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Detection of *Helicobacter* DNA in Bile from Bile Duct Diseases

Several species of *Helicobacter* colonize the hepatobiliary tract of animals and cause hepatobiliary diseases. The aim of this study is to investigate *Helicobacter* found in the biliary tract diseases of humans. Thirty-two bile samples (15 from bile duct cancer, 6 from pancreatic head cancer, and 11 from intrahepatic duct stone) were obtained by percutaneous transhepatic biliary drainage. Polymerase chain reaction analysis using *Helicobacter* specific *urease A* gene and 16S rRNA primers, bile pH measurement, and *Helicobacter* culture were performed. *Helicobacter* DNA was detected in 37.5%, and 31.3% by PCR with *ureA* gene, and 16S rRNA, respectively. The bile pH was not related to the presence of *Helicobacter*. The cultures were not successful. In conclusion, *Helicobacter* can be detected in the bile of patients with bile duct diseases. The possibility of pathogenesis of biliary tract diseases in humans by these organisms will be further investigated.

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INTRODUCTION

Helicobacter pylori has been established as a causative factor in the pathogenesis of active chronic gastritis (1) as well as peptic ulcer diseases (2, 3) since its discovery in 1983 (4). It has also been linked to the development of gastric adenocarcinoma (5, 6) and gastric mucosa associated with lymphoid tissue lymphoma (7). *Helicobacter pylori* was reported as only a human pathogen existing in the gastric antrum (8, 9), dental plaque (10), feces (11), and gastric juice (12, 13). Recently, several new species of *Helicobacter* have been isolated from the stomach, intestinal tract, and liver of various mammals. Some of the species could cause hepatobiliary disease in animals (14-19), which is similar to those in humans. Therefore data raises the potential that these hepatic *Helicobacter* or bile resistant *H. pylori* may cause hepatobiliary diseases in humans. *H. pylori* has been shown to be sensitive to both deoxycholic acid and chenodeoxycholic acid in vitro (20), which are the major free bile acids in human bile, however the inhibitory effect of bile acids on the survival of *H. pylori* is still unclear. It can be guessed that under certain pathologic conditions such as bile duct obstruction, bile composition can be altered, and thereby the inhibitory effect on the growth of *H. pylori* might decrease or disappear.

We investigated the existence of *Helicobacter* in bile of patients with obstructed bile duct disease by polymerase chain reaction using *Helicobacter*-specific 16S rRNA and *ureA* gene primers, and observed the relationship between the existence of the organism and bile pH.

MATERIALS AND METHODS

Materials

Thirty-two bile samples from the patients with biliary tract diseases were obtained by percutaneous transhepatic biliary drainage to avoid contamination by the gastrointestinal route. The mean age of the patients was 64.7 ± 15.8 years old (range: from 35 to 82), and 20 of them were male. Diseases included 15 cases of histologically proven bile duct adenocarcinomas, 6 cases of pancreatic head adenocarcinomas and 11 of intrahepatic duct stones.

pH measurement

Bile pH was measured by pH metry (ϕ 45, Beckman, USA) just after obtaining the bile samples.

Helicobacter culture

The culture for *Helicobacter* species was performed from

fresh bile samples. The media used for culture was brucella agar with 5% horse serum, trimethoprim, vancomycin, polymyxin, and amphotericin B impregnated media. In addition, selective antibiotic medium was prepared as follows: brucella agar, 5% horse serum, 50 μg/mL amphotericin B, 100 μg/mL vancomycin, 200 μg/mL polymyxin B, 200 μg/mL bacitracin, and 10.7 μg/mL nalidixic acid (Sigma Chemical Co., St Louis, MO). The plate was incubated in a microaerophilic condition at 37℃ and anaerobic condition at 90% N_2, 5% CO_2, and 5% H_2, an environment previously proved to be ideal for the growth of other rodent *Helicobacter*. The bacterial cultures were carried out for up to 7 days. Half of the bile sample was filtered through a 0.22 μm filter onto two selective plates, and was frozen at -20℃ until assayed for polymerase chain reaction analysis.

DNA extraction

Samples of 1 ml bile were pelleted by centrifugation for 10 min at 15,000 *g*, incubated with lysis buffer [20 mM Tris-HCl (pH 8.0), 1% Triton X-100, and proteinase K (10 mg/mL)] for 4 hr at 55℃, and extracted twice with equal volumes of phenol-chloroform. After extraction with chloroform, the DNA was precipitated with 3 M sodium acetate and washed with ethanol. DNA pellets were dissolved in 100 μL ddH_2O.

PCR amplification

All reactions were performed in a 50 μL volume with an automated Perkin-Elmer 2400 thermocycler (Perkin-Elmer, U.S.A.). Reaction mixtures contained each oligonucleotide primer at 1 μm, PCR buffer [50 mM Tris-HCl, 50 mM NaCl, 15 mM $MgCl_2$ (pH 8.3)], 2.5 U of *Taq* polymerase (Boehringer Manheim, Germany), and 10 μL of template DNA, unless otherwise stated. For the bile samples, *Helicobacter*-specific primer pairs H276f and H676r were used to generate 16S rRNA amplicons of approximately 375 base pairs (21). The primers have the following sequences: H276f, 5'-CTATGACGGGTATCCGGC-3' (sense); H676r, 5-ATTCCACCTACCTCTCCCA-3' (antisense). The reaction was performed with a step-cycler program of 94℃/2s, 53℃/2s, 72℃/30s for 35 cycles. The sequence of primers for detecting *Helicobacter urease A* (*ureA*) gene were HPU1, 5'GCCAATGGTAAATTAGTT-3' (sense) and HPU2, 5'-CTCCTTAATTGTTTTTAC-3' (antisense) (22). The PCR amplication conditions were denaturated at 94℃ for 30 sec, annealed at 45℃ for 30 sec, and elongated for 30 sec. The PCR reaction ended at the 40th cycles. The size of final *ureA* gene product was 411 base pairs. PCR products (10 μL) were electrophoretically separated in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. DNA markers were run on each gel to determine reaction product sizes.

Statistics

Results for pH measurement were analyzed by the Student's *t*-test. $p<0.05$ was considered significant.

RESULTS

Culture

Helicobacter sp. from the bile sample were not cultured even after prolonged incubation under both microaerophilic and anaerobic conditions.

PCR amplification with *Helicobacter* specfic primers

Among the 32 bile samples subjected to PCR analysis using the *ureA* gene primer, 12 of them (37.5%) were positive (Fig. 1). PCR positive bile samples were as follows: 7 cases of bile duct cancers, 2 cases of pancreatic cancers, and 3 cases of intrahepatic duct stones. Using the *Helicobacter* spe-

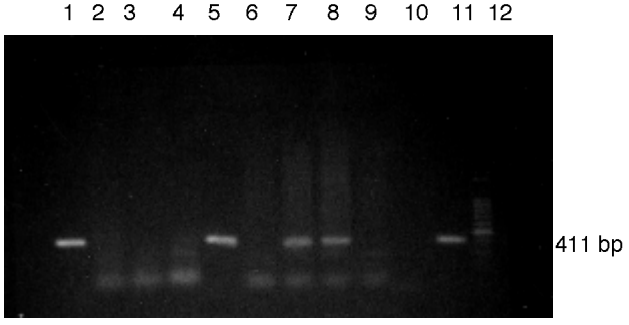

Fig. 1. PCR products of ure A (411 bp) from the bile samples. Lane 1-9, bile samples. Lane 10, negative control. Lane 11, positive control (*H. pylori* type strain ATCC 43504). Lane 12, 100 bp DNA ladder marker.

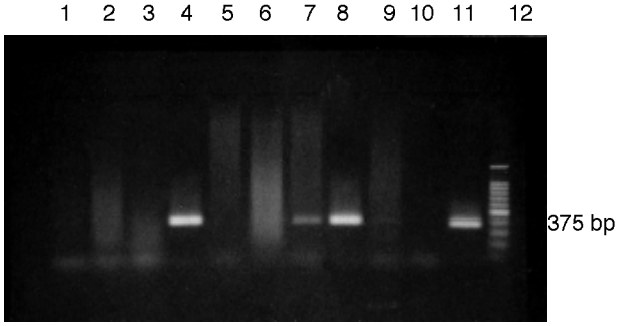

Fig. 2. PCR products of *Helicobacter* specific 16S rRNA (375 bp) from the bile samples. Lane 1-9, bile samples. Lane 10, negative control. Lane 11, positive control (*H. pylori* type strain ATCC 43504). Lane 12, 100 bp DNA ladder marker.

cific 16S rRNA primer, amplicons were positive in 10 cases of 32 samples (31.3%). Six cases were diagnosed as bile duct cancers, 1 case as pancreatic cancer, and 3 as intrahepatic duct stones (Fig. 2).

Bile pH

The pH in the *Helicobacter* DNA positive bile was 7.83 $\pm$0.41, and 7.78 $\pm$0.48 in *Helicobacter* negative bile samples. There was no correlation between the existence of *Helicobacter* and bile pH (p=NS).

DISCUSSION

Helicobacter pylori is generally believed to be very sensitive to bile, with some investigators reporting that 96% of the organisms are killed in duodenal fluid containing bile (20). Other investigators have reported that 76% of *H. pylori* survived against treatments of 5% bile for 30 min (23). But actually, inhibitory effects on *H. pylori* could be less significant in vivo. Some studies have reported *H. pylori* can be detected in bile reflux gastritis (24), in the residual stomach after gastric resection (25), and in the duodenum containing abundant bile acids (9). Under pathologic conditions such as bile duct obstruction, it has been suggested that the alteration of bile composition could cause *H. pylori* to grow in the bile duct.

Recently *H. pylori* (22) and other *Helicobacter* species such as *H. bilis, H. rappini*, and *H. pullorum* were detected by PCR using *Helicobacter* DNA in the diseased bile or gallbladder tissue (26). But all the studies about *Helicobacter* culture in bile have not been successful, so exact identification of *Helicobacter* species has been impossible until now. In rats, unidentified gram negative bacterium has been observed in the livers experimentally infected with *Fasciola hepatica*, when compared to the control rats (27). The study shows that bacterial infection is possible by alteration of the bile through liver fluke infestation. That bacterium has been confirmed as *H. rappini*, belonging to the genus *Helicobacter* (28). Recently, additional *Helicobacter* sp. have been isolated from the gastrointestinal tracts of animals, including hepatobiliary tract, *H. hepaticus* (16, 18, 19), *H. pullorum* (29), *H. rappini* (30), *H. cholecystus* (31), and *H. bilis* (32). These organisms are considered to cause hepatobiliary diseases similar to those in humans.

Helicobacter DNA detected in human bile could be *H. pylori* or another hepatic *Helicobacter* identified in animals because these bacteria have not been grown in vitro until now. Detection of *H. pylori* DNA in gallbladder and bile has been reported. Three of 7 bile samples obtained by percutaneous transhepatic cholangiodrainage were positive for *H. pylori* by PCR using *ureA* gene. Investigators suggested that this organism can cause asymptomatic cholangitis (22). In another study, a microorganism closely resembling *H. pylori* was detected by PCR of the *ureB* gene and immunohistochemical staining in the resected gallbladder tissue (33). Interestingly, hepatic *Helicobacter* sp. were identified in 13 of 23 bile samples and 9 of 23 gallbladder tissues by PCR using *Helicobacter*-specific 16S rRNA. And these investigators have reported that after sequencing about 8 of the *Helicobacter* genus-specific PCR amplicons, 5 sequences represented strains of *H. bilis*, two strains of *H. rappini*, and one strain of *H. pullorum*. They also reported that *Helicobacter* sp. in human bile might be hepatic *Helicobacter* sp. rather than *H. pylori* for two reasons: first, the sequence for the urease genes of hepatic *Helicobacter* are unknown, so it is not possible to exclude the occurrence of a nearly identical urease gene in one of these *Helicobacter* sp., and secondly, *H. pylori* is unable to grow in vitro bile acids. They have suggested that hepatic *Helicobacter* organisms, especially *H. bilis, H. rappini, H. pullorum*, play a role in the development of hepatobiliary disease in humans, and these *Helicobacter* sp. in humans can induce bile duct injury, chronic inflammation, and proliferation as well as possibly being responsible for gallbladder or biliary tissue neoplasia (26). We have reported that *Helicobacter* DNA was detected in 13 of 52 bile samples (13.5%) by PCR using 26 kDa surface antigen (34). And in this study, *Helicobacter* DNA was detected in more than 30% of bile samples from the diseased bile duct by PCR using *ureA* gene or 16S rRNA. From our previous and present studies, we think the reason for different detection rates of *Helicobacter* depends on the kinds of primers used for detection. Several different primers, 16S rRNA, *ureA, ureB*, or 26 kDa antigen have been described and validated for *H. pylori* (35, 36). But these primers are not confined to detect *H. pylori* and can detect other *Helicobacter* sp. similar to *H. pylori*. Therefore, it is recommended that the investigators do not rely on a single pair of primers. Confirmatory amplication using a second pair of primers from a different gene should be used whenever possible.

Helicobacter sp. were not cultured from bile samples in this study because of the possible effect of antibiotics on the culture. Antibiotics were administered to treat cholangitis in most of the patients before obtaining sample, so the *Helicobacter* could not have grown even though the culture procedure was performed rapidly as soon as bile was obtained. But repetitive trials to culture will be necessary. In our previous study *H. pylori* could grow in 90% human biles of some cases with biliary tract disease (37). Therefore, we think that *H. pylori* might also survive in diseased bile duct of humans because *H. pylori* could grow in human bile. Also the actual effects of bile acid in the diseased state of the biliary tree can not be well known in vivo. So, we think *Helicobacter* sp. in biliary tree could be *H. pylori* or another species of *Helicobacter*, and recognize the possibility of bile resistant *H. pylori* as well as bile resistant *Helicobacter* species. We found

the existence of *Helicobacter* DNA in diseased human bile duct, but we do not know if *Helicobacter* sp. found in the biliary tree are viable.

In summary, although the clinical significance of *Helicobacter* detected in the biliay tree is not clear, these results should stimulate further studies to ascertain whether these *Helicobacter* might play a role in the pathogenesis of bile duct diseases in humans.

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