Etiologic and clinical studies in primary sclerosing cholangitis
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Chapter V

*Helicobacter* species in primary sclerosing cholangitis

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Chapter V

**SUMMARY**

*Background & Study Aims:* The cause of primary sclerosing cholangitis (PSC) is unknown. Recently, several novel murine hepatotropic *Helicobacters* have been described. The aim of this study was to study the presence of these hepatotropic *Helicobacters* in bile and by serology in PSC patients.

*Patients & Methods:* Bile aliquots from 34 patients with well-established (PSC) were subjected to culture and PCR. Serum IgG antibodies against *H. hepaticus, H. bilis, H. pullorum,* and *H. rappini* from 16 PSC patients were determined. All results were compared with a control group of 24 patients, who underwent endoscopic retrograde cholangiopancreatography for unrelated disorders.

*Results:* All bile cultures were negative. PCR analysis revealed a 400 bp *Helicobacter*-specific amplicon in 12/34 PSC patients and 12/24 controls (odds ratio 0.55, CI 0.16-1.80). In 3 samples a 1200-bp was sequenced showing high homology with *H. hepaticus* in 1 PSC patient and 1 control, and with *H. pylori* in 1 PSC case. Serum antibody titers against *H. hepaticus, H. bilis, H. pullorum* and *H. rappini* were similar in both groups.

*Conclusion:* This controlled study could not incriminate hepatotropic *Helicobacters* as a risk factor for primary sclerosing cholangitis.

Key words: primary sclerosing cholangitis; polymerase chain reaction; *Helicobacter hepaticus; Helicobacter bilis; Helicobacter pullorum; Helicobacter rappini*
INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic progressive disease characterized by fibro-obliterative lesions that can be distributed throughout the entire biliary tree. Its etiology is essentially unknown. In the past several bacterial pathogenetic causes have been implicated, but were never proven. Based on the high correlation with inflammatory bowel disease, Boden et al. postulated portal bacteremia (1). They treated their PSC patients with long-term tetracycline with some success (2). However, their results could not be confirmed by others and portal bacteremia has never been demonstrated in colitis patients (3-6). Moreover, extensive histologic studies have never revealed micro-organisms in the periductular mainly lymphocytic portal infiltrate in the liver. Another argument against this hypothesis - and also against the theory that toxic substances generated by colonic bacteria would easily permeate through an inflamed bowel wall and induce a toxic cholangioliitis - is the fact that PSC can occur without inflammatory bowel disease, precede it, or become manifest many years after proctocolectomy (7).

Recently, two novel Helicobacter species have been isolated in mice: Helicobacter hepaticus and Helicobacter bilis (8, 9). Both micro-organisms colonize the bile, liver, and intestine of mice. Infection leads to chronic hepatitis and, interestingly, H. hepaticus infection in A/JCr mice is associated with liver tumors (10-12). In immunodeficient mouse strains, H. hepaticus infection is linked with inflammatory bowel disease (13).

Because these infections in laboratory animals bear some striking similarities to PSC in humans, we endeavoured to detect the presence of Helicobacter species by culture and polymerase chain reaction (PCR) in bile from PSC patients and controls. Moreover, antibodies against these species were measured in serum.

MATERIALS AND METHODS

Bile samples were collected during endoscopic retrograde cholangiopancreatography (ERCP) from 34 PSC patients and 24 controls.

In the majority of cases bile samples were contaminated with megluminejoxitalamate, which was used as a contrast agent to opacify the biliary tree. The bile specimens were immediately stored at -18 °C for a maximum of 48 hours, and further on kept at
- 20 °C for 1-81 months.

Corresponding sera stored at - 80 °C were available from 16 PSC patients and 22 controls.

The randomly selected control group consisted of five patients with pancreatic head tumor, three patients with chronic pancreatitis, 10 cases of choledocholithiasis, two cholangiocarcinomas, one gallbladder cancer, two ampullary tumors, and one metastatic bile duct stenosis.

Bile samples were cultured as previously described (14).

Bile specimens were subjected to PCR analysis using Helicobacter-specific 16S rRNA primers as described previously (15). As initial primer a 400-bp oligonucleotide specific for all known Helicobacter spp was chosen. A second PCR amplification using a 1200 bp primer was performed on all positive samples. Positive samples were subsequently cloned and sequenced.

Antibodies against the outer membrane protein (OMP) of H. hepaticus, H. bilis, and H. pullorum, as well as against whole cell sonicate (WCS) of H. bilis, H. pullorum, and H. rappini were determined on the available 16 PSC and 22 control sera with an ELISA technique. ELISA tests were performed utilising Immulon 2 HB flat bottom, 96 well, microtiter plates (Dynex Technologies, Inc., Chantilly, VA, USA). Wells were coated with 100 ng of bacterial antigen in carbonate buffer, pH 9.6. Antigens used included whole cell sonicate and outer membrane protein antigen preparation of pooled Helicobacter bilis and pooled H. pullorum bacterial strains. Additional plates were coated with a whole cell sonicate antigen preparation of pooled H. rappini strains and an outer membrane protein antigen preparation of H. hepaticus 3B1 strain.

Antibody to H. pylori was tested using the H. pylori IgG ELISA kit (Wampole Laboratories, Cranbury NJ, USA) according to manufacturer’s instructions. For our in-house ELISA, sera from PSC patients was diluted 1:200 in phosphate buffered saline (PBS). Sera were tested against each antigen preparation. One hundred μl of diluted sera was placed into each well. A negative control well was included on each plate. A known anti-H. pullorum and anti-H. hepaticus serum was used as a positive control. Plates were incubated for 1 hour at 37°C and then washed 5 X with PBS containing 0.05 % Tween20 (Sigma Chemical Co., St. Louis, MO 63178) using semi-automated equipment. Anti-human IgG alkaline phosphatase conjugate (Sigma) was diluted 1:1000 in PBS and 100 μl added to each well. Plates were incubated again for 1 hour at 37°C and then washed 5 X. Alkaline phosphate substrate, p-nitrophenyl phosphate 5 mg (Sigma) in 10 ml of 10 % diethanolamine, pH 9.8, was then added, 100 μl per well. An automated ELISA reader, Dynatech MR 7000
(Dynex Technologies, Inc.) was used to read the plates with a double filter setting at 450 \( \lambda \) and 540 \( \lambda \). Plates were read after a 15-minute incubation period at room temperature.

**Statistical analysis**
Odds ratios were determined and confidence intervals calculated as described by Mehta et al (16). To check for possible confounders, Chi-square testing and stepwise forward logistic regression analysis were performed using the SPSS Package 8.0 (SPSS Inc., Chicago, Ill, USA). Results of the serology were analysed using independent sample \( t \)-test. When necessary, loge-transformation was performed to correct for skewness of sample distribution.

**RESULTS**
Cultures of bile samples were negative in all specimens. PCR testing with the 400-bp primer was positive in 12/34 PSC bile specimens (35 %) and in 12/24 control samples (50 %). The odds ratio was 0.55 with a confidence interval of 0.16-1.80. Neither Chi-square testing nor stepwise forward logistic regression analysis revealed any significant confounding influence of factors such as sex, age of the patients, age of the samples, or previous CBD cannulation. Testing the 24 400-bp Helicobacter positive samples in the 1200-bp assay revealed 7/24 positive results (4 PSC specimens, 3 controls). Sequencing and cloning of specific Helicobacter amplicons was successful in three specimens. One amplicon from a control sample was typed as *H. hepaticus*, one from a PSC specimen was typed *H. hepaticus*-like, and another one from a PSC patient was typed *H. pylori*-like.

Nine of 16 PSC (56 %) sera and 11 out of 22 control (50 %) sera were positive in the *H. pylori* ELISA, so no difference was found in detectable antibodies against *H. pylori* between the two groups. The mean antibody titers against *H. hepaticus*-OMP, *H. bilis*-OMP and -WCS, *H. pullorum*-OMP and -WCS, and *H. rappini*-WCS in the PSC sera were not different from those in the control group. The results are depicted in figure 1. Height of the titers did not correspond with positive 400-bp status of the patient (data not shown).
DISCUSSION

The genus *Helicobacter* has experienced considerable expansion over the last decade (17). Apart from *H. pylori*, there are only anecdotal reports of some members of this genus causing disease in humans. *Helicobacter rappini* and *H. pullorum* have been associated with gastro-enteritis (18, 19). In addition, using sensitive PCR techniques, high homology to the 16S rRNA sequence of *H. rappini* and *H. pullorum* was found in gallbladder tissue in 4 of 23 Chilean patients with chronic cholecystitis and in liver tissue and bile in 2 of 8 PSC patients (15, 20). The two most recently added species to the *Helicobacter* genus, *H. hepaticus* and *H. bilis* were isolated from the liver, biliary tract and intestine of laboratory mice and are responsible for inducing chronic hepatitis, hepatocellular tumors and enterocolitis (9-12, 21). So far these two microorganisms have not been implicated in human disease. The present study was initiated to test the hypothesis that chronic or past infection with one of the above-mentioned *Helicobacter spp* could be involved in the pathogenesis of primary sclerosing cholangitis. Since neither the PCR nor the serology have been validated and lack a golden standard with cut-off values in humans, a control group was introduced. Our results indicate that neither *H. hepaticus*, *H. bilis*, *H. pullorum*, nor *H. rappini* can be considered as a risk factor in PSC. *Helicobacter* specific 16S rRNA sequences were found in 22 of 58 bile specimens. This finding did not correspond with *H. pylori* seropositivity (Chi-square p=0.428) Only in three samples specific *Helicobacter* amplicons could be determined. This leaves 19 400-bp samples unexplained. It may
be that the PCR using the 400-bp primer is not entirely specific for *Helicobacter spp.* or that the sensitivity of the 1200-bp amplification is too low.

The negative results of the bile cultures were in a way anticipated, since the initial freezing of the specimens was done without adding DMSO to prevent cell lysis. Furthermore, bile acids are known to inhibit growth of *Helicobacter spp.*. The negative culture results are in accordance with the findings of Rudi *et al.* who also failed to culture and amplify various *Helicobacter spp.* from bile (22). Although we could only demonstrate the presence of a *Helicobacter hepaticus*-like organism in one PSC patient, this does not mean that a pathogenetic role for hepatotropic *Helicobacters* is entirely ruled out. It may be that an initiating noxious event in the form of a subclinical infection of the biliary tree induces a chronic immune response in genetically susceptible individuals, which in turn leads to overt disease only after many years. This hypothesis would comply with the general contention that PSC is an autoimmune disease. Traces of such an initiating infection could possibly be demonstrated by specific serum IgG antibody determination. However, antibody testing in humans against novel *Helicobacters* has not been validated. Moreover, *H. pylori* serum antibodies may become negative months to several years after eradication of the organism. Many PSC patients will have used antibiotics, which would constitute a possible negative confounder.

In conclusion: The results of this controlled study cannot incriminate *Helicobacter hepaticus, H. bilis, H. pullorum,* or *H. rappini* as a risk factor for PSC. The possibility of a previous infection initiating a chronic autoimmune response is not ruled out.

**REFERENCES**

Chapter V


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