

## SHORT REPORT

# Identification of *Helicobacter pylori* DNA in Iranian patients with gallstones

SH. FARSHAD<sup>1</sup>\*, A. ALBORZI<sup>1</sup>, S. A. MALEK HOSSEINI<sup>2</sup>, B. OBOODI<sup>1</sup>,  
M. RASOULI<sup>1</sup>, A. JAPONI<sup>1</sup> AND J. NASIRI<sup>1</sup>

<sup>1</sup> Professor Alborzi Clinical Microbiology Research Centre, Shiraz University of Medical Sciences, Shiraz, Iran

<sup>2</sup> Department of Surgery, Nemazi Hospital, Shiraz University of Medical Sciences, Shiraz, Iran

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## SUMMARY

In order to identify *Helicobacter* in gallstones of Iranian patients with biliary disease, gallstone and bile samples from 33 patients were subjected to rapid urease test, culture and Multiplex PCR using primers based on 16s rRNA and isocitrate dehydrogenase genes for the identification of *Helicobacter* genus and *H. pylori* respectively. This PCR was also done on bile samples from 40 autopsied gallbladders with normal pathology (control group). In 18·1% of stone and 12·1% of bile samples, *H. pylori* DNA was detected using PCR. Rapid urease and culture tests were negative for all samples. The PCR was negative in the control group. In conclusion, *H. pylori* DNA was detected in stone samples of Iranian patients with gallstones but we are not sure of their viability. To clarify the clinical role of *Helicobacter* in gallbladder diseases, studies using accurate tests on larger patient and control groups are needed to ascertain whether this microorganism is an innocent bystander or active participant in gallstone formation.

It was recently proposed that bile-resistant *Helicobacter* spp. identified in bile and gallbladder tissues might be associated with gallbladder disease [1, 2]. Bacteria closely resembling *H. pylori* have been detected in resected gallbladder mucosa [3] and bile samples [4–6]. Furthermore, Figura and co-workers proposed that *H. pylori* present in bile samples might represent a risk factor in gallstone formation [5]. A possible consequence of colonization by *Helicobacter* spp. is a chronic inflammation in the gallbladder mucosa. This inflammation may impair gallbladder mucosa acid secretion and acidification of the content [7] reducing the solubility of calcium salts in gallbladder bile and increase the risk of their precipitation in the lumen [8]. The presence of DNA from *H. pylori*

has also been shown in cholesterol gallstones [9]. This study was undertaken to evaluate the presence of *Helicobacter* genus in general, and *H. pylori* spp. in particular, in human gallstones by means of culture and PCR methods.

A total of 33 patients (age 18–70 years) scheduled for laparoscopic cholecystectomy in Dena and Nemazi Hospitals in Shiraz, Iran, were included in this study. Their gallbladders were pathologically studied and the patients were categorized to two groups according to pathology findings: mild chronic cholecystitis ( $n=25$ ) and asymptomatic gallstone ( $n=8$ ). Two samples including gallstone and bile were provided from each patient. From each sample one part was put into a tube containing transfer media (brain heart infusion broth supplemented with 20% glucose) and sent to the laboratory to be cultured for *Helicobacter* as soon as possible (<2 h). The other part of each sample was put into a sterile bottle and kept

\* Author for correspondence: Dr Sh. Farshad, Professor Alborzi Clinical Microbiology Research Centre, Shiraz University of Medical Sciences, Shiraz 71935, Iran.  
(Email: s\_farshad@yahoo.com)

frozen at  $-70^{\circ}\text{C}$  until subsequent preparation for PCR.

Bile samples from 40 autopsy gallbladders with normal histology from persons aged 20–67 years were obtained and considered as normal control samples in PCR.

Gallstones from patients were gently homogenized and cultured on rapid urease test media and *Brucella* agar base (Merck, Darmstadt, Germany) supplemented with 10% lysed horse blood and the following antibiotics: amphotericin B (2 mg/l), trimethoprim (5 mg/l) and nalidixic acid (10 mg/l) (Sigma Chemical Co. Deisenhofen, Germany). A total of 50  $\mu\text{l}$  of each bile sample was also directly transferred to the above-mentioned media. The *Brucella* agar media were kept in a microaerophilic atmosphere at 6%  $\text{O}_2$ , 7.1%  $\text{CO}_2$ , 7.1%  $\text{H}_2$ , 79.8%  $\text{N}_2$  at  $37^{\circ}\text{C}$  for 5–10 days. The bile samples and homogenized stones were also evaluated for presence of *H. pylori* by direct Gram staining.

The frozen stones were put in 1.5  $\mu\text{l}$  Eppendorf tubes and 200  $\mu\text{l}$  sterile digestive buffer [Tris–HCl, 20 mM (pH 8.0); EDTA– $\text{Na}_2$ , 2 mM; Triton X-100, 1.2%] was added to each tube. The content of the tubes was homogenized using a sterile plastic pestle. A total of 7  $\mu\text{l}$  lysozyme (50 mg/ml) was added to each tube and incubated at  $37^{\circ}\text{C}$  for 30 min followed by the addition of 10  $\mu\text{l}$  proteinase K (20 mg/ml) and incubated at  $56^{\circ}\text{C}$  for 2 h. The homogenates were kept at  $37^{\circ}\text{C}$  overnight. Finally, the lysates were centrifuged and the supernatants were transferred to new microtubes.

In total, 100  $\mu\text{l}$  of each bile sample was washed  $2 \times 3$  times with PBS until its pH was adjusted to 7.4–7.6. The pellet was resuspended in 170–200  $\mu\text{l}$  lysis buffer [Tris–HCl, 50 mM (pH 8.0); EDTA, 100 mM; NaCl, 100 mM; SDS, 1%] + 0.5 mg/ml proteinase K followed by 2 h incubation at  $56^{\circ}\text{C}$ . The samples were then kept at  $37^{\circ}\text{C}$  overnight. After centrifugation, the supernatants were transferred to new microtubes. DNA was isolated from the supernatants obtained from previous steps by a sequence of procedures including respectively: extraction via phenol chloroform, precipitation with ethanol, and dissolution in sterile double-distilled water. Then 10  $\mu\text{l}$  of the final DNA samples were tested to investigate the existence of the genes specific for *Helicobacter* genus and *H. pylori* spp.

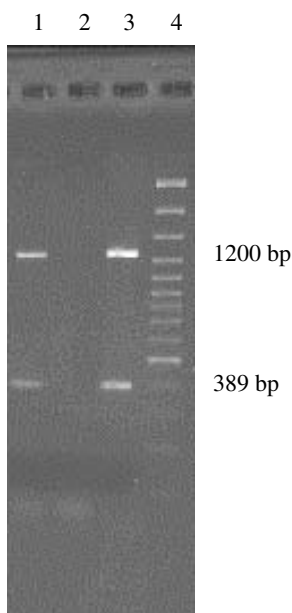
DNA from pure *H. pylori* isolates that were confirmed by their morphology upon Gram staining and by positive oxidase, catalase and rapid urease tests

was extracted using phenol chloroform protocol for use as a positive control in our PCR.

In order to improve simultaneous detection and identification of *Helicobacter* genus in general and *H. pylori* specifically and reduce number of amplifications needed, we established a Multiplex PCR using two sets of primers (TIB morLBI, Syntheselabor, Berlin, Germany). A primer pair (Hcom1 and Hcom2) on the basis of 16s rRNA gene sequence of *Helicobacter* genus, were with the sequences 5'-GTA AAG GCT CAC CAA GGC TAT-3' and 5'-CCA CCT ACC TCT CCC ACA CTC-3' to amplify a fragment of 389 bp [10]. The second set of primers (Hicd1 and Hicd2) on the basis of an isocitrate dehydrogenase gene sequence of *H. pylori* spp., were with the sequences 5'-ATG GCT TAC AAC CCT AAA ATT TTA CAA AAG CC-3' and 5'-TCA CAT GTT TTC AAT CAT CAC GC-3' to amplify a fragment of 1200 bp [11]. To optimize the condition, DNA extracted from positive control (pure *H. pylori*) was used. The optimized reaction was performed in a volume of 50  $\mu\text{l}$  comprising 50 pm each primer, 10  $\mu\text{l}$  of chromosomal DNA from positive control, 2 U of *Taq* DNA polymerase, 0.2 mM deoxynucleoside triphosphates and 2 mM  $\text{MgCl}_2$  in a gradient thermal cyclor (Eppendorf, Germany). The cycle profiles were set as follows: initial denaturation at  $94^{\circ}\text{C}$  for 5 min, denaturation at  $94^{\circ}\text{C}$  for 1 min, primer annealing at  $55^{\circ}\text{C}$  for 2 min, and 2 min extension at  $72^{\circ}\text{C}$ . The samples were amplified for 30 cycles followed by 10 min at  $72^{\circ}\text{C}$ . PCR products were analysed by electrophoresis of a 10  $\mu\text{l}$  aliquot using 1.5% (w/v) agarose gel. The sizes of the PCR products were estimated by comparison with 100-bp DNA size markers (MBI Fermentas, Hanover, MD, USA). Negative control reactions with distilled water were performed with each batch of amplification to exclude the possibility of contamination.

A determined concentration of DNA extracted from pure *H. pylori* was serially diluted (dilution from  $10^{-1}$  to  $10^{-6}$ ) and optimized Multiplex PCR was done on these dilutions. According to the genomic DNA molecular weight of *H. pylori* [12] sensitivity of this PCR assay was determined based on the highest dilution of DNA in which the primers could amplify their specific sequences.

The specificity of optimized Multiplex PCR was tested using DNA extracted from nine non-*Helicobacter* control bacteria including: *E. coli*, *Enterobacter* spp., *Klebsiella* spp., *Shigella* spp., *Streptococcus viridans*, *Campylobacter* spp., *Proteus*



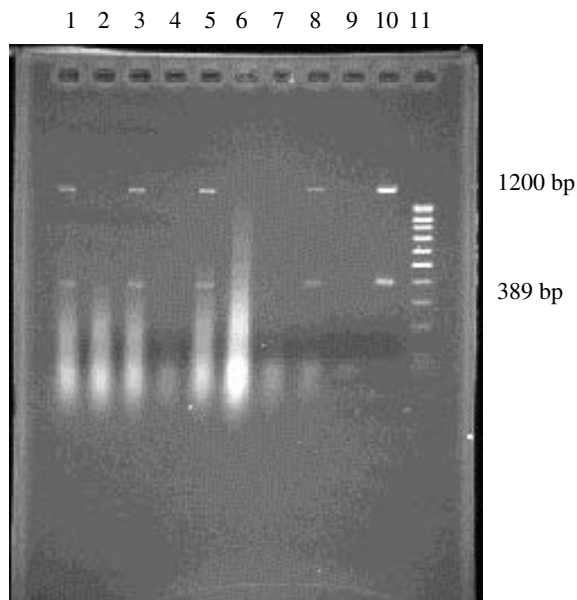
**Fig. 1.** Multiplex PCR amplification of the 16s RNA region (389 bp) and isocitrate dehydrogenase gene (1200 bp) of *H. pylori* in positive control (1, 3) negative control (2) and molecular marker (4).

*mirabilis*, *Salmonella typhi*, and *Haemophilus influenzae*.

The established sensitive and specific Multiplex PCR was performed on DNA extracted from control normal samples (bile) and patient samples (bile and gallstone) to determine the existence of genomic DNA of *Helicobacter* genus member in general, and *H. pylori* spp. in particular, in these samples.

To test the inhibitory effect of bile components on PCR procedure we spiked five randomly selected negative bile samples with pure *H. pylori*. For that we prepared a standard suspension of bacteria according to the 0/5 McFarland tube. Ten-fold serial dilutions of the *H. pylori* cell suspension were made and bile samples were spiked with these concentrations. These samples were processed and DNA extraction was performed in parallel with other samples as explained above.

None of the gallstone and bile samples from patients was positive in the rapid urease test, Gram staining and culture for *Helicobacter*. Our optimized Multiplex PCR amplified two fragments of the expected sizes of 389 bp and 1200 bp from a DNA preparation of *H. pylori*-positive control, using Hcom1–Hcom2 and Hicd1–Hicd2 primers respectively (Fig. 1). Our Multiplex PCR proved to be 100% specific for *Helicobacter* genus and *H. pylori* spp.-specific genes and did not result in a false-positive



**Fig. 2.** Amplification of the 16s RNA region (389 bp) and isocitrate dehydrogenase gene (1200 bp) of *H. pylori* in positive bile (1), negative bile (2), positive gallstones (3, 5, 8), negative gallstones (4, 6, 7), negative control (9), positive control (10), and molecular marker (11).

with *E. coli*, *Enterobacter* spp., *Klebsiella* spp., *Shigella* spp., *Streptococcus viridans*, *Compylobacter* spp., *Proteus mirabilis*, *Salmonella typhi* and *Haemophilus influenzae*. The 389 bp and 1200 bp fragments were amplified by this Multiplex PCR assay from a minimum of 0.03 pg of *H. pylori* DNA equivalent to 150 organisms. *H. pylori* DNA was identified in 18.1% (6 out of 33) of stone and 12.1% (4 out of 33) of bile samples from patients (Fig. 2). All these patients were categorized in the mild chronic cholecystitis group ( $n=25$ ). None of bile samples from the control group was positive in PCR for *Helicobacter* DNA. Two expected fragments of 389 bp and 1200 bp were amplified in all five bile samples spiked with a lower detection limit of 500 *H. pylori* per ml. No amplification was observed in DNA extracted from parallel non-spiked negative bile samples.

Several authors have reported that *H. pylori* DNA has been found in human bile [4–6]. It has been concluded that the presence of *H. pylori* in bile may represent an increased risk of gallstone formation [5]. One study from Mexico failed to detect an association between gallstones and *Helicobacter* colonization [13]. Furthermore, the results of two different studies in Korea and Hong Kong did not substantiate a definite role of *Helicobacter* spp. in the

pathogenesis of biliary diseases [5, 14]. Chen et al. concluded that *Helicobacter* infection alone may not play a significant role in the formation of gallstones [15]. However Monstein et al. reported the presence of *H. pylori* DNA in 55% of cholesterol gallstones [9]. In our study, we could not isolate *Helicobacter* from gallstone and bile samples using culture method. All other studies on *Helicobacter* culture in gallbladder samples were not successful [2]. This may be because the strains identified by other methods in gallbladder are not culturable. However, the process in gallbladder gallstone formation may take a longer time, and the embedded bacteria may be destroyed or killed. The PCR technique has a high sensitivity and specificity compared to routine bacterial culture. In order to identify *Helicobacter* DNA in our samples, we established a Multiplex PCR using two pairs of primers based on the 16S rRNA gene (for genus) and isocitrate dehydrogenase gene, *icd* (for species) of *H. pylori* [2, 11]. Argyros et al. showed that the *icd* gene was very specific in identifying *H. pylori*, and also excluding any false-positive amplification with other bacteria close to *H. pylori* following species-specific protein antigen primers [11]. Using our sensitive and specific Multiplex PCR protocol we identified *H. pylori* DNA in 18.1% of stone and 12.1% of bile samples. All our positive patients were categorized in the mild chronic cholecystitis group. None of bile samples from the control group was positive in PCR. It seemed that the results obtained from different parts of world are very discordant. Some of these inconsistencies may be attributed to geographical or regional variations in the distribution of bile-resistant *Helicobacter* spp. The other possible explanation for these discrepancies is due to the accuracy of different tests. Several different kinds of primers, 16S rRNA, *ureA*, *ureB* or 26 kDa antigen have been described and validated for *H. pylori* [16, 17]. These primers are not confined to detect *H. pylori* and can detect other *Helicobacter* spp. or even some other genus members similar to *H. pylori*. On the basis of these data Roe et al. [2] suggested confirmatory amplification using a second pair of primers from a different gene. In the present study, the PCR amplifications were done on the basis of a Multiplex PCR using two pairs of primers, 16S rRNA and isocitrate dehydrogenase genes. So using a unique PCR in different parts of world is recommended to better compare the results. Another reason, which can be important in establishing an association between *Helicobacter* infection and gallstones, is that in

different studies different samples have been tested. In most studies, the samples of bile and tissue from patients with gallstones have been considered [15, 18], while in others only stone samples were subjected to PCR [9]. In the present study, we tested bile samples from patients and a control group in parallel with stone samples to exclude any possibility of contamination of the stones with the organisms if they existed in the bile as normal flora. On the other hand, to exclude any inhibitory effects of bile components on PCR, we spiked five randomly selected negative bile samples with pure *H. pylori* and no inhibitory effects were demonstrated. Therefore, according to our data, it seems that the existence of *H. pylori* in the stone samples is more dominant in comparison with the bile samples, moreover, this organism cannot be a normal flora of the environment of gallbladder, because we did not identify its DNA in the samples of the control group. So where is *H. pylori* DNA in gallstones from? Molecular detection of organisms in bile may simply be due to the enterohepatic circulation of DNA. On the other hand *H. pylori* infection may serve as an initiating factor or play other important roles in the development of gallstones. In both situations *H. pylori* or its DNA will be more concentrated and captured inside gallstones during the stone formation.

In conclusion, in this study *H. pylori* DNA could be detected in stone samples of Iranian patients with gallstones. But we are not sure if these organisms are viable. To clarify the clinical role of *Helicobacter* spp. in gallbladder disease studies with accurate tests on larger patient and control groups are needed to ascertain whether this microorganism is an innocent bystander or active participant in gallstone formation.

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