control (*H. pylori* DNA).

Table (3): *Helicobacter pylori* isolates identified by semi nested PCR in relation to the patient gender

Patient gender	Number of *H. pylori* isolates*	Helicobacter isolates other than *H. pylori*
Male	**1**	**15**
Female	**4**	**30**
Total	**5**	**45**

*** Interpretation was based on the results of inner run of the nested PCR.**

When compared with the traditional methods, PCR showed that only 10% of the Helicobacter isolates were *H.pylori*.Considering all the collected samples (n=250),only 2% were confirmed as *H. pylori* by PCR (n=5). However, 12 isolates resulted in the production of the suspected size (1054 bp) after the outer PCR amplification Fig. (3).

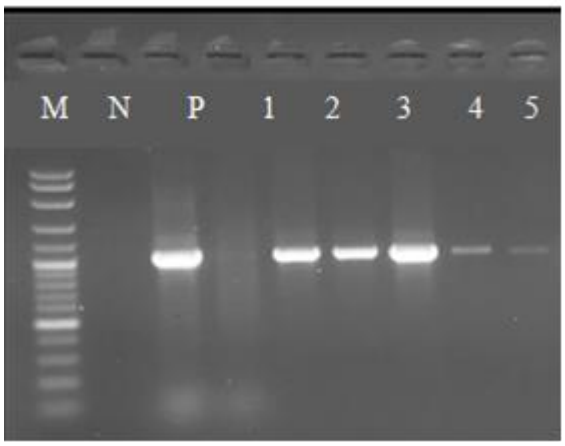

Fig. (3): Agarose gel electrophoresis of the outer run of semi nested PCR using *H. pylori*-16S RNA specific primers. Lanes (M: 1000 bp DNA size marker, N: negative control (no template), P: positive control (*H. pylori* DNA), 1: a negative result and lanes 2-6: positive 1054 bp products.

From the literature, the conserved genes used for detection of *H. pylori* are urease operon (*urea*) and *gym*, also known as *ureic***(Clayton *et al.*, 1991; De Reus *et al.*, 1997)**, the *16S rRNA, 23S rRNA* **(Hoshina *et al.*, 1990; Ho *et al.*, 1991; Maeda *et al.*, 1998; Rimbara *et al.*, 2013)** and *Hsp60* **(Singh *et al.*, 2008)** genes. Of the above mentioned genes, the *16s rRNA* gene of *H. pylori* was targeted in this study. Unfortunately, only 5 isolates were confirmed to be *H. pylori* after sequencing of the semi nested PCR products. Many speculations can explain the obtained molecular identification carried out in the present investigation. First of all, the highly conserved *16S rRNA* gene in bacteria exhibits sequences which are shared by different species of Helicobacter. For example, a 109 bp amplicon, specific to *H. pylori 16S rRNA*, has also been amplified in human tissues, thus compromising its relevant diagnosis of *H. pylori* **(Chong *et al.*, 1996)**.There are two major pitfalls in the performance of PCR: Presence of polymerase inhibitors in the biological samples and the possibility of contamination during the process of collection of specimens. Special kits are commercially available and have been found able to counteract contaminations **(Wang *et al.*, 1993; and Son *et al.*, 1999).**

Also, the gel documentation of PCR products is another source of contamination. Therefore, different separated chambers should be used along with separate micropipettes and change of gloves at every step wherever, chances of contamination, is highly recommended. Specificity of the PCR based detection of *H. pylori* is another issue. It has been proposed that any specimen may be designated positive for *H. pylori* when there are amplifications of two different conserved target sequences **(Cirak *et al.*, 2003; Rocha *et al.*, 2005)**.

As compared to our results, a nested PCR was carried out by **Mishra *et al.* (2008)** where 62.5% patients were positive for *H. pylori* targeting *Hsp60* gene. This suggested the *Hsp60* gene to be good alternative as a non-invasive method for detecting *H. pylori* infection in fecal specimens. Conclusively, the choice of diagnostic tests to diagnose *H. pylori* infection depends on the sensitivity, specificity, reproducibility, cost and rapidity of the test **(Mégraud, 1996)**. There is an urgent need for a reference method to be used as "gold standard" to truly-infected patients. Unfortunately, none of the currently used methods is able to achieve this objective. Therefore, it is recommended to carry out two or more assays in parallel or in cascades and compare the obtained results.

The sensitivity of nested or semi nested PCR can be superior to other PCR assays. However, the researcher must consider the possibility of crossover contamination and detection of DNA from dead bacteria.

The low positivity rate of the culture method performed in this study may be due to a low bacterial burden, presence of non-cultivable coccid forms **(Andersen *et al.*, 2000)**, absence of microorganisms in the gastric biopsies, loss of viability during transport, fastidious growth requirements or contamination by other bacteria suppressing the growth of *H. pylori* or antibiotic intake. Therefore, it is preferred to compare culture results with results of other diagnostic methods on the same samples. Also, false positive nested PCR results may not be true false positives, rather the results obtained by the relatively less sensitive culture method may be false negatives **(Saez *et al.*, 2012)**.

Employing either invasive or non-invasive test is almost equally important depending upon the given clinical situation. Due to poor sensitivity or specificity, none of the available diagnostic approaches can be considered as gold standard for detection of *H. pylori* infection. However, combinations of more than one test usually give the quite satisfactory diagnosis **(Patel *et al.*, 2014).**

Ethical issue:

This study was approved by the Research Ethics Committee process number (HAM00116) Informed consent was obtained from all patients.

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الكشف البكتريولوجي والجزيئي عن هيليكوباكتر بايلوري في بزلات منظارية لمرضى تخمة المعدة والمترددين على مستشفى حكومي بالقاهرة، مصر

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الملخص العربي

تعتبر بكتيريا هيليكوباكتر بايلوري مسبباً أساسياً لمشاكل المعدة في الإنسان وإلى الآن لم يتم الاتفاق على اختبار كمعيار ذهبي لتشخيص الإصابة بتلك البكتيريا. ولقد تم في هذه الدراسة تجميع 250 عينة بزل بالمنظار المعدي لمرضى ذكور وإناث من أعمار مختلفة يعانون من مشاكل بالمعدة والمترددين على وحدة الجهاز الهضمي بمستشفى أحمد ماهر الحكومية والتي تقع غرب القاهرة (في الفترة من نوفمبر 2019م إلى ديسمبر 2020م). وتم إجراء دراسات بكتريولوجية على العينات بغرض عزل بكتيريا هيليكوباكتر والتعرف عليها. وقد تم التعرف على 50 معزولة على أنها تنتمي لجنس هيليكوباكتر طبقا لنتائج الفحوصات الشكلية والبيوكيميائية. وعند إجراء اختبار تفاعل بوليميريز تلسلسلي نوعي من النوع شبه العشي تبين أن 5 عزلات من نوع هيليكوباكتر بايلوري بينما تم اعتبار باقي العزلات هيليكوباكتر غير بايلوري حيث تم في دراسات سابقة عزل بعضها من حالات آدمية تعاني من مشاكل بالمعدة. وعليه فإنه من الضروري اعتماد طريقة تشخيص معينة والاتفاق عليها للتخلص الجاد وتحديد العلاج الشافي من إصابات المعدة بتلك البكتيريا في الإنسان والحيوان. ويفضل الجمع بين نتائج أكثر من اختبار على نفس العينة لإدماغ الدليل على سبب الإصابة الآدمية وكذلك الكشف عن بكبتربا هيليكوباكتر في الحيوانات.

كلمات دالة:

هيليكوباكتر بايلوري، بي سي آر، التشخيص البيوكيميائي، الزرع البكتيري.