

## Detection of *Helicobacter pylori* in Paraffin-Embedded Specimens from Patients with Chronic Liver Diseases, Using the Amplification Method

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**Abstract** *Helicobacter* DNA has been detected in the liver specimens of patients with various hepato-biliary diseases. The aim of this study was to investigate the presence of *H. pylori* DNA in the liver tissue of Iranian patients with chronic liver diseases (CLD). Genomic DNA was extracted from the paraffin sections of 46 liver biopsies of patients with CLD and 13 from patients with metastatic adenocarcinoma as a control group. Polymerase chain reaction (PCR) analysis was carried out using primers for *H. pylori* 16S rRNA and *cagA* genes. On analysis, 17 of the 46 patient samples were positive in *H. pylori* 16S rRNA PCR and 2 of the 13 were positive from the control group. None of the samples were positive for the *cagA* gene. This study showed the greater presence of *H. pylori*-like DNA in the liver samples from patients with CLD than in controls.

**Keywords** *Helicobacter pylori* · Chronic liver diseases · Paraffin-embedded samples · Polymerase chain reaction

### Introduction

*Helicobacter pylori* is a gram-negative and microaerophilic microorganism that can cause chronic gastritis, peptic ulcers, and gastric adenocarcinoma [1, 2]. Besides these gastroduodenal diseases, *H. pylori* has been implicated in extra-gastric disorders, including ischemic heart disease [3], neurologic diseases, iron deficiency anemia [4], and respiratory diseases [5]. *Helicobacter* spp. infections have been reported to be associated with certain liver diseases in some animal species, such as *H. canis* in dogs [6], *H. pullorum* in poultry [7], and *H. hepaticus* and *H. bilis* in mice [8, 9].

Firstly, the possible colonization of the human hepato-biliary system by *Helicobacter* spp. have been reported by Fox et al. [10], who have shown the presence of *H. bilis* and *H. pullorum* in the bile of Chilean patients with chronic cholecystitis. Recently, *Helicobacter* DNA has been found in the liver of patients with different chronic liver diseases (CLD), such as primary sclerosing cholangitis (PSC) [11], hepatocellular carcinoma (HCC) [12], hepatitis C virus (HCV)-related chronic hepatitis, and cirrhosis [13, 14]. CLD is an inflammatory disease and each inflammatory process is characterized by increased levels of pro-inflammatory cytokines, such as interleukins 1 and 6 (IL-1 and IL-6), tumor necrosis factor (TNF), and by the presence of lympho-mono cellular infiltrate and lymphoid follicle formation [12].

A *Helicobacter*-positive diagnosis based on *H. pylori* and *Helicobacter* genus-specific polymerase chain reaction (PCR) assays and the sequencing of PCR products showed a high homology to *H. pylori* [12–15]. The aim of this study was to determine whether genomic *H. pylori* sequences were detectable in the archival samples of Iranian patients with CLD.

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## Methods

### Tissue Collections

Paraffin blocks of 46 liver biopsies from patients with CLD were evaluated. These included patients with chronic hepatitis ( $n = 28$ ), non-alcoholic fatty liver diseases (NAFLD,  $n = 11$ ), cirrhosis ( $n = 4$ ), and HCC ( $n = 3$ ). The age range of the patients was from 17 to 85 years, with 19 women and 25 men. Nine patients were positive for anti-hepatitis B virus (HBV) and five patients were anti-HCV positive. Paraffin blocks of 13 liver biopsies from patients with metastatic adenocarcinoma were used as controls. All paraffin blocks were collected from the Pathology Department of the Mehr Hospital and Milad Hospital, in Tehran, Iran.

### DNA Extraction

The liver tissue samples were de-embedded by washing in xylene for  $2 \times 30$  min. The specimens were then rehydrated through graded ethanol (99%, 80%, 60%, and 40%) for 5 min, and finally washed for 5 min in double-distilled water. DNA was extracted from sections using the QIAamp DNA Mini Kit (Qiagen, Germany), according to the instructions of the manufacturer. The DNA was stored frozen at  $-20^{\circ}\text{C}$  until its use as the template in the PCR.

### PCR Amplification

The oligonucleotide primers used to amplify a segment of the *H. pylori* genes were selected to produce a small amplicon, since specimens that had been fixed with formalin were the source of the DNA [16, 17]. Two sets of primers (Metabion International AG, Germany) were used in this protocol. Initially, samples were amplified by *H. pylori* 16S rRNA primer, as described by Weiss et al. [18]. The sequence of the sense primer (JW21) was 5'-GCGACCTGCTGGAACATTAC-3' (position 691–710) and the antisense primer (JW22) was 5'-CGTTAGCTGCAT TACTGGAGA-3' (position 829–809). The forward and reverse primers amplified a product of approximately 139 bp. The amplification conditions were:  $95^{\circ}\text{C}$  for 5 min; 35 cycles at  $95^{\circ}\text{C}$  for 30 s,  $55^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 30 s; and finally  $72^{\circ}\text{C}$  for 2 min.

Samples generating a positive result from the first step of PCR were subsequently analyzed with *cagA* primer as described by Pellicano et al. [12]. The *cagA*-1 (5'-ATAATGCTAAATTAGACAACCTTGAGCGA-3') and *cagA*-2 (5'-TTAGAATAATCAACAAACATCACGCC AT-3') amplified a 290-bp product from the *cagA* gene. The amplification conditions were:  $94^{\circ}\text{C}$  for 5 min; 35 cycles at  $94^{\circ}\text{C}$  for 1 min,  $61^{\circ}\text{C}$  for 1 min, and  $72^{\circ}\text{C}$  for 1 min; and finally  $72^{\circ}\text{C}$  for 2 min.

The PCR reaction mixture for the two sets of primers contained: 5  $\mu\text{l}$  PCR buffer  $10\times$  (Roche, Germany, 25 mM  $\text{MgCl}_2$ ), 200  $\mu\text{M}$  deoxyribonucleotide triphosphate (Roche, 1 mM), 10 pmol of each primer, 2.5 U *Taq* DNA polymerase (Roche, Germany, 5 U/ $\mu\text{l}$ ), and 10  $\mu\text{l}$  of DNA in a total volume of 50  $\mu\text{l}$ . The PCR products were analyzed on 2% (w/vol) agarose gel, which were stained with ethidium bromide and observed under short-wavelength ultraviolet light.

We used two positive controls; the first was DNA extracted from paraffin-embedded gastric biopsy specimens from patients with a known peptic ulcer caused by *H. pylori* and the second was DNA from pure *H. pylori* culture that was isolated from gastric biopsy and confirmed by microbiology tests. Each amplification contained positive (*H. pylori* genomic DNA) and negative (distilled water) controls to exclude cross-over contamination.

## Results

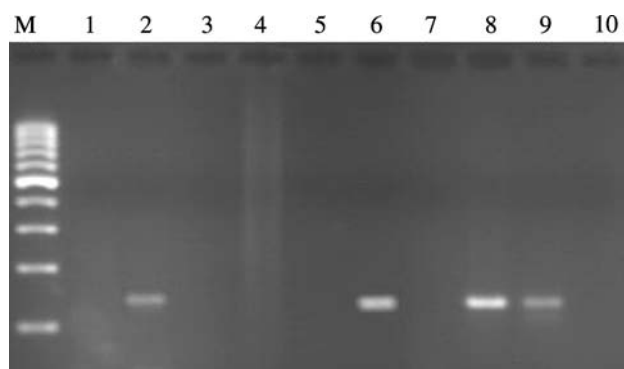
PCR analysis using *H. pylori* 16S rRNA primer was positive in 17 of 46 (36.9%) samples with CLD and in 2 of 13 (15.3%) controls. The distribution of *H. pylori*-positive samples among the different patient groups is shown in Table 1. Under ultraviolet illumination, the size of the PCR product corresponded to the expected 139 bp (Fig. 1). *H. pylori* DNA was detected in 7 of 14 (50%) patients with HBV and HCV infection. None of the 17 *H. pylori*-positive samples reacted in the PCR assays using primers for the *cagA* gene.

## Discussion

The postulated role of *H. pylori* in the pathogenesis of extra-gastric manifestations is based on the fact that: (1) local inflammation has systemic effects; (2) *H. pylori* gastric infection is a chronic process lasting for decades; and (3) persistent infection induces a chronic inflammatory and immune response which are able to induce lesions both

**Table 1** Results of the *Helicobacter pylori* polymerase chain reaction (PCR) assays in the different patient groups

| Pathological groups               | No. of patients | <i>H. pylori</i> 16S rRNA-positive (%) |
|-----------------------------------|-----------------|--|
| Chronic hepatitis                 | 28              | 9 (32.1)                               |
| Non-alcoholic fatty liver disease | 11              | 5 (45.4)                               |
| Cirrhosis                         | 4               | 1 (25)                                 |
| HCC                               | 3               | 2 (66.7)                               |
| Total                             | 46              | 17 (36.9)                              |
| Controls                          | 13              | 2 (15.3)                               |



**Fig. 1** Amplification of a 139-bp 16S rRNA DNA fragment of *H. pylori*. Lane M: molecular size marker (100–1,000 bp); lane 2, 6: positive samples; lane 8, 9: positive controls (*H. pylori* DNA from paraffin-embedded gastric tissue and pure culture); lane 10: negative control (double-distilled water)

locally and remote to the primary site infection [13, 19]. In addition, *Helicobacters* are strong inducers of inflammatory cascade; infection with them could lead to the accumulation of an extraordinary number of lymphocytes and polymorphonuclear cells in the infected tissue. It has been shown that *Helicobacter* spp. could also secrete a liver-specific toxin that causes hepatocyte necrosis in cell culture, and might, therefore, also be involved in damaging liver parenchyma in vivo [12].

Recently, several separate research groups have detected *Helicobacter* spp. in the bile, gallbladder [20, 21], and liver tissue of patients with different hepato-biliary diseases. Nilsson et al. [22] have identified *H. pylori* in human liver samples patients suffering from primary sclerosing cholangitis and primary biliary cirrhosis. Avenaud et al. [23] have shown the presence of *Helicobacter* spp. genomic sequences in the liver tissue of eight patients with primary liver carcinoma. Tolia et al. [24] have demonstrated, by PCR, the presence of genomic sequences of *Helicobacter* spp. in the liver tissue of 40 patients with miscellaneous liver diseases; a further analysis by sequencing revealed that most of these species were *H. pylori*. Furthermore, a high prevalence of antibodies to *H. pylori* in the serum of patients with chronic hepatitis and in patients with cirrhosis have also been reported [25, 26]. Iranian investigators have detected *H. pylori* DNA in 4 of 36 bile samples from patients with biliary diseases [27]. These observations suggested that *Helicobacter* spp., including *H. pylori*, might play a role in the development of hepato-biliary diseases.

In the present study, which composed of two groups, we detected *H. pylori*-like DNA in 17 of 46 (36.9%) liver samples from patients with CLD, whereas only 2 of 13 (15.3%) samples were positive in the controls. Moreover, 7 of 14 (50%) samples from patients with HBV or HCV infection were positive for *H. pylori* DNA. These data are

in agreement with the published studies, which have shown a higher frequency of *Helicobacter* spp. in the liver samples of patients with HCV or HBV infection [24].

The cytotoxin-associated gene (*cagA*), which encodes an immunodominant antigen and is a marker for the presence of the *cag* pathogenicity island (*cag* PAI), is not present in all *H. pylori* strains [28]. Xuan et al. [29] have detected the *cagA* gene in only 3 of 17 (17.6%) positive samples from patients with HCC. Stalke et al. [30] have identified *Helicobacter* species in the liver and stomach tissue of Polish patients with CLD PCR denaturing gradient gel electrophoresis and immunohistochemistry. Their results have shown that *H. pylori*-like organisms appeared to dominate the gastric mucosa and liver tissue patients. The prevalence of the *cagA* gene in positive samples was 36% (23 of 62) and 12% (3 of 25) for the stomach and liver, respectively [30]. In our study, none of the positive samples reacted in PCR assays using primers for the *cagA* gene. This discrepancy may be explained by the selection criteria of the patients. On the other hand, our results confirm that the *cagA*-negative strain has a role in liver diseases, as previously described [30].

To the best of our knowledge, this is the first report on the detection of *Helicobacter* spp. in patients with CLD in Iran. The main limitation of this study was its retrospective nature, which did not allow us to determine whether *H. pylori* was present in the stomach of these patients or to gather all of the clinical information and duration of infection, which is needed for a more accurate analysis.

In conclusion, we noticed that patients with various CLD had a greater probability of positive *H. pylori*-like DNA compared with the control group. To confirm these data, we need to sequence the PCR products and study other genes, such as *vacA*, *ureA*, and *ureB*. Indeed, to make clear the exact clinical role of *Helicobacter* species, namely, *H. pylori* in CLD, more studies on a larger population of patients and certain populations (e.g., those with HBV, HCV, and HCC) are needed.

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