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Detection of *Helicobacter Pylori* Infection in Biliary System in Egyptian Patients with Calcular Obstructive Jaundice

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Abstract

Background: Bacterial infection was described as one of the precipitating factor in cholesterol gallstone formation and many studies have confirmed the presence of *Helicobacter* species in the hepatobiliary system. The aim of our study was to detect the presence of *Helicobacter* species in bile juice by polymerase chain reaction (PCR) in patients presented with calcular obstructive jaundice.

Methods: Bile samples from 75 patients presented with calcular obstructive jaundice were obtained during Endoscopic retrograde cholangiopancreatography (ERCP) and subjected to nested PCR for *H. pylori* DNA detection and bacterial culture. Gastric biopsies were also taken for *H. pylori* rapid urease and culture.

Results: *Helicobacter* DNA was detected in 11 out of 75 bile sample by nested PCR, 0 were positive by bile culture, 30 were positive by rapid urease test from gastric biopsy and 5 were positive by gastric biopsy culture.

Conclusion: *H. pylori* was found in the biliary system, in patients with calcular obstructive jaundice suggesting that these bacteria may be of etiological importance in gallstone formation.

Keywords: Gallstone disease; *Helicobacter*; Obstructive jaundice-nested PCR

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Introduction

Helicobacter pylori (*H. pylori*), is a gram-negative microaerophilic bacterium found usually in the stomach, was first identified by Warren and Marshall [1] in 1982 and soon after it was linked with chronic gastritis and gastric ulcers [2]. Although *H. pylori* is recognized as a human pathogen associated with gastric lesions, studies have revealed the presence of *Helicobacter* species with some extragastric diseases such as cardiovascular diseases, lung diseases, hematologic diseases, eye and skin diseases, hepatobiliary diseases, diabetes mellitus, and neurological disorders [3].

Gallstone disease is one of the common problems affecting the digestive tract however, the cause of gallstone formation beginning with a change in the composition of bile, leading to stones formation [4]. Different studies suggest that bacterial infection has an important role in the formation of brown pigmented gallstones and that the formation of pure cholesterol gallstones depends mainly on cholesterol saturation and solubility [5].

In many studies, the presences of different *Helicobacter* species were shown in the hepatobiliary system [4,6]. Bile-resistant hepatic *Helicobacter* species such as *H. bilis*, *H. pullorum*, and *F. rappini* were isolated from gall bladder (GB) mucosa and bile juice of patients with chronic cholecystitis and gallstones so these agents may be the key elements in the development of various GB-related diseases, especially GB cancer [7].

Diagnosis of *H. pylori* infection includes invasive (culture of Biopsy specimens taken by endoscopy) and non-invasive (serologic testing of the patient's serum antibody response to the organism) methods [8,9]. Recently Polymerase chain reaction (PCR) has been a reliable and highly sensitive tool for detection of *H. pylori* gene sequences in clinical specimens [10,11].

Several studies suggest *Helicobacter* species as an etiological agent in gallstone formation, while another study showed no association [12].

In this study, we aimed to detect specific *Helicobacter* strains identified by PCR assay uses a nested set of primer in biliary system from Egyptian patients with calcular obstructive jaundice.

Material and Methods

Subjects

This prospective study was conducted from January 2014 to April 2016 after permission was granted from Cairo university ethical committee. Our study included 75 patients referred to El-Ibrashi unit of Gastroenterology and Endoscopy Kaser El-Eini Hospital Cairo University complaining of calcular obstructive jaundice proved with imaging either transabdominal ultrasound or Magnetic resonance cholangiopancreatography (MRCP). All patients undergo ERCP examination who agreed to participate in the study were appointed to the endoscopy room on the day of the procedure.

Informed consent

The patients were 100% compliant to the study. For confidentiality, their names were omitted and replaced by numerical codes.

Clinical specimens

Bile juice was collected by intra-procedure aseptic aspiration using catheter through the inner endoscopic channel for bacterial culture and PCR analysis. Bile samples used for PCR were directly stored at -20°C. Gastric Biopsy specimens were also used for rapid urease test and bacterial culture.

Microbiological culture

Bile juice and part of gastric biopsy were cultured immediately on Columbia blood agar (CM331, Oxoid Ltd, Basingstoke, UK) with Dents *H. pylori* selective supplement. Plates were incubated under a microaerophilic atmosphere at 37°C for 5-7 days. The microorganism was identified as *H. pylori* by colony morphology, Gram staining, and positive urease, catalase and oxidase test [13].

During endoscopy, part of gastric biopsy was taken and tested for urease activity by rapid urease test using Kimberrly-Clark, Utah, USA test, the test was read at 2 min, 30 min, 2 h and 24 h, the change in color from a light yellow to pink indicating positive results.

DNA extraction from bile juice

The refrigerated bile samples were pelleted by centrifugation for 10 minutes at 14,000 rpm, then incubated with lysis buffer 10 mM Tris-HCl (pH 8.5), 10 mM EDTA, 100 mM NaCl, 0.5% SDS and proteinase K at 55°C for 8 hours. Then DNA was extracted twice with phenol-chloroform, and purified using a QIAamp DNA kit (QIAGEN, Hilden, Germany).

Polymerase chain reaction of DNA

The PCR method was used to amplify 16S rRNA of *Helicobacter* species from the bile samples, the PCR

product was re-amplified with nested PCR primers designed by Qiagen-Germany. The sequence of primers for *Helicobacter* 16s rRNA are (5'-GCTATGACGGGTATCC-3') and (5'-GATTTTACCCTACACCA-3'). The primers of *H. pylori* (26 kDa surface antigen) are (5'-TGGCGTGTTCATTGACAGCGAGC-3' and (5'-CCTGCTGGGCATACTTCACCATG-3'). All reactions were 50 µL in volume and performed with an automated Gene Amp PCR system 9600 (Perkin Elmer, Norwalk, CT, USA). Reaction mixtures contained 0.2 mM of each oligonucleotide primer, 10x PCR buffer [25 mM TAPS (pH 9.3), 50 mM KCl, 2 mM MgCl₂, 1 mM 2-mercaptoethanol], 0.5 U of Taq polymerase, 0.4 µL of each primer, and 5 µL of template DNA. The PCR products were denatured at 94°C for 1 minute, annealed at 55°C for 1 minute, and elongated at 72°C for 1 minute. The PCR reaction ended at 35 cycles. Then, 50µL mixture containing 3 µL of PCR product was reamplified by nested PCR. Final nested PCR products were electrophoretically separated in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light in comparison with 100 bp molecular weight ladder markers (Gibco BRL).

Results

Culture of *H. pylori* from gastric biopsy and bile specimens

Our prospective study included 32 men and 43 women with ages ranged from 31 to 63 years (mean age 51.4 years). All patients complaining of calcular obstructive jaundice were proved with imaging modality either trans-abdominal ultrasound or MRCP. Therapeutic ERCP was done and bile juice specimens were obtained during the procedure. Bile juice was collected by aseptic aspiration using catheter through the inner endoscopic channel and bacterial cultures were performed immediately in all patients. Gastric biopsy was taken during the procedure for *H. pylori* culture and rapid urease testing. Bile samples culture showed no growth for *H. pylori* however 5 gastric biopsy samples (6.7%) were positive. The rapid urease test in the gastric mucosa was negative in 45 (60%) and positive in 30 (40%) of these patients **Table 1**.

Detection of *H. pylori* using nested PCR assay

Helicobacter specific 16S rRNA PCR product expected at 400-bp were positive for 11(14.7%) out of 75 bile juice samples **Figure 1**. To identify *H. pylori*, specific primer pairs HPF and HPR were used to generate amplicons of approximately 298-bp in samples positive for *Helicobacter* 16S rRNA **Figure 2**. Patient samples were considered to be positive for *H. pylori* by PCR amplification if the 400 bp band was seen in the initial reaction and the 298 bp was seen in the internal nested reaction **Table 1**.

Table 1 Percentage of patient's different tests.

Test	Percentage %
Bile juice culture	0
Gastric biopsy culture	6.7
Gastric biopsy rapid urease	40
<i>Helicobacter</i> specific 16S rRNA PCR	14.7
<i>H. pylori</i> 26 kDa surface antigen PCR	14.7

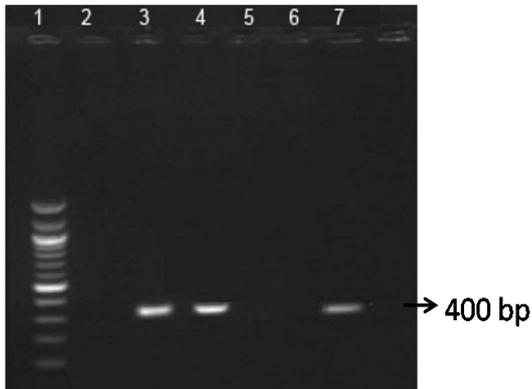


Figure 1 *Helicobacter* specific 16S rRNA PCR products in bile giving a 400 bp amplicons. Lanes: 1, 100 bp molecular weight ladder; 2, negative control; 3, positive control; 4-7, positive patient samples; 5-6, negative patient samples.

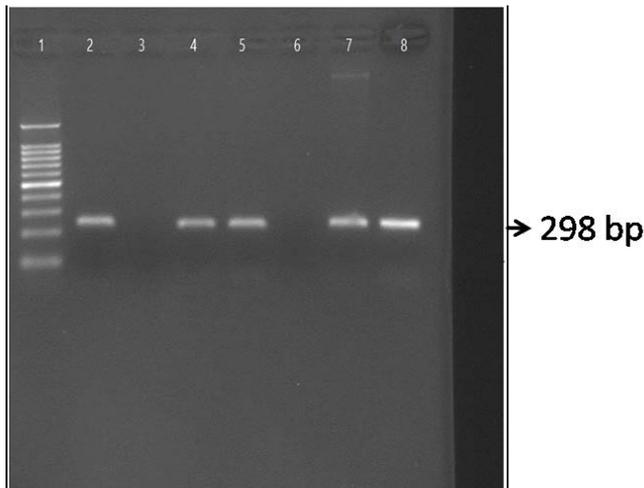


Figure 2 PCR products of 26 kDa surface antigen for *H. pylori* in bile giving a 298 bp amplicons. Lanes: 1, 100 bp molecular weight ladder; 2, positive control; 3, negative control; 4-5-7-8, positive patient samples; 6, negative patient sample.

Discussion

Extragastric diseases have been suggested in many studies due to *Helicobacter* species infection. The *Helicobacter* species that have implicated as a cause of hepatobiliary diseases are *H. pylori*, *H. rodentium*, *Flexispira rappini* and *H. pullorum* [14,15]. There is evidence that many *Helicobacter* species such as *F. rappini*, *H.*

bilis, *H. canis*, *H. hepaticus*, *H. cholecystus* and *H. pullorum* may be able to survive in bile juice [16,17].

In this study, *Helicobacter* DNA was detected in 14.7% of the patient bile juice samples. In addition, all bile samples positive for *Helicobacter* DNA were also positive for *H. pylori* identified using specific 26 kDa surface antigen primers. In accordance to our result Lee et al. 2010 study show that *Helicobacter* DNA was detected in 6 out of 48 bile samples [18], in contrast to Fallone et al. 2003 study which could not detect any *Helicobacter* DNA in bile samples [11].

Different studies used enzyme-linked immunosorbent assay to identify *Helicobacter* species in bile samples, however this method is not as specific or sensitive in detecting *Helicobacter* as PCR and may lead to false positive results due to cross-reactivity between different bacterial organisms or proteins [11,19]. Furthermore, Nested PCR against 16s rRNA *Helicobacter* genus and *H. pylori* specific surface antigen used in this study is more specific rather than other studies using genes being limited to *Helicobacter* species such as urease A that may cross-react with other organisms giving false positive test [20,21].

Since *Helicobacter* species inhabit the gastrointestinal tract, the most likely source of these organisms is ascending infection from the duodenum [22]. Therefore, the detection of *H. pylori* using rapid urease test was done on the patient's gastric biopsy. 40% of the gastric biopsies were positive for *H. pylori* urease test including the positive samples for *H. pylori* in the bile detected by PCR. These results suggest that bile regurgitation may have a role to allow surviving of *H. pylori* that are resistant to bile salts and causing inflammation and stone formation.

Unfortunately, we could not isolate any *H. pylori* from bile samples in this study and others studies probably because the difficult culture of this fastidious organism and the high sensitivity to the bile salts that may inhibit its viability [23,24].

In summary, our data suggest that bacterial infection of *H. pylori* may have a role in the formation of gallstones that may be due to cholesterol or calcium precipitation [25]. Future studies focusing in the route of *H. pylori* infection and the effect of bacterial eradication on the development of gallstone will be needed.

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Conflict of Interest

The authors declare they have no conflict of interest.

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