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SHORT COMMUNICATION

Lack of Association between *Helicobacter pylori* Infection and Biliary Tract Diseases

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Abstract

There are ambiguous results about the involvement of *Helicobacter species* in production of hepatobiliary diseases. This study was aimed to investigate any possible association between the presences of *Helicobacter* spp., their genotypes and occurrence of different biliary diseases. Cultures of 102 bile samples for *Helicobacter* spp. did not show any growth, but the presence of *Helicobacter* genus specific DNA (*16s rRNA* gene) was detected in 3.92% of them. No significant association was found between development of the diseases and presence of the bacteria. All the *Helicobacter* genus positive samples belonged to *H. pylori* species and showed *vacA*$^+$ (s_1/m_2), *cagA*$^-$ genotypes.

Key words: *Helicobacter pylori*, bilary diseases

Helicobacter pylori had been found by Marshall in 1986 in gastric biopsy specimens. The bacterium is associated with many diseases in the gastrointestinal tract. *H. pylori* DNA had been detected in human liver tissue samples of patients with primary sclerosing cholangitis and primary biliary cirrhosis (Warren and Marshall, 1983, Kawaguchi *et al.*, 1996, Roe *et al.*, 1999). There are also some reports confirming the presence of non *Helicobacter pylori* species, such as *H. pullorum, H. canis, H. cholecystus, H. rappini, H. hepaticus,* and *H. bilis* in the liver, bile and gallbladder tissues (Vorobjova *et al.*, 2006, Matsukura *et al.*, 2002).

Several species of *Helicobacter* genus are believed to play major roles in the causation of gallbladder cancer. Previously, it was found that *H. pylori* was not associated with the gallbladder diseases (Roe *et al.*, 1994), but in some studies it was found to be associated with the biliary tree and gallbladder cancers (Cover *et al.*, 1992, Presser *et al.*, 2003).

Free bile acids in the human bile can kill *H. pylori*, however the inhibitory effect of bile acids on the survival of this bacterium is still unclear (Hanninem, 1991). It can guess that at numbers of certain pathological conditions such as bile duct obstruction, bile composition can be altered and thereby its inhibitory effect on the growth of *H. pylori* might decrease or disappear (Roe *et al.*, 1994).

Two major important virulence markers of *H. pylori*, cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA) had been well described (Xiang *et al.*, 1995). It has been reported that the cagA is present in approximately 60% of *H. pylori* strains from Western populations but over 90% of the strains from Southeast Asian populations (Truong *et al.*, 2009).

In contrast to *cagA*, *vacA* is present in nearly all the *H. pylori* strains around the world, only half of which express *cagA*, concurrently (Atherton, 1998). CagA is considered as the sole bacterial oncoprotein responsible for gastric carcinogenesis and VacA is a virulence marker that induces cell vacuolation. Colonization of *Helicobacter* spp. in the biliary tract has been implicated as a possible cause of hepatobiliary diseases ranging from chronic cholecystitis and primary sclerosing cholangitis to gallbladder cancer and primary hepatic carcinoma (Mishra *et al.*, 2010). Although Helicobacter species have identified in the bile, tissue and stones of patients with benign biliary diseases, due to differing results that have been obtained from different studies in diverse geographical regions, no causative relationship could be established for their roles in the disease occurrence (Neri *et al.*, 2005, Abayli *et al.*, 2005, Francavilla *et al.*, 2000).

In this study, we investigated the presence of *Helicobacter* species in the bile samples of patients with

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gallstone disease. We also evaluated any probable associations between the presence of *Helicobacter* DNA and the biliary diseases. From August 2010 through February 2011, 102 bile samples were collected with ERCP (Endoscopic Retrograde Cholangiopancreatography) from patients referred to Taleghani hospital in Tehran, Iran. Obstruction of bile duct, bile duct cancer, gallstone and related inflammatory disease were considered as reasons for the ERCP. All the biles samples were obtained from biliary drainage tubes; at least two milliliters of the bile were taken by needle aspiration from each patient during the operation and were collected in a sterile container. Bile samples were cultured on Brucella agar supplemented with 10% (v/v) sheep blood and selective supplement (vancomycin 2.0 mg, polymyxin B 0.05 mg, trimethoprim 1.0 mg) (Merck). The cultured plates were incubated at 37°C for three to five days in a microaerophilic atmosphere (5% O 2, 10% CO 2, 85% N 2) in a CO_2 incubator (Innova-Co 170; New Brunswick Scientific, Edison, NJ, USA). The remainders of the bile samples were stored at –20°C for further analysis. DNAs from the bile samples were extracted by using phenol- chloroform method (Wilson *at al.*, 1995). To detect the bacterial DNA, the 16S *rRNA* gene of the *Helicobacter* genus was amplified by PCR assay. PCR was also used for seeking the presence of *H. pylori* DNA using *glmM*, *cagA*, and *vacA* gene specific primers compared to positive control *H. pylori* strain RIGLD-133 (Table I). For *vacA* genotyping among the positive samples, signal region s_1/s_2 alleles and midregion m_2 allele of the gene were determined by multiplex PCR (Table I). The PCRs were performed in applied thermal cyclers (Eppendorf, Hamburg, Germany). All PCRs in this study were performed in a volume of 25 µL containing: 1X PCR buffer, 500 nM of each primer, 2 mmol/L of $MgCl_2$; 200 µM of each deoxyribonucleotide triphosphate (dNTP), 1.5 U of Taq DNA polymerase, and 200 ng of DNA sample under the following conditions: initial denaturation for 5 min at 94°C followed by 30 cycles of 93°C for 1 min, 58°C for 30 s and 72°C for 1 min. The amplified products were identified by electrophoresis in 1.0% agarose gel.

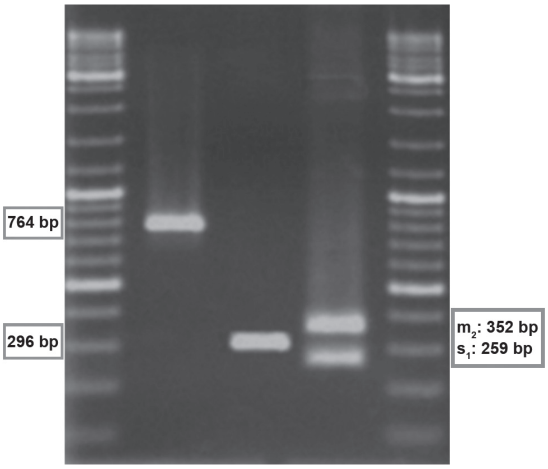

Fig. 1. PCR results for 16S rDNA, *glmM* and *vacA* genes.
Lane 1 and 5 ladder mix; lane 2: PCR product for *16S rRNA*; lane 3: PCR product for *glmM* and lane 4: PCR product for *vacA* gene.

Among 102 patients under the study, highest clinical problems were belonged to biliary stone disease. The biliary stones were detected in 52% of the patients. All the cultured bile samples for *Helicobacter* spp. did not show any growth for the bacterium, but *Helicobacter* sp. specific PCR result for *16S rRNA* gene were positive in four samples (3.92%). PCR for *glmM* gene demonstrated all of the identified *Helicobacter* spp. as *H. pylori* species (Fig. 1). No significant association was detected between the type of diseases and presence of the bacterium. Although *vacA* gene was found in all of the *H. pylori* DNA samples (100%), but they did not harbor *cagA* gene, interestingly (Table II).

Table I
Primer sequences that used in this study

Gene	Gene specific primer sequences (5'→3')	Product size (bp)	Annealing temperature (°C)
vacA s_1/s_2	**F:** ATGGAAATAC AACAAACACAC **R:** CTGCTTGAATGCGCCAAAC	259/286	55
vacA m_2	**F:**:GGAGCCCCAGGAAACATTG **R:** CATAACTAGCGCCTTGCAC	352	55
glmM	**F:** GGATAAGCTTTTAGGGGTGTTAGGGG **R:** GCTTACTTTCTAACACTAACGCGC	296	58
cagA	**F:** AATACACCAACGCCTCCAAG **R:** TTGTTGCCGCTTTTGCTCTC	349	57
16S rRNA	**F**: GGCTATGACGGGTATCCGGC **R:** GCCGTGCAGCACCTGTTTTC	764	57

Table II
Association of *Helicobacter* spp. and biliary diseases

	Gallstone disease (74)	Biliary– pancreatic Malignancy (15)	Other disease (13)	Total (102)
Positive culture[1]	0	0	0	0
16S rDNA	2	1	1	4
glm gene	2	1	1	4
vacA gene	2	1	1	4
cagA	0	0	0	0
s1-s2	S1	S1	S1	–
m1–m2	m2	m2	m2	–
H. pylori positive samples	2	1	1	4

[1] Culture of samples was done on *Helicobacter* specific media.

Benign diseases of the hepatobiliary system and the stone diseases are encountered as clinical problems in all parts of the world. Correlation of bacterial infections and their products with these diseases are of the major concerns. Presence of *Helicobacter* DNA has been investigated in the bile and biliary tissue of human beings with diverse biliary diseases (Warren and Marshall, 1983, Kawaguchi *et al.*, 1996, Roe *et al.*, 1999). Presser Silva *et al.*, 2003 investigated the presence of *Helicobacter* species by culture of gallbladder tissue and bile samples. Result of this investigation was similar to our results, as their efforts for culture of *Helicobacter* spp. from the bile samples had not been successful (Presser *et al.*, 2003). Some species of *Helicobacter* genus may be unculturable in common culture media. Viability of these bacteria strictly affected in the bile duct during their infections that limits their detection in these samples by conventional methods. Molecular studies can confirm the existence of *Helicobacter* spp. DNA in the culture negative samples. Some studies from Germany and Mexico failed in detecting the presence of DNA of *Helicobacter* spp. (Mendez-Sanchez *et al.*, 2001, Rudi *et al.*, 1999), but Matsukura *et al.* had detected different non *H. pylori* strains by analyzing the *16s rRNA* gene in the bile samples (Matsukura *et al.*, 2002). Frequency of this presence was 59.2%. Lowered risk of the infection by the pets, major reservoirs of non- *Helicobacter pylori* species, in Iran can explain absence of non *Helicobacter pylori* species in our study among the different bile samples. However, in comparison to results obtained by Farshad *et al.*, (18.2%) and Abayli *et al.*, (9.1%), frequency of *H. pylori* in our bile samples was lower (3.92%) (Abayli *et al.*, 2005, Farshad *et al.*, 2004).

In addition to the direct role of *H. pylori* in biliary diseases, it may also promote the risk of stone formation by acting as a foreign body to form a nidus around which the stone may develop or it may produce hydrolyzing enzymes or nucleating proteins like immunoglobulins. CagA protein of *H. pylori* has been found to have a homology with aminopeptidase and hence can increase the risk for gallstone formation (Maurer *et al.*, 2005). In our study, 50% of the bile positive samples were belonged to patients with bile stone and 50% to patients with malignancy or other diseases. There wasn't any statistically significant association between the presence of *H. pylori* and the bile diseases. All of the *H. pylori* isolates in our study were *cagA* negative which can to some extent explain the lack of this association. No other studies have yet analyzed this association.

According to several studies on gastric biopsies, s_1/m_1 is the most frequent *vacA* gene subtype in Mexico (Mendez-Sanchez *et al.*, 2001) and Japan (Ito *et al.*, 1997), in contrast to other countries such as Iran. In a recent study that was conducted in our research center on gastric biopsy samples, the s_1m_2 genotype was a frequently observed genotype in Iranian strains while s_1m_1 was more common in strains isolated from Afghani patients (Dabri *et al.*, 2010). Similarity of the common *vacA* genotypes between the gastric and biliary tract isolates could propose their gastric source of infection. This relationship was established in studied patients, as all of the positive samples showed *vacA* s_1m_2 and $cagA^-$ genotypes. In Asian countries, such as Japan (Maeda *et al.*, 1998) and Korea (Miehlke *et al.*, 1996), the proportion of cagA+ *H. pylori* strains was usually over 90% in all of the isolates that is higher than the isolates in Iran (~60%). Additional studies in this field are needed to clear more details about roles of *non-pylori* and *H. pylori* genotypes and diversity in their virulence factors in the production of biliary diseases.

In conclusion, according to our results the relationship between *Helicobacter* spp. infections and biliary tract diseases was not supported in our patients. Homology of the identified virulence gene markers of *H. pylori* in the positive samples proposed their initial roles for pathogenesis of the biliary tract. Low rates of *H. pylori* infection among the studied samples propose a possible role for other bacteria or other predisposing factors that need future analysis.

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