Identification of Helicobacter pylori in the Gallbladder of Egyptian Patients with Chronic Calculous Cholecystitis

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ABSTRACT
The relationship between the H. pylori infection and cholelithiasis still remains controversial. Some authors reported strong positive correlation while, others indicated totally negative results. Most of the studies carried out so far were mainly molecular and histopathological studies and failed to establish association between the colonization of H. pylori in the biliary tract and stone diseases. On these bases the present study was carried out to investigate the presence of H. pylori in the gallbladder (GB) tissues, bile samples and gallstones of Egyptian patients with chronic calculous cholecystitis by microbiological and molecular methods, also, we tried to postulate on the probable etiological association of H. pylori with the disease. Our data showed that H. pylori was detected in 11.7% of GB mucosa and 12% of bile samples by microscopic examination and was successfully isolated from 11.7% and 10% of gallbladder tissues and bile respectively. Moreover, H. pylori DNA was detected by RT-PCR in 28.3%, 26% and 3.3% of gallbladder tissues, bile and stones respectively. Our results support a cause-and-effect association between H. pylori infection of the gallbladder and cholelithiasis.

Key words: H. pylori- cholelithiasis - gallbladder - bile - chronic calculous cholecystitis

INTRODUCTION

Helicobacter pylori (H. pylori) represent one of the most common and medically prominent infections worldwide. At least half of the world’s population are infected by H. pylori, making it the most widespread infection in the world\(^{[1,2]}\). The prevalence of infection is different worldwide, depending on the socioeconomic status and sanitation conditions; being fewer than 40% in the developed countries and more than 80% in developing countries\(^{[3,4]}\). The problem is more complicated in Egypt as the prevalence of the organism exceeds universal rates reaching 91.7%\(^{[5]}\).

H. pylori is implicated in the pathogenesis of gastric and duodenal ulcer\(^{[6]}\) and has been proposed as a risk factor for gastric cancer development\(^{[7,8]}\). Helicobacter pylori infection has also been associated with various extragastric diseases, including lesions of the gallbladder\(^{[9,10,11,12]}\). Since Kawaguchi et al.\(^{[13]}\) first reported the presence of bacteria resembling H. pylori in the gallbladder's mucosa of a patient with calculous cholecystitis and suggested a contribution between infection and gallstone formation. Following this initial report, H. pylori has been identified in the bile\(^{[14,15,16,17]}\), the liver\(^{[18]}\) and the biliary epithelium of humans\(^{[19,20,21,22,23,24,25,26,27]}\) and its presence has been proposed as related to several hepatobiliary diseases ranging from chronic cholecystitis and primary sclerosing cholangitis to gall-bladder carcinoma and primary hepatic carcinomas\(^{[12,26,28]}\). The relationship between the H. pylori infection and cholelithiasis still remains controversial. Some authors reported strong positive correlation while, others indicated totally negative results\(^{[9,10,19,27,28,29,30,31,32]}\). Most of the studies carried out so far were mainly molecular and histopathological studies and failed to establish association between the colonization of H. pylori in the biliary tract and stone diseases. On these bases the present study was carried out to investigate the presence of H. pylori in the GB tissues, bile samples and gallstones of Egyptian patients with chronic calculous cholecystitis by microbiological and molecular methods, also, we tried to postulate on the probable etiological association of H. pylori with the disease.

PATIENTS & METHODS
From March to September 2011 a study was carried out on patients who were referred to Sayed Galal University Hospital, Bab El Shaareya, Cairo, Egypt for cholecystectomy. A total of 60 subjects (24 male and 36 female) with a diagnosis of chronic calculous cholecystitis were enrolled in this study. Their age range was 28 to 63 years for males and 38...
to 55 years for females. Patients were evaluated by routine ultrasonography and admitted to hospital 24 hours before procedures. No antibiotics were prescribed before surgery. Patients with acute cholecystitis and those who had used antibiotics 2-4 weeks prior to cholecystectomy were excluded from the study.

**Specimens processing:**

Resected gallbladders were taken under sterile conditions and immersed in thioglycolate broth in tightly closed sterile containers, kept at 4°C and were transported to the microbiology laboratory for processing.

Under aseptic conditions the gallbladders were punctured and 2 ml bile samples were aspirated and collected in sterile vials. Bile samples were used for microscopic examination, culture and about 0.5 ml was immediately frozen at -20°C for further molecular analysis. The gall bladders were opened in sterile Petri dishes and small pieces of mucosal tissues were cut using sterile blades and were efficiently ground using sterile glass mortar and pestle and used for microscopic examination, culture and PCR as described before (33). As for the stones; the nucleus of each gallstone was crushed using sterile motor and pestle and a part was collected in a sterile vial and used for microscopic examination and culture. Small portions were also stored at -20°C for PCR.

**Classification of gallstones** (34):

Gallstones were classified according to visual appearance or color. The stones were separated into two groups: (1) Black and brown stones were regarded as pigment stones, and (2) yellow stones were regarded as cholesterol stones.

**Microscopic examination:**

The gallbladder mucosa, bile samples smears were prepared and fixed with 90% methanol and were stained with LMB as described by Misra et al. (35) and were examined microscopically for detection of H. pylori like bacilli.

**Microbiological culture and identification of H. pylori** (36-38):

GB tissue specimens, bile samples and crushed stones were enriched in brain heart infusion broth (Oxoid) sublimed with 10% horse serum and Dent’s supplement containing trimethoprim, vancomycin, Cefsulodin and polymyxin B (39). All plates were incubated at 37°C in an anaerobic jar containing Campy Gen Gas generating sachet (Oxoid) for 3-10 days. Plates were checked every other day for growth. If the plates require re-incubation a new Campy Gen sachet was used. Growths were identified as H. pylori based on their colonial morphology on selective Dent’s medium and Belo Horizonte medium supplemented with 2, 3, 5-triphenyltetrazolium chloride indicator media (40), cellular morphology observed by microscopic examination of Gram stained smears using basic fuchsin as a counter stain and biochemical identification by oxidase, catalase, and urease tests (41,42).

**Real time PCR amplification:**

H. pylori DNA was extracted from prepared gall bladder mucosa, bile and stone samples using the QIAamp DNA Mini Kit (QIAGEN, Germany) automatically using Qiacube instrument (QIAGEN, Germany) according to manufacturer instructions. The extracted DNA was stored at -20°C until PCR amplification. Amplification of DNA was carried out using Fast SYBR® Green kits (Applied Biosystems, USA) which includes Fast SYBR® Green Master Mix and primers for Ure C gene (F, AAGCTT TTAGGGGTGTTAGGGGTT and R, AAGCTTACTTTCTAACACTAAAGC).

Standard H. pylori ATCC 43526 was used as positive control and RNAse/DNAse free is used instead of template as negative control. The cycling conditions were: 20 sec at 95°C, 40 cycle of 3 sec at 95°C, and 30 sec at 60°C. The amplicon is detected via amplification curve or dissociation curve (melting curve) using SDS software supplied with the real time PCR instrument. The crossing points (Cp), the cycles when the fluorescence of a given sample significantly exceeded baseline signal, were recorded and expressed as a function of the cycle number. Melting curve analysis was also performed to assess the specificity of the amplicon.

**RESULTS**

Out of 60 resected gall bladders of patients diagnosed with chronic calculous cholecystitis 35 cholesterol stones, 25 pigment stones, 60 GB tissue specimens and 50 bile samples were collected. Collection of bile samples was not possible from 10 Stone-filled gallbladders (table 1)
Table (1): Samples collected from resected gallbladders:

<table>
<thead>
<tr>
<th>Studied Samples</th>
<th>Number</th>
</tr>
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<tbody>
<tr>
<td>GB mucosa</td>
<td>60</td>
</tr>
<tr>
<td>Bile sample</td>
<td>50</td>
</tr>
<tr>
<td>Pigment stones</td>
<td>25</td>
</tr>
<tr>
<td>Cholesterol stones</td>
<td>35</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>170</strong></td>
</tr>
</tbody>
</table>

Microscopic detection of *H. pylori* like bacilli in GB mucosal specimens and bile:

Microscopic examination of LMB stained GB mucosal specimens, bile and crushed gallstone smears revealed about 3-4 um curved bacilli stained blue in 7 of GB mucosal specimens and 6 of the examined bile samples but were not detected in any of the examined stone samples. (figure 1)

Isolation and identification of *H. pylori* from GB tissues, bile samples & stones:

*H. pylori* was successfully isolated from 7 GB tissue specimens and 5 bile samples but not from any of the cultured stone samples. Resultant colonies showed typical, circular, entire, convex, smooth and gray colonies on selective Dent’s medium and when these colonies were streaked on Belo Horizonte medium glossy colonies with golden pigmentation were produced after microaerophilic incubation at 37°C for 3 days confirming the identity of *H. pylori* (figure 2). Microscopic examination of suspected *H. pylori* isolates after Gram’s stain and by images by transmission electron microscope are presented in figures (3) and (4). Also oxidase, catalase, and urease tests were positive for the tested isolates.

Figure (1): LMB staining showing *H. pylori* curved bacilli in stained GB mucosa (a) and in bile samples (b) and (c)

Figure (2): Growth Characteristics on Belo-Horizonte indicator medium: typical glossy colonies with golden pigmentation caused by reduction of tetrzolium chloride in Belo-Horizonte indicator medium after microaerophilic incubation at 37°C for 3 days confirming the identity of *H. pylori*. 
Molecular detection of *H. pylori* in GB tissues, bile samples and stones:

Molecular analysis of GB tissues, bile samples and stones showed that bacterial DNA was found in 10 (16.6%) of 60 gallstone samples. RT-PCR assay was run on DNA extracted from the 10 gallstones with positive bacterial DNA only 2 samples showed positive PCR results for *H. pylori* Ure C gene, 1 was cholesterol stone (yellow) and the other was pigmented mixed cholesterol stone (orange and yellow orange). Bacterial DNA was also detected in 38 (76%) of 50 bile samples and 13 of these 38 samples were positive for *H. pylori* by RT-PCR. Also, bacterial DNA was detected in 47 (78.3%) of the 60 GB tissue specimens and 17 of the 47 samples were positive for *H. pylori* by RT-PCR. Positive results by real time PCR are illustrated in figure (5).

In summary, our data showed that *H. pylori* was detected in 11.7% of GB mucosa and in 12% of bile samples by microscopic examination of LMB stained smears and was successfully isolated from 11.7% and 10% of gallbladder tissues and bile samples respectively. Moreover, *H. pylori* DNA was detected by RT-PCR in 28.3%, 26% and 3.3% of gallbladder tissues, bile and stones respectively. The Incidence of *H. pylori* in GB tissue, bile and gallstones samples by different methods applied in the study is summarized in table (2).
Table (2): Detection of H. pylori in GB mucosa, bile and gallstones samples by microscopic examination, culture and RT-PCR

<table>
<thead>
<tr>
<th>Type of specimens (N)</th>
<th>Microscopic examination N (%)</th>
<th>Microbiological culture N (%)</th>
<th>RT-PCR N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GB mucosa (n = 60)</td>
<td>7 (11.7%)</td>
<td>7 (11.7%)</td>
<td>17 (28.3%)</td>
</tr>
<tr>
<td>Bile (n = 50)</td>
<td>6 (12%)</td>
<td>5 (10%)</td>
<td>13 (26%)</td>
</tr>
<tr>
<td>Stones (n = 60)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>2 (3.3%)</td>
</tr>
</tbody>
</table>

DISCUSSION

In this study H. pylori bacilli were demonstrated in 11.7% of GB mucosa and 12% of bile samples by microscopic examination of LMB stained smears and it was successfully isolated from 11.7% and 10% of gallbladder tissues and bile samples respectively. Moreover, molecular investigation by RT-PCR showed that the incidence of H. pylori was 28.3%, 26% and 3.3% in gallbladder tissues, bile and stones samples respectively (table 2). These results were consistent with the findings of a recent Egyptian study conducted by Ghazal et al. in which H. pylori DNA was detected by nested PCR in the gallbladder tissues and bile of 28% and 18% respectively of patients with gallstone disease. On the other hand, our results were contradictory to the results of Karagin et al. who detected H. pylori in only 1% of 100 GB tissue specimens of cholelithiasis patients in Sewed and to the results of another two study, one was carried out in Turkey by Bostanoglu et al. while the other was conducted in Canada by Fallone et al. In the later two studies the DNA of the Helicobacter genus was not detected in the bile or GB tissues of patients with biliary diseases. Although regional variations might be considerable, it has to be mentioned that DNA from H. pylori was consistently detected in biliary specimens only when a sensitive PCR method was used. Results of the later studies are also in disagreement with others that have identified Helicobacter DNA in the bile or gall-bladder tissue of patients with biliary diseases in the same geographic regions.

In the current study the incidence of H. pylori in either GB tissues or bile samples was higher than that was reported in several other studies from different countries such as Turkey, Pakistan, Korea and Greece. On the other hand, it was lower than the incidence reported in other populations such as Serbians, Italians, Brazilians, Japanese and Ukrainians. The latter studies, following different investigational approaches, concluded that the incidence of H. pylori in the biliary tree is as high as 60%. This broad variation in the colonization rate of the biliary system cannot be explained only by the differences in the H. pylori seroprevalence among different populations but also due to methodological and experimental variability and differences in sensitivity and specificity of the applied molecular techniques used for the detection of H. pylori.

Our results showed that many of the tested GB tissue and pile samples that were negative for H. pylori by culture were positive by RT-PCR. A possible explanation for this is that H. pylori exist in two forms, an actively dividing spiral form and a coccoid form. The coccoid was first identified in the human stomach in 1993 by Chan et al. Growing evidence supports the concept that the coccoid form of H. pylori is not simply a degenerate morphological manifestation but is alive and metabolically active, although difficult to detect by bacterial culture but can be detected by PCR. Additionally, RT-PCR using primers designed for amplification of ureC gene has a high specificity and sensitivity for detection of H. pylori in clinical samples.

Although that, the human biliary system is thought to be sterile this sterility can be broken under certain conditions. H. pylori, which is probably the most common chronic bacterial infection of humankind may colonize the biliary tract and it has been implicated as a possible cause of hepatobiliary diseases. Several investigators proposed that colonization of the mucosa of the gallbladder by H. pylori is a potential risk factor for gallstone formation. The background theory is that colonization of the mucosa by H. pylori, as is the case with other bacteria, may cause chronic inflammation, impairing the acid secretion, reducing the solubility of calcium salts in the bile and increasing the risk of their precipitation in the lumen of the gallbladder, thus favoring gallstone formation. In this study although H. pylori was detected in only 3.3% of stone samples by RT-PCR yet it was detected in the
gallbladder tissues and bile samples by microbiological and molecular methods suggesting that presence of *H. pylori* in the gallbladder may act as a lithogenic component in the context of gallstone formation.

The relationship between the *H. pylori* infection and cholelithiasis is controversial. Some studies have supported a cause-and-effect in the context of gallstone formation. Nevertheless, further studies that include healthy control subjects, patients with biliary diseases that also harbor *H. pylori* in their stomachs, isolation of the bacterium from both locations, and molecular analysis of the isolated strains are required in order to establish a significant correlation.

**REFERENCES**


العثور على الجرثومة الحزازونية في الحويصتات المرارية للمرضى المصريين المصابين بالتهاب المرارة العصي العصبي

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تظل العلاقة بين العدوى بالجرثومة الحزازونية والتهاب الحويصتات المرارية المصحوب بتكون الحصوات مثيرة للاهتمام. فقد اقر بعض الباحثين وجود علاقة قوية بينهما وكانت تجلي الاختلافات بينهما. وقد اعتمد على دراسات الدراسة التي أجريت حتى الآن على طرق معتمدة في دراسة الدرجة الجزيئية ودراسة دور الجزيئات الأخرى. ولن تكون علاقة بين استجابة الجرثومة الحزازونية للعوامل الحساسية وتكون الحصوات. ولنا على ما سبق فقط تم إجراء هذه الدراسة لبحث تواجد الجرثومة الحزازونية في الحويصتات المرارية. العصارية الصفرائية وحصوات المرارة بالمرضى المصريين المصابين بمرض التهاب المرارة الحزازوني بطرق ميكروبيولوجية جزيئية وذلك لما قد يكون في شأن دعم وجود علاقة سببية بين الجرثومة الحزازونية. وهذا المشروع أظهر النتائج وجود الجرثومة الحزازونية في أنواع المرارة التي تم فحصها بواسطة الميكروسكوب في 11.7% من الحويصات المرارية. 12% من عينات العصارية الصفرائية وتلك تمكننا من قياس الجرثومة بوزنها من الحويصبات المرارية. 12% من عينات العصارية الصفرائية. بالإضافة إلى ذلك تم الكشف عن وجود الحمض النووي للميكروكوب باستخدام تفاعل الانصهار المتسلسل في 18.3% و 21% و 3.2% من عينات الحويصبات المرارية. العصارية الصفرائية وحصوات المرارة تابعاً. ولنا على ما سبق فأن هذه النتائج من شأنها أن تمتلك عواقب قوية لوجود علاقة سببية بين العدوى بالجرثومة الحزازونية والتهاب الحويصتات المرارية المصحوب بتكون الحصوات.