Abstract. – OBJECTIVE: Gallstone disease is a common surgical ailment. Helicobacter pylori has a role in upper gastrointestinal disorders, including gallstones. This study aimed to determine the association of H. pylori with gallstones, so developing a preventative strategy for gallstone formations.

PATIENTS AND METHODS: A prospective study was conducted on 95 patients referred to the surgical clinic of Al-Meeqat General Hospital, Al-Madinah Al-Munawarah, Saudi Arabia, with gallstone disease. Detection of H. pylori antibodies (IgG) in serum was done in all the patients who underwent cholecystectomy. H. pylori stool antigen (HPSA) using stool samples was done for IgG sero-positive patients prior to the cholecystectomy. The bile collected from the gall bladder during operation was examined for the presence of H. pylori by Gram stain, culture and HPSA assay. Gallbladder mucosa was examined for urease A gene by polymerase chain reaction (PCR) in patients proven to be positive for stool or bile serology.

RESULTS: Of the 95 patients, 75 (79%) were positive for H. pylori antibodies. Twenty-six (34.7%) patients were positive with H. pylori antigens in bile and 21 (28%) with H. pylori antigens in the stool samples. Among these 47 patients, PCR was positive in 29 (62%) subjects. H. pylori couldn’t be detected among the studied patients by either Gram stain or culture.

CONCLUSIONS: The presence of H. pylori in bile may indicate a significant risk for cholelithiasis. PCR is a rapid reliable method for the detection of H. pylori DNA in bile. This rapid molecular approach together with culture and immunological methods could help clinicians to effectively manage patients at high risk of developing gallstones at an earlier stage.

Key Words: Helicobacter pylori, Cholecystitis, Gallbladder stones, Urease gene, PCR.

Introduction

Cholelithiasis is considered to be one of the most frequent surgical ailments that the general surgeons encounter during their clinical practice. Gallstones develop more frequently among fatty females under the age of fifty, after which they occur equally in both sexes. Once formed, gallstones may predispose to a wide variety of clinical conditions including acute cholecystitis, chronic cholecystitis, obstructive jaundice and acute pancreatitis that may lead to chronic pancreatitis. Symptoms of gallstone-related clinical conditions vary from nausea, vomiting and fatty dyspepsia to severe right hypochondrial and epigastric pain, jaundice, fever and shock. However being frequently seen, the diagnosis is not difficult and can be established by a variety of diagnostic tools including ultrasonography, CT scan, etc.
ERCP, liver function tests and pancreatic enzymes. Factors contributing to gallstone formation include a change in the cholesterol/bile salts ratio that may be secondary to hypercholesterolemia, chronic liver disease or ileal disease. Others include haemolytic anaemia or biliary infection. Interestingly, a significant number of patients develop primary common bile duct stones after cholecystectomy (more than two years post-surgery), which may pose a diagnostic challenge to the treating physicians.

The hypothesis of the presence of *H. pylori* in the biliary epithelium of the patients with hepatobiliary ailments has been sporadically investigated. Literature is replete with the suggestive evidence of *H. pylori* DNA components in bile, gallbladder tissue and/or cholesterol gallstones. However, Monstein et al reported that detecting bacterial DNA of *H. pylori* in cholesterol gallstone may indicate that *H. pylori* is a normal flora in the gallstone or, alternatively, the formation of cholesterol gallstone is maybe predisposed by the colonization of *H. pylori* in the biliary tract.

More studies on cases and data of large number of patients having different hepatobiliary diseases should be performed in many research centers all over the world in order to verify the correlation of *Helicobacter* species with cholelithiasis. Earlier reports attempting to identify the DNA of *Helicobacter* in the gallbladder tissue of patients with various biliary diseases have shown discordant findings. Some results did not rule out the chance of *Helicobacter* infection being as a contributing agent or cofactor in the development of biliary diseases. One study demonstrated the significant relation between the detection of *H. pylori* in the gallbladders and symptomatic gallstones in these patients. Another research showed a significant correlation of *H. pylori* infection with mucosa of both the gallbladder and stomach.

Infection caused by *H. pylori* in the gallbladder may lead to cholelithiasis and subsequent cholecystitis. This observation underscores the need to establish appropriate measurements for prevention and eradication against the overgrowth of *H. pylori* in the gallbladder. Unfortunately, the available literature in this area is hampered by the lack of gold standard methods in diagnosing *H. pylori* species. Most reported studies have been based on molecular techniques that detect *Helicobacter* species in bile, rather than the evidence of variable bacteria in the bile. Recently tested various pertinent methodological strategies include microbiological cultivation, histopathologic PCR, automated DNA sequencing, bacterial profiling by temporal temperature gradient gel electrophoresis, and southern blot analysis using a *H. pylori* species specific primer.

Over the last decade, an escalating number of studies have reported the association of *H. pylori* infection with extra-digestive conditions. Majority of these studies have identified infection caused by *H. pylori* based on immunological assays and urease breath test (UBT), but rarely by using molecular approaches. Interestingly, no one could isolate any *H. pylori* using culture-based methods.

The present study was designed to elucidate the etiological association of *H. pylori* species with gallstone disease in Al-Madinah Al-Munawarah region in Saudi Arabia, which will help the management of this common surgical problem by using different diagnostic and molecular modalities to detect *H. pylori* in the bile. So, the findings of this study may help to develop a preventive framework for gallstone formation, thus arresting the development of the disease process from the onset. The study also aimed at finding a rapid and reliable technique for detecting and identifying *H. pylori* in the bile using molecular techniques as culture-based methods usually require 2-4 days.

### Patients and Methods

#### Study Design

A prospective cross-sectional study was performed over the period from January to October, 2014 at Al-Meeqat General Hospital, Al-Madinah Al-Munawarah, Kingdom of Saudi Arabia. Ninety-five consecutive patients with established symptomatic gallstone disease, requiring cholecystectomy, were recruited in the study. Thirty subjects were selected as control group in this study. The demographic data, nutritional history, drug and past medical history were obtained by the surgical registrar using a well-structured data sheet designed for this purpose. Consent was taken from all patients to use their data in the current research work. Detection of the serum *H. pylori* antibodies (IgG) was done in both patients and control groups. Stool samples were collected from patients prior to the operation and were then kept in a cool box containing ice cubes until transferred to the microbiology laboratory. *H. pylori* stool antigen (HPSA) using stool samples were done for IgG sero-positive patients only. Laparoscopic cholecystectomy was done under general anesthesia using the standard 4-port ap-
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The patients had uneventful postoperative recovery and were discharged within 2 days of the surgical procedure. The gallbladder specimen, and bile samples were taken during the operation from IgG sero-positive patients only. Bile retrieved from the gallbladder was transported in ice from the operating room to the microbiology laboratory in 1 ml horse serum supplemented brain heart infusion broth to be processed within 2 hours. Specimen taken from the bile secretion was divided into three parts and examined for the presence of *H. pylori* by direct Gram stain, culture and *H. pylori* antigen assay. During the operation, a small part of gall bladder mucosa was collected from each sample under sterile technique and was placed into sterile containers. The tissue was then kept directly in liquid nitrogen for consequent DNA extraction and detection of urease A gene by PCR for cases proven to be positive for stool and bile serology.

**Detection of Serum *H. pylori* IgG Antibodies**

Serum *H. pylori* IgG antibodies was detected using indirect quantitative enzyme immunoassay (HeliSAL serum, Cortecs Diagnostics Ltd., London, UK). Enzyme immunoassay were then done and analyzed based on manufacturer’s guidelines. For each laboratory run, standard curve was plotted by comparing the concentrations of IgG in tested samples that were expressed as units against results of their optical densities. The cut-off value for positive *H. pylori*-specific IgG antibodies in the serum is any value of ≥ 1 units/ml.

**Detection of *H. pylori* Stool Antigen (HpSA) in Stool Samples**

The stool samples were tested and analyzed by HpSA test (Generic Assay, Dahlewitz, Germany) based on its manufacturer’s instructions. HpSA test is a qualitative, sandwich Enzyme-Linked Immunosorbent assay (ELISA) using polyclonal *H. pylori* antibodies adsorbed to microwells as capture antibody. The cut-off value was obtained by the mean OD of negative control at 450 nm, plus 0.1. OD ≤ cut-off was defined negative, and OD > cut-off was considered positive.

**Detection of *H. pylori* Antigens (HpSA) in the Bile**

The detection of *H. pylori* antigen in bile was performed by Primer Platinum HpSA assay (Meridian Bioscience Inc., Cincinnati, OH, USA), using a polyclonal anti-*H. pylori* capture antibody adsorbed to microwells. The immunoenzymatic assay validation was carried out based on the manufacturer recommendations. A clear positivity reflected a value of optical density ≥ 1.923.

**Gram Stain**

The bile specimens were directly smeared using Gram stain method and examined for Gram-negative curved bacilli.

**Microbiological Culture**

Collected bile specimens were cultured by streaking on Columbia blood agar (CM331; Thermo Fisher Scientific Inc., Glasgow, UK) and Columbia blood agar with Dents *H. pylori* selective supplement (SR147, Thermo Fisher Scientific Inc., Glasgow, UK). The plates were then incubated under microaerophilic conditions of 5% O₂, 7.5% CO₂, 7.5 H₂, and 80% N₂ using Camyp-Pak microaerophilic system envelopes (Columbia Diagnostics, Springfield, VA, USA) at 37°C for up to 10 days for detection and identification of *H. pylori* based on colonial morphology, Gram staining, and the production of urease, catalase, and oxidase enzymes.

**DNA Extraction from Gall Bladder Tissue**

Bacterial DNA was extracted from gallbladder tissue specimens using Nucleo Spin Tissue Kit (Macherey-Nagel GmbH & Co., Duren, Germany) based on the provided manufacturer’s instructions. In Brief, about 25 mg of gallbladder tissue specimen was suspended in 180 µL of lysis buffer and homogenized. After that, suspension was incubated with 25 mg/mL of proteinase K solution and 20 mg/mL of RNase A followed by precipitation by ethanol and binding of DNA to silica membrane. Finally, elution of DNA was performed by adding 50 µL of sterile distilled water. The concentration of bacterial DNA was determined by measuring the optical density at 260 nm.

**DNA Amplification**

The PCR was performed using specific published primers for urease A gene. The primer sequence used was HPU126 (5’-GCC AAT GGT AAA TTA GTT-3’) and HPU226 (5’-CTG TTT AAT TGT TTT TAC-3’) and the size of amplified product was 411 bp. One µg of the extracted DNA was amplified in 50 µL of the reaction mixture.
Each PCR reaction consisted of 1 unit Taq Polymerase (Promega, Madison, WI, USA), 2 mM MgCl2, 0.2 mM dNTP (Roche Diagnostics, Mannheim, Germany) and 20 µl of HPU specific oligonucleotide primers (Promega, Madison, WI, USA). Extracted DNA of known H. pylori isolate was used in PCR run as a Positive control. Negative control (non-template control) was also used in the PCR run. The samples were then overlaid with 100 µL of mineral oil, and were then subjected to PCR amplification in the DNA thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). The PCR amplification’s conditions for 30 cycles were as follow: denaturation for 2 minutes at 94°C, primers annealing for 1 minute at 55°C, and extension of primer for 1 minute at 72°C. After the last cycle, the PCR tubes were incubated for 7 minutes at 72°C. The PCR products after amplification were visualized by ultraviolet light transilluminator (Bio-Rad Laboratories Inc., Hercules, CA, USA)26.

Statistical Analysis

Statistical analysis was done using SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA). The correlation between two variables was evaluated using chi-square, Fisher’s exact and Student t-tests, where, \( p \leq 0.05 \) as considered significant.

Results

Among the 95 patients, the mean age was 48.5 ± 16.1 years (mean ± SD) for males and (51.2 ± 15.9 years) for females. Fifty four percent presented with nausea, 47% with vomiting, and 67% with abdominal pain; whereas in 18% the presenting complaint was hematemesis. There was statistical significance correlation of gallstones with smoking as shown in Table I. Serum H. pylori IgG was detected in 75 (79%) in patients and 12 (40%) in control groups (Table II). Of the 75 IgG sero-positive patients, 21 (28%) were positive for the presence of H. pylori antigen in their stools and 26 (34.7%) were positive for H. pylori antigen in the bile samples (Table III, and Figure 1). H. pylori could not be detected from bile specimens among studied cases using either di-

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients group</th>
<th>Control group</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>Mean ± SD</td>
<td>41 ± 11.3</td>
<td>0.001</td>
</tr>
<tr>
<td>44 ± 10.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>55</td>
<td>22</td>
</tr>
<tr>
<td>Female</td>
<td>40</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Symptoms:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nausea</td>
<td>51 (54%)</td>
<td>31 (33%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Vomiting</td>
<td>45 (47%)</td>
<td>25 (26%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>67%</td>
<td>34 (36%)</td>
<td>0.005</td>
</tr>
<tr>
<td>Hematemesis</td>
<td>17 (18%)</td>
<td>4 (4%)</td>
<td>0.001</td>
</tr>
<tr>
<td>High school education</td>
<td>71%</td>
<td>73%</td>
<td>0.001</td>
</tr>
<tr>
<td>Cigarette smoking</td>
<td>62 (65%)</td>
<td>21 (22%)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Table I. Demographic characteristics of the patients and control groups.

<table>
<thead>
<tr>
<th>Age range</th>
<th>Patients group</th>
<th>H. pylori IgG sero positive</th>
<th>Control group</th>
<th>H. pylori IgG sero positive</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 20</td>
<td>2</td>
<td>2 (100%)</td>
<td>1</td>
<td>0 (0%)</td>
<td>0.05</td>
</tr>
<tr>
<td>21-30</td>
<td>31</td>
<td>24 (77%)</td>
<td>12</td>
<td>4 (33%)</td>
<td>0.005</td>
</tr>
<tr>
<td>31-40</td>
<td>27</td>
<td>23 (85%)</td>
<td>5</td>
<td>3 (60%)</td>
<td>0.001</td>
</tr>
<tr>
<td>41-50</td>
<td>25</td>
<td>19 (76%)</td>
<td>7</td>
<td>3 (42%)</td>
<td>0.001</td>
</tr>
<tr>
<td>&gt; 51</td>
<td>10</td>
<td>7 (70%)</td>
<td>5</td>
<td>2 (40%)</td>
<td>0.005</td>
</tr>
<tr>
<td>Total</td>
<td>95</td>
<td>75 (79%)</td>
<td>30</td>
<td>12 (40%)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table II. Distribution of serum H. pylori IgG antibodies in the patients and control groups.
The correlation of *Helicobacter Pylori* with the development of cholelithiasis and cholecystitis

It has been empirically proven that the colonization of *H. pylori* in the gall bladder causes inflammation in the gallbladder and it is considered as important etiological factors that lead to cholecystitis. In addition, there is an association between the presence of *H. pylori* in the biliary tract and an increased risk of gallstone formation. Colonization by *H. pylori* in chronically inflamed gallbladder mucosa may impair gallbladder acid secretion and acidification of the content, decreasing the solubility of calcium salts in the bile and maximizing the risk of their precipitation in the gallbladder lumen. Damage to the epithelial cells of gallbladder mucosa caused by *H. pylori* may be related to the specific virulence characteristics of *H. pylori* such as cytotoxin-associated protein (CagA) and vacuoles toxin (VacA), as well as urease, lipopolysaccharides and mucus enzyme of *H. pylori*.

Although one of the recommended ways to detect the presence of *H. pylori* is the culture in microbiology laboratory, but *H. pylori* is very difficult to grow on culture because of the microaerophilic characteristics of this organism as it dies if it has any contact with air. This may explain why we couldn’t detect *H. pylori* among studied cases using either direct Gram stain or culture methods in our study. Another explana-

Table III. Prevalence of *H. pylori* antigen in stool and bile samples of IgG sero-positive patients.

<table>
<thead>
<tr>
<th>Sample</th>
<th>HpSA +ve</th>
<th>HpSA -ve</th>
<th>(p)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stool</td>
<td>21 (28%)</td>
<td>54 (72%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Bile</td>
<td>26 (34.7%)</td>
<td>49 (65.3%)</td>
<td>0.001</td>
</tr>
</tbody>
</table>
The presence of \textit{H. pylori} in the bile may represent an increased risk for gallstones formation with resultant cholecystitis. Furtherm ore, PCR technique is a feasible, rapid and reliable diagnostic technique for the detection of \textit{H. pylori} DNA in bile. This rapid molecular approach together with culture and immunological methods could help clinicians to effectively treat and manage patient who is at high risk to have gallstones and cholelithiasis at an earlier stage than is possible with only current immunological and culture methods. Further evidence-based studies are required to establish the link, and to determine whether \textit{H. pylori} is a causative agent of, or a co-factor for the gallstone formation. This may help us to devise a strategy to tackle the etiopathogenesis of the disease at an early stage.

**Limitations of the Study**

The significance of the presence of DNA of \textit{H. pylori} in the biliary system needs to be validated by further clinical trials. Mere presence of the \textit{H. pylori} DNA in the bile does not testify the pathogenic role of this bacterium in gallstone formation.

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The correlation of Helicobacter Pylori with the development of cholelithiasis and cholecystitis

**Ethical Approval**
Ethical Committee of Al-Meeqat General Hospital, Almadina Almunawarah, KSA and Scientific Research Deaney of Taibah University approved the study.

**Conflict of Interest**
The Authors declare that they have no conflict of interests.

**References**


