

MOLECULAR DETECTION OF HELICOBACTER PYLORI DNA IN PATIENTS WITH GALLSTONE

Mohammad Hassanpour^{1, 2}, Reza Ghotaslou^{1, 2}, Mansur Khakpoor³,
Ahmad BabazadehBedoustani⁴, Saeid Shirgir²

1. *Liver and Gastrointestinal Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*
2. *Department of Microbiology, School of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.*
3. *Department of Pathobiology, Tabriz branch, Islamic Azad University, Tabriz, Iran.*
4. *Biotechnology Research Center, Tabriz branch, Islamic Azad university, Tabriz, Iran.*

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ABSTRACT

We aimed to study the presence of *H. pylori* in gallstones in Azerbaijan (Iran) by molecular method. A total of 60 gallbladder samples were collected from patients who underwent cholecystectomy. The bile samples and gallbladder tissues with gallstones were considered the experimental group, while subjects without any gallstones (according to pathologist diagnosis) were considered the control group. In total, there were 30 experimental cases and 30 control cases. To determine the presence of *H. pylori* we performed multiplex PCR. 3 cases (10%) were positive in terms of *H. pylori* DNA through PCR, of 30 gallbladder samples from patients suffering from gallstones, whereas the PCR results were all negative in the control group. The findings suggested that there was no statistically significant difference between the experimental and control groups in terms of *H. pylori* DNA ($P > 0.05$). This study showed that *H. pylori* DNA can be found in patients with gallstones. A better understanding of the role of *H. pylori* will require further studies on larger numbers of patients and controls. However, our results did not reject the likelihood of *H. pylori* infection as a cofactor in the development of gallstones.

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Introduction

Gallstones are the most common human diseases, which become more prevalent with age, observed in 30 to 40 percent of people until the age of 80 years old. The prevalence of biliary diseases is 2-3 times higher in women than in men, reported more frequently in multiparous and obese women over 40 years old. Gallstones often are asymptomatic, identified accidentally during surgery or assessment of other problems. The symptoms usually are caused by the presence of stones in the gallbladder or obstruction of the bile duct [1-4].

Helicobacter pylori (*H. pylori*) are microaerophilic, a Gram-negative bacterium, capable of causing chronic inflammation of the stomach, duodenal ulcer and gastric adenocarcinoma [5-7]. Moreover, *H. pylori* account for the most common infection in humans, affecting more than half the world's population. There are high rates of *H. pylori* infection in the afflicted third world countries. *H. pylori* can be observed in many patients with digestive problems in the gastric and duodenal mucosa. The role of *H. pylori* has been reported in diseases originating outside the abdomen. Some researchers have identified *H. pylori* from stomach, intestines, and liver of humans and various animal species [8, 9]. Considering the presence of *H. pylori* in the duodenum and the bile duct, scientists think the *H. pylori* may be associated with the biliary disease. Previous studies have been proved the relation between *H. pylori* and occurrence of gallstones [1, 5], but the main cause and mechanism involved in

gallstones are unknown [2, 5, 11-13]. In this study, considering the importance of gallstones, we aimed to study the presence of *H. pylori* in gallstones in Azerbaijan (Iran) by molecular methods.

Materials and Methods

Patients: A total of 60 patients (30 controls and 30 experimental) with gastrointestinal symptoms referred to Sina Hospital (Tabriz University of Medical Sciences) and QamarBaniHashem Hospital (Khoy city, Uremia University of Medical Sciences), from January 2015 to May 2016 were randomly selected. The levels of liver enzymes including Serum glutamic-oxaloacetic transaminase (SGOT), SGPT (Serum glutamic-pyruvic transaminase), and ALK (Alkaline Phosphatase) were measured. Abdominal and gallbladder sonography were performed in all subjects before cholecystectomy (surgery or laparoscopy). The gallbladder tissue or bile liquid samples were collected in sterile containers. The samples stored at -20 °C [4]. The part of samples sent to the pathology laboratory. This study was approved by the local Ethics Committee (TBZMED, REC, 1394, 587 dated 13 October 2015).

DNA extraction: The (Deoxy Ribonucleic Acid) DNAs of samples were extracted by the phenol-chloroform method with minor modifications. The surface and mucosal layers of the gallbladder were scratched by a surgical blade and homogenized. The samples solved in a 1000 µL the TB lysis buffer including NaCl 100 mM, Tris-HCl 10mM [pH,8.0], SDS 0.5%, and EDTA 25mM, and vortexed with 300 µL of blended homogeneous tissue for 45 minutes at 60 °C. The samples were centrifuged. The supernatant liquid was transferred to another tube contain 500 µL of phenol and 500 µL of chloroform-isoamyl alcohol (1 to 24). After mixing and centrifuging, the supernatant liquid was discarded slowly and the DNA-containing deposit was exposed to air until alcohol evaporated. Thirty µL of deionized water was added to the tube. The involvement of DNA was qualitatively measured through a Nanodrop spectrophotometer (Thermo serial No: 7663) at a wavelength 260-280 nm.

PCR: In this study, two pairs of primers were used. The first primer (H1-1-891, H1-2-891) was based on *Helicobacter* genus 16S rRNA gene sequence, (gene sequence *Helicobacter* 3' GTA AAG GCT CAC CAA GGC TAT- 5' and 3' CCA CCT ACC TCT CCC ACA CTC- 5') (389-base pair, bp). The second primer is based on the specific gene of *H. pylori* (isocitrate dehydrogenase), sequence: 3'- ATG GCT TAC AAC CCT AAA ATT TTA CAA AAGCC- 5' and 3' TCA CAT GTT TTC AAT CAT CACGC- 5' (1200-bp). In this study, the DNA of *H. pylori* ATCC (American Type Culture Collection) 43504 was used as a positive control, while distilled water was used as a negative control [10].

Statistical analysis:

The data were analyzed using SPSS (Statistical package for social science) software for windows ver. 20 SPSS, Inc., Chicago, IL. The X² and Fisher exact tests were used for examine the relationship between gallstones and the *H. pylori*.

Results

This study was done on 30 patients with gallstones (28 females and 2 males) with an average age 42-60 years old as the cases group and 30 patients without gallstones (26 females and 4 males) with an average age 50-53 years old as the control group. Ten percent of patients with biliary diseases were used antibiotics in the past two months, 13.3% were smokers and 1.7% had a history of peptic ulcer infection and positive *H. pylori* Immunoglobulin G (IgG). However, these above mentioned factors were negative in all biliary patients with positive DNA of *H. pylori* (Table 1).

There was no significance difference between the positive-PCR (Polymerase Chain Reaction) and negative-PCR in the biliary patients and no the control group in terms of fever and blood pressure. The liver enzymes (SGOT, SGPT, and ALK) in patients with biliary diseases and the control group were normal and was no statistically significant difference between the presence *H. pylori* DNA and the liver enzymes in the two groups (P>0.05).

According to the pathologic findings, the patients were classified into four groups, one sample belonging to a 47-year-old woman diagnosed with gallbladder adenocarcinoma (Table 1).

This study was performed using the Multiplex-PCR (Figure 1). DNA of *H. pylori* was observed in 10% (3 of 30) of the gallbladder samples in the experimental group. None of the samples were positive-PCR in the control group. However, there was no statistically significant difference between the experimental and control groups in terms of *H. pylori* DNA (P>0.05)

Table 1: The patients with biliary diseases based on the pathologic findings and PCR results

Studied groups, N (%)	Positive DNA of <i>H. pylori</i> , N (%)
Chronic cholecystitis and cholelithiasis, 19 (31.7)	0
Chronic cholecystitis, 20 (33.3)	2 (6.66)
Acute-chronic cholecystitis, 20 (33.3)	1 (3.33)
Gallbladder adenocarcinoma, 1 (1.7)	0
Total, 60 (100)	10

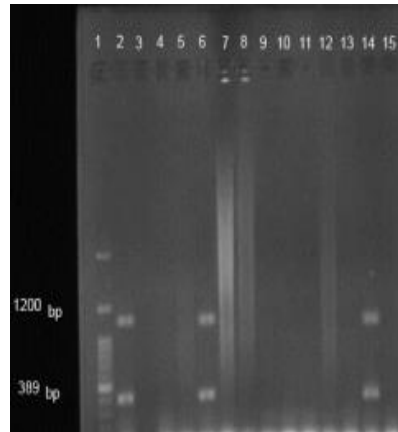


Figure1: Multiplex PCR with *H. pylori* primers 16SrRNA (389 bp) and isocitrate dehydrogenase (1200 bp). Lane 1) 100 bp as molecular marker, Lane 2) positive control (containing both genes), Lane 3) negative control, Lanes 4, 5, 7, 8, 9, 10, 11, 12, 13, 15) negative *Helicobacter* and *H. pylori*, Lanes 6 and 14) positive *Helicobacter* and *H. pylori* samples.

Discussion

This study demonstrated that *H. pylori* may be involved in the formation of gallstones. Previous studies have been proved the relation between *H. pylori* and occurrence of gallstones [1, 5], but the main cause and mechanism involved in gallstones are unknown [2, 5, 11-13]. Although the detection of *H. pylori* DNA in the bile was confirmed according to various reports, the organism and its link to various gastrointestinal and hepatobiliary diseases in humans need to be identified [2, 12, 14, 15]. The presence of *H. pylori* DNA in bile and gallbladder tissue samples in humans has been reported from Taiwan [9], Japan [1], South Korea [16, 17], Italy [16], Mexico [18], Sweden [19] and Iran [20]. It has been reported that various strains of *H. pylori* may cause gallstones and bile duct cancer [1]. Most of the studies confirmed the presence of *Helicobacter* in the gallbladder, but some studies do not confirm [2, 5, 9, 12, 13, 21, 22]. Previous studies have so far reported positive for control *Helicobacter* strains by 0 to 40.5% [13, 17, 22, 23]. In the current study, *H. pylori* DNA was positive in 10% of bile samples, and the results were consistent with those of previous reports [2, 9, 17, 22, 23]. The *Helicobacter* members, particularly the specific strains of *H. pylori*, have already been identified through Multiplex-PCR [20]. Given the sensitivity of Multiplex-PCR protocol, the DNA of *H. pylori* in this study was identified in three bile samples from patients with chronic and acute cholecystitis, while all controls indicated negative results. Other studies, however, identified and reported DNA of *Helicobacter* strains in bile samples of up to 56.5% of patients with acute cholecystitis [2, 9, 15, 24]. Nonetheless, Fallon et al. from German reported that there is no relationship between *H. pylori* DNA in chronic and acute cholecystitis [21]. A study identified the simultaneous presence of *H. pylori* (13.9%) in the stomach and gallbladder mucosa [2]. Another study reported *H. pylori* in the gastric mucosa (43.1%) and in patients with cholelithiasis (33.8%). Although higher infection rates have been reported in patients with gallstones, there was the statistically significant difference [11]. Similarly, this study found no statistically significant difference between the experimental and control groups in terms of *H. pylori* DNA ($P > 0.05$).

The difference in the prevalence of *H. pylori* in various reports may be due to the selection of experimental groups, primer types, PCR procedures and *H. pylori* infection rate in the region. *H. pylori* are not the normal flora of the gallbladder, but it might be involved in chronic cholecystitis [2, 20]. Different results obtained from various parts of the world about the prevalence of *H. pylori* in the gallbladder have been associated with regional differences in terms of *H. pylori* infection rates. The prevalence of *H. pylori* infection has been reported from different geographical areas. In some reports, there was no relationship between the presence of *Helicobacter* DNA in bile and presence of *H. pylori* in the antrum [5, 12, 14, 23, 25]. Some scholars believe that there is no relationship between the distribution of *Helicobacter* strains in the biliary duct and its distribution in gastric diseases in different parts of the world [9, 24]. Such difference may be due to the sensitivity of PCR in experiments and geographic changes in human exposure to *H. pylori* [9, 15, 17, 26].

In this study, we managed to identify the presence of *H. pylori* DNA only in biliary patients in Azerbaijan (Iran). In the previous studies were confirmed the presence of several *H. pylori* strains in the human bile duct using PCR [12, 25]. There have also been reports on the involvement of *Helicobacter* strains other than *H. pylori* such as *H. bilis*, *H. pullorum*, *H. rodentum*, *H. flexispira* and *H. rappini* in the liver and biliary tracts [9, 14, 26]. The *H. pylori* DNA in this study was examined based the genus-specific primer 16SrRNA for *Helicobacter* and specific primer for *H. pylori*. The 16SrRNA is the general gene of *H. pylori* genus, which can be found in all *Helicobacter* strains and was once used to detect *H. pylori* [9, 13, 14, 26]. In this study, *Helicobacter* species did not found in the gallbladder other than *H. pylori*.

The minimum level of *Helicobacter* DNA detectable through PCR is about 100 fg or 20 *H. pylori* in theory [27]. According to the results, the *H. pylori* could not have been identified through PCR due to their low number of patients with biliary diseases.

There are also differences in specific serum antibody responses in 61% of biliary patients for identification of *Helicobacter*. According to various reports, there is no significant relationship between *H. pylori* DNA in the gallbladder and anti-bacterial antibodies (IgG) in serum and bile [15].

In the previous studies, the incidence of biliary diseases was reported to be 2-3 times higher in women than in men, usually covering multiparous women over 40 years of age. Patients with biliary diseases may have symptomatic gallstones accompanied by pain or no symptoms and no pain, the symptoms are associated with the gallbladder disease or the bile duct obstruction by stones [1-4]. A few studies reported pain in the right upper abdomen in all patients (100%), fever in 75%, nausea, and vomiting in 68% [4] and smoking in 23.2% [2, 3]. In this study, patients with biliary diseases had pain in the upper right abdomen (98.3%), fever (53.3%), nausea (76.7%), vomiting (58.3%) and smoking (13.3%). The results were consistent with previous reports. One study pointed to a significant difference in the high blood pressures of subjects with biliary diseases [3]. In this study, the blood pressures in none of the groups (experimental and control) were significantly different. In another study, the levels of jaundice and liver enzymes indicated no statistically significant difference between patients with biliary stone and non-stone biliary patients [13]. This was consistent with our results since the liver enzymes in patients with biliary disease (experimental and control) were normal.

It is hypothesized that the presence of *H. pylori* DNA in patients with gallstones can confirm the role of *H. pylori* in the formation of gallstones [5, 14, 16]. Previous studies found other strains of bacteria such as *E. coli*, Enterobacteriaceae and Propionibacterium spp. in gallstones [1]. However, the exact role of bacteria in the pathogenicity of biliary diseases is unknown. There also little known about the important involvement of *H. pylori* DNA and other *Helicobacter* strains in biliary diseases [5, 9, 14]. Scholars have not yet managed to culture and isolate *Helicobacter* from bile [9, 11, 15]. Since *H. pylori* are extremely sensitive to biliary acids (acid deoxycholic acid and chenodeoxycholate) found in the gallbladder bag, it is unlikely that live bacteria are involved in the gallbladder [28]. It is promising that the confirmed relationship between *H. pylori* and gallbladder stones in the future would allow the prescription of anti-*Helicobacter* drugs to treat biliary diseases.

To determine a relationship between *H. pylori* and biliary diseases we recommend further research in different laboratories by more samples and a unique PCR protocol such as quantitative real-time PCR.

Conclusions

The results of this study suggested that *H. pylori* DNA can be identified from the gallbladder tissues of patients with biliary problems. It cannot be argued that bacterial genes are the only factor involved in the formation of gallstones. However, our results did not reject the likelihood of *H. pylori* infection as a cofactor in the development of gallstones.

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Conflict of Interest

There is no conflict of interest.

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