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The correlation of *Helicobacter Pylori* with the development of cholelithiasis and cholecystitis: the results of a prospective clinical study in Saudi Arabia

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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, 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 using 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[1]. 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[2]. 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,

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ERCP, liver function tests and pancreatic enzymes[2,3]. 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[4]. Interestingly, a significant number of patients develop primary common bile duct stones after cholecytectomy (more than two years post-surgery)[5], 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[6,7]. Literatures is replete with the suggestive evidence of *H. pylori* DNA components in bile, gallbladder tissue and/or cholesterol gallstones[8,9]. However, Monstein et al[10] reported that detecting bacterial DNA of *H. pylori* in cholesterol gallstone may indicates 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[11]. 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[12]. 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[13].

Infection caused by *H. pylori* in the gallbladder may lead to chlolelithisais and subsequent cholecystitis[14]. 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[15-18].

Over the last decade, an escalating number of studies have reported the association of *H. pylori* infection with extra-digestive conditions[19]. 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[19].

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-

proach[20]. 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 immunoassays 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 [21].

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[22].

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 ≥ 1.9[23].

Gram Stain

The bile specimens were directly smeared using Gram stain method and examined for Gram-negative curved bacilli[24].

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% O_2, 7.5% CO_2, 7.5 H_2, and 80% N_2 using Campy-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[24,25].

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[23].

DNA Amplification

The PCR was performed using specific published primers for urease A gene[26]. The primer sequence used was HPU1[27] (5′-GCC AAT GGT AAA TTA GTT-3′) and HPU2[27] (5′-CTC CTT 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.

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

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

Each PCR reaction consisted of 1 unit Taq Polymerase (Promega, Madison, WI, USA), 2 mM MgC12, 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 extention 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 II. Distribution of serum *H. pylori* IgG antibodies in the patients and control groups.

Age range	Patients group		Control group		
	No.	*H. pylori* IgG sero positive	No.	*H. pylori* IgG sero positive	*p*-value
< 20	2	2 (100 %)	1	0 (0%)	0.05
21-30	31	24 (77%)	12	4 (33%)	0.005
31-40	27	23 (85%)	5	3 (60%)	0.001
41-50	25	19 (76%)	7	3 (42%)	0.001
> 51	10	7 (70%)	5	2 (40%)	0.005
Total	95	75 (79%)	30	12 (40%)	0.001

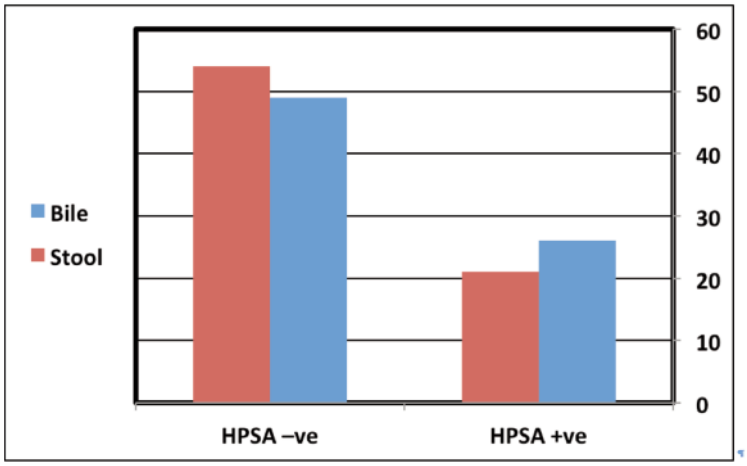

Figure 1. Prevalence of *Helicobacter pylori* antigen in stool and bile samples of IgG sero-positive patients.

rect Gram stain or culture-based methods. Among the 47 patients proved to be positive with stool and bile serology results, 29 (62%) were found to have a positive PCR results (Figure 2).

Discussion

H. pylori infection is prevalent in Saudi Arabia[27]. *Helicobacter pylori* is believed to be a mediating factor for gastric and extragastric disease. The gallbladder and bile duct may be two of the targets of chronic *H. pylori* infection[28]. In the present study, the mean age of patients was found to be 48.5 ± 16.1 years for males and 51.2 ± 15.9 years for females. These findings are in agreement with other results, which reported that the mean ± SD age of such patients was 42.4 ± 11.1 years[29]. Of the 95 patients in the current study, 75 (79%) were positive for the presence of serum *H. pylori* IgG antibodies. Among these sero-positive patients, 26 (34.7%) patients were positive for the presence of *H. pylori* antigens in bile and 21 (28%) patients for *H. pylori* antigens in the stool samples. These results match with another clinical trial where antigen for *H. pylori* organism had been detected in the stool samples of 16 (21.9%) patients, and in the gallbladder bile of 14 (19.2%) patients[30].

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[29]. 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[23]. 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*[31].

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[19]. 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.

Sample	HpSA +ve	HpSA –ve	*p*-value
Stool	21 (28%)	54 (72%)	0.001
Bile	26 (34.7%)	49 (65.3%)	0.001

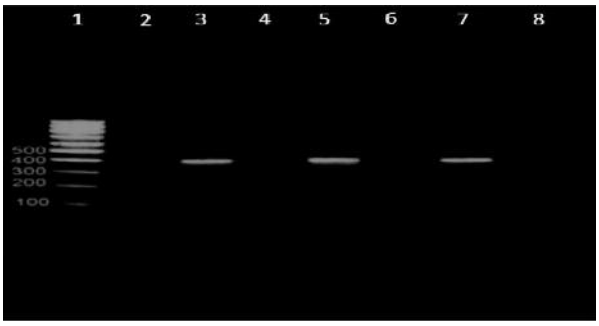

Figure 2. PCR amplification product detecting urease A gene (411 bp). Lane 1 showing molecular weight marker (100 bp Ladder). Lanes 2 and 4 are PCR positive and Lanes 3, 5 and 6 are PCR negative. Lane 7 and 8 shows the positive and negative controls, respectively.

tion is that *H. pylori* exists predominantly in a non-culturable coccoid form outside the stomach. This is because bile has a chemorepellent effect on *H. pylori* as well as an oxygen concentration under the optimum value (< 7%)[23].

Many studies have reported that various PCR techniques could be used as a diagnostic tool to detect *H. pylori* DNA in the bile and gallbladder mucosa. PCR should provide a rapid means of detecting non-culturable *H. pylori.* So, PCR amplification of *H. pylori* DNA sequences has the potential to be a rapid and highly sensitive and specific method for the laboratory diagnosis of *H. pylori* infection[29].

In this study, using PCR technique for detection of *H. pylori* urease A gene, 29 (62%) gallbladder mucosa samples gave positive results among 47 examined cases proven to be positive with stool or bile serology results. The reason for why the other 18 gallbladder mucosa samples were PCR negative could be due to a possible low-grade infection, which could not be detected by PCR approach due to low number of colony forming units (CFU) of bacteria in the sample. Most molecular approaches lack sensitivity in terms of limit of detection (LOD) when infection is at very low rate. Therefore, one of the recomendation for future studies based on results of the current study is to determine the analytical sensitivity of PCR used for detection of urease A gene in order to know the limit of detection (LOD) of this assay[23].

Similarly, others detected *H. pylori* DNA in 32.6% of the gallbladder mucosa of their patients[26]. On the other hand, Fallone et al[32] could not detect DNA of the *Helicobacter* species in bile specimens collected from Canadian patients with biliary disorders; whereas a completely discordant result was reported by Silva et al[33] who detected bacterial DNA of genus *Helicobacter* in most of the Brazilian specimens with biliary diseases. Regional differences due to variable infection rates and the changing sensitivity of the different molecular approaches used may be responsible for the differences in the published studies[33].

Conclusions

The findings of this report summarize that the presence of *H. pylori* in the bile may represent an increased risk for gallstones formation with resultant cholecystitis. Furthermore, PCR technique is a feasible, rapid and reliable diagnostic technique for the detection of *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 *H. pylori* is a causative agent of, or a cofactor 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 *H. pylori* in the biliary system needs to be validated by further clinical trials. Mere presence of the *H. pylori* DNA in the bile does not testify the pathogenic role of this bacterium in gallstone formation.

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Ethical Approval

Ethical Committee of Al-Meeqat General Hospital, Almadinah Almunawarah, KSA and Scientific Research Deanery of Taibah University approved the study.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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