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Original Article

Detection of *Helicobacter* DNA in Bile Samples of Patients with Biliary Diseases Living in South of Iran

S. Farshad, A. Alborzi, S.A. Malekhosseini[1], B. Gramizadeh[2], B. Oboodi, M. Rasouli, A. Japoni, M. Kalani, B. Pourabbas

Abstract

Background: It has been reported that several species of Helicobacter colonize the biliary tract of animals and human, but their participation in hepatobiliary diseases are not established. This study is undertaken to determine if Helicobacter genus members, especially with regard to *H. pylori*, could be detected in the bile and gallbladder diseases.

Methods: Thirty-eight gallbladder tissues and thirty-six bile samples recovered from 38 patients with different gallbladder diseases were subjected to rapid urease test, culture and an established Multiplex-PCR using two pairs of primers based on 16s rRNA and isocitrate dehydrogenase genes specific for Helicobacter genus and *H. pylori* species, respectively. Bile and tissue samples from 40 autopsy gallbladders with normal pathology were tested by PCR as control group.

Results: In 4 of 36 bile samples *H. pylori* was identified using PCR. None of the tissue samples of the patients and controls were positive in PCR for Helicobacter DNA. We were not successful in isolation of Helicobacter using culture method.

Conclusion: This study showed the presence of *H. pylori* DNA in the bile samples of a small sample size of patients with biliary diseases in south of Iran. We could find no pathogenetic role for *H. pylori* in the formation of hepatobiliary disease. To establish a clinical role for Helicobacter species in the hepatobiliary diseases, more studies on a larger group of patients and control groups are needed to ascertain whether Helicobacter species or their component might have a role as a causative agent or cofactor in the pathogenesis of biliary tract diseases in human.

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Keywords ● *H. pylori* ● biliary diseases ● Cholecystitis ● human

Professor Alborzi Clinical Microbiology Research Center, Departments of Surgery[1], and Pathology[2], Nemazee Hospital, Shiraz University of Medical Sciences, Shiraz, Iran.

Correspondence:
Shohreh Farshad PhD,
Professor Alborzi Clinical Microbiology Research Center,
Nemazee Hospital,
Shiraz University of Medical Sciences,
Shiraz, Iran.
Tel: +98 711 6262225
Fax: +98 711 6287071
E-mail: s_farshad@yahoo.com

Introduction

Helicobacter pylori (*H. Pylori*) is a gram negative and microaerophilic microorganism that can cause chronic gastritis, gastric and duodenal ulcers and gastric adenocarcinoma.[1,2] In the last few years, the scientists have been interested in studying the relationship between *H. pylori* infection and various extra-digestive diseases.[2,3] Bile acids are generally

known to have inhibitory effects on the adherence and growth of *H. pylori* in vitro.[4,5] The in vitro bacteriostatic effect of bile has not been demonstrated to the same degree in vivo, suggesting the adaptive conditioning of *H. pylori*. It has been shown that a lower pH is more conducive to the survival of *H. pylori*.[4] According to the animal models, it seems that various *H. Pylori* species and possibly other newly bile adapted species which can cause disease in dogs, mice and other rodents, may also invade the human bile tree and liver.[6]

While studies in Taiwan and Korea regularly seem to detect Helicobacter in human bile in chronic cholestatic bile tract diseases, conflicting negative PCR results on bile have been reported.[7,8] On the other hand, recently a correlation between presence of *H. pylori* DNA in bile and bile duct malignancies has been shown.[9] Considering the results about the role of Helicobacter microorganism as a causative agent in biliary tract diseases, supportive studies are needed to clarify this hypothesis. The present study was performed to investigate if members of *H. Pylori* genus in general and *H. pylori* species in particular, can be detected by PCR method in bile and gallbladder samples of Iranian patients with gallbladder diseases.

Materials and Methods

Patients and normal controls

A total of 38 patients, scheduled for laparoscopic cholecystectomy in Dena and Nemazee Hospitals of Shiraz, Iran were included in this study. Two samples of gallbladder tissue and bile were provided from each patient. Totally 38 gallbladder tissues and 36 bile samples were provided and examined. One part of each sample was transferred into a tube containing Brain Heart Infusion Broth, supplemented with 20% glucose, and cultured in the laboratory for Helicobacter as soon as possible (less than 2 hours). Other part of each sample was put into a sterile bottle and kept frozen at -70°C for subsequent preparation for PCR. Finally, a piece of gallbladder tissue was transferred to formalin and sent for histopathological studies. The gallbladder tissues and bile samples from 40 with normal histology were obtained and considered as normal control samples for PCR.

Culture

The gallbladder tissue samples provided from patients were gently homogenized and cultured on rapid urease test media and brucella agar base (Merck; Germany) supplemented with 10% lysed horse blood and amphotericin B, trimethoprim and nalidixic acid. 50 µl of each bile sample was directly transferred to the above-mentioned media. The brucella agar media were kept in a microaerophilic atmosphere at 37°C in a gas pack jar for 5-10 days. The bile samples and homogenized tissues were also examined for presence of *H. pylori* by direct gram staining.

DNA extraction

Preparation of the tissues

The superficial cell layers and mucous were scraped from each gallbladder sample with a surgical blade and then homogenized. In a 1.5 µl micro tube 300 µl of digestion buffer (NaCl 100 mM, Tris-HCL 10mM [pH, 8.0], SDS 0.5%, EDTA 25mM) was mixed with 100µl of homogenized tissue followed by addition to each tube of 7 µl of proteinase K at concentration of 20mg/ml (MBI, Fermentans, USA). The tubes were then vortexed and incubated at 56°C for 2 hours and then kept at 37°C while shaking. After 4-5 days, all tissue lysates were centrifuged and the supernatants were separated.

Preparation of bile samples

One hundred µl of each bile sample was washed 2-3 times with Phosphate Buffer Saline (PBS) until pH was adjusted to 7.4-7.6. The pellet was re-suspended in 170-200 µl lysis buffer [all in mM; Tris-HCL (50); pH 8.0], EDTA (100), NaCl (100), SDS 1%) + 0.5 mg/ml Proteinase K followed by two hrs incubation at 56°C. The samples were then kept at 37°C overnight. After centrifugation, the supernatants were transferred to new micro tubes.

DNA was extracted from the supernatants obtained from previous steps by a sequential procedure including extraction with phenol chloroform, precipitation with ethanol, and dissolution in sterile double distilled water. 10 µl of the final DNA samples were tested for investigation of the presence of the genes specific for Helicobacter genus and *H. pylori* species.

PCR for Helicobacter in the samples

In order to improve simultaneous detection and identification of Helicobacter genus in general and *H. pylori* specifically and reduce the number of amplifications needed, we used a previously established Multiplex-PCR.[10]

This sensitive and specific Multiplex PCR was performed on DNA extracted from bile and gallbladder tissue of autopsied gallbladders as normal control samples as well as samples of bile and gallbladder tissues from patients, to determine the presence of genomic DNA of the member of Helicobacter genus in general, and *H. pylori* species in

particular. Two sets of primers (TIB MOL-BIOL, Syntheselabor GmbH, Berlin, and Germany) were used in this protocol. A primer pair (Hcom1 and Hcom2) on the basis of 16s rRNA gene sequence of Helicobacter genus, denoting sequences of 5′-GTA AAG GCT CAC CAA GGC TAT-3′ and 5′-CCA CCT ACC TCT CCC ACA CTC-3′ were used to amplify a fragment of 389-bp.[11]

The second set of primers (Hicd1 and Hicd2) based on an isocitrate dehydrogenase gene sequence of H. pylori species, having the sequences of 5′-ATG GCT TAC AAC CCT AAA ATT TTA CAA AAG CC-3′ and 5′-TCA CAT GTT TTC AAT CAT CAC GC-3′ were used to amplify a fragment of 1200- bp.[12] DNA from pure *H. pylori* isolated from gastric biopsy and confirmed by their morphology after gram staining and by positive oxidase, catalase and rapid urease tests, was extracted using phenol chloroform protocol and used as a positive control in our PCR.

Bile inhibitory test

To test the inhibitory effect of bile components on PCR procedure, we spiked 5 randomly selected negative bile with pure *H. pylori* and 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 the bile samples were spiked with these concentrations. These samples were processed and DNA extraction was performed in parallel with other samples.

Results

According to the pathological findings we categorized our patients into 4 groups (Table 1). In pathology examination, all 40 control tissues recovered from autopsy gallbladders were normal. None of the gallbladder tissues and the bile samples from patients with gallbladder diseases was positive for *H. pylori* using culture methods. But using specific and sensitive PCR, Helicobacter DNA was detected in 11% (4 from 36) of the bile samples of 25 patients that were categorized as being in the mild chronic cholecystitis group. *H. pylori* DNA was not detected in tissue and bile samples of control group and tissue samples of patients (Table 2).

Table 1: Categorization of patients with biliary diseases according to their pathological findings

Pathological Groups	n (%)
Mild Chronic Cholecystitis	25 (65.8)
Moderate Chronic Cholecystitis	2 (5.6)
Mild acute Cholecystitis	3 (7.9)
Gallstone with no pathological changes	8 (21.0)
Total	38 (100)

Table 2: Percents of Bile and Tissue samples obtained from patients with different biliary diseases positive for *H. pylori* in different diagnostic tests.

Diagnostic Test	Sample			
	Bile		Tissue	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)
Culture	0	100	0	100
RUT	0	100	0	100
PCR	11	89	0	100

Rut= Rapid Urease Test

Discussion

Since the first detection of *H. pylori* in bile, there are many different reports of identification of this organism from different hepatobiliary diseases in human which could not establish any certain correlation between *H. pylori* infection and these diseases yet.[13] In this regard PCR results for Helicobacter identification in bile samples are varying from negative to a maximum of 56.5%.[14-17] For the tissue samples, rates of 3% to 39% have been reported for detection of Helicobacter DNA.[17-19] The control samples had 0-40.5 % of positivity for Helicobacter species DNA.[16,20,21] All the studies about Helicobacter culture in bile have not been successful.[22]

Our results obtained from the present study were consistent with some of the reports mentioned above. We couldn't isolate any *H. pylori* microorganism from bile and gallbladder tissue samples using culture method, even that fresh samples were transferred to the lab in less that 2 hours in transport media specific for *H. pylori*.

The culture of this fastidious organism is extremely difficult, if not impossible and maybe the strains have been identified by other methods in gallbladder are not culturable. It is also possible that after entrance of sensitive Helicobacter to the bile system it loses its viability in the presence of salt components of bile but its DNA and antigens persist there for a longer time. Of course the inhibitory effects of bile alone may inhibit the growth of the organisms, producing a false negative result. Therefore, repetitive efforts to culture would be necessary. The first report of successful culture from a liver biopsy specimen has been reported only recently.[23]

We used a previously established multiplex-PCR to identify Helicobacter members in general, and *H. pylori* species, in particular in our samples.[10] Two pairs of primers based on 16S rRNA gene for genus and isocitrate dehydrogenase gene, icd, for species of *H. pylori* were employed in this protocol.[11,22] It was showed that the icd gene is very specific to identify *H. pylori* and to exclude any false positive amplification with other bacteria close to *H. pylori* followed by using species-specific protein antigen primers.[11]

Using our sensitive and specific PCR protocol we identified *H. pylori* DNA in 11.1% (4 of 36) of bile samples from patients. All these 4 patients had Mild Chronic Cholecystitis. PCR was negative for tissue samples of patients and bile and tissue samples of control group. In other studies Helicobacter species DNA has been detected in bile samples of 56.5% of patients with Chronic Cholecystitis.[17,19]

According to these data it seems that *H. pylori* cannot be a normal flora of gallbladder but maybe a relationship exists between the conditions in Chronic Cholecystitis and the presence of *H. pylori*. However, Fallone et al. reported a negative result for any correlation between the presence of Helicobacter DNA and acute and Chronic Cholecystitis in German patients.[8] This study pointed out that one reason for these discordant results obtained from different parts of the world might be due to regional differences.

Different infection prevalence rates have been reported in different geographical regions. As no correlation was shown between the presence of Helicobacter species DNA in bile and the presence of *H. pylori* in the antrum,[20] the regional differences among distribution of Helicobacter species in biliary tract could not be related to the distribution of this organism in gastric diseases. As the presence of some species of Helicobacter other than *H. pylori* such as *H. bilis, H. pullorum, H. rodentum,* and *Flexispira. rappini*, in hepatobiliary tree has been reported,[13,17,19] their distribution in different parts of the world could be different. So it seems to establish an association between *H. pylori* colonization and biliary diseases, a larger number of samples is required to be tested in different laboratories.

The other possible explanation for these discrepancies is that several different kinds of primers similar to 16S rRNA, *ureA, ureB* or 26 kDa antigen have been described and validated for diagnosis of *H. pylori*.[24,25] However, these are not solely confined to the detection of *H. pylori* because these antigens can detect other Helicobacter species or even some other genus members similar to *H. pylori*. On the basis of these data, confirmatory amplification using a second pair of primers from a different gene has been suggested.[22] In the present study, PCR amplifications were carried out on the basis of a Multiplex-PCR using two pairs of primers based on 16S rRNA and icd genes. Therefore, application of a unique Multiplex-PCR protocol and primers in different parts of the world is recommended to validate and interpret the results more accurately.

Conclusion

PCR technique can detect Helicobacter DNA in bile samples of Iranian patients with mild chronic cholecystitis without having a pathogenic role in hepatobiliary diseases. To clear the clinical role of Helicobacter species in the hepatobiliary diseases, more studies on larger populations of patients and control groups are needed to ascertain whether they have a role as a causative agent or cofactor in the pathogenesis of biliary tree diseases. Experimental infection in biliary tract of animal models is also recommended.

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