ACTA MEDICA LITUANICA. 2004. VOLUME 11 No. 3. P. 31–35

Detection of *Helicobacter* spp. in liver biopsy specimens

Vitalija Petrenkienė[1], Astra Vitkauskienė[2], Laimas Jonaitis[1], Limas Kupčinskas[1], Torkel Wadström[3]

[1] *Department of Gastroenterology*
[2] *Department of Microbiology, Kaunas Medical University Hospital, Kaunas, Lithuania*
[3] *Department of Medical Microbiology, Lund University, Lund, Sweden*

Background. *Helicobacter hepaticus* in mice causes chronic hepatitis and hepatocellular carcinoma. Recently *Helicobacter* spp. has been detected in liver of patients suffering from cholestatic diseases and hepatocellular carcinoma. However the role of *Helicobacter* spp. in human hepatic and biliary diseases remains unclear.

Aim. To detect in human hepatic tissue *Helicobacter* spp. which might be involved in liver diseases.

Materials and methods. Fifty-four paraffin-embedded liver samples from patients with various liver diseases (chronic viral hepatitis, fatty liver, steatohepatitis, viral liver cirrhosis, primary biliary cirrhosis and primary sclerosing cholangitis, extrahepatic cholestasis and other chronic liver diseases) were tested by polymerase chain reaction for presence of genomic 16S ribosomal RNA of *Helicobacter*, using specific primers and for a gene encoding a 26 kDa surface protein. Immunoglobulin G antibodies against *Helicobacter pylori* in sera by enzyme-linked immunosorbent assay were performed.

Results. 87% of patients were *Helicobacter pylori*-seropositive. In 10.6% of *Helicobacter pylori*-seropositive patients, *Helicobacter* spp. in liver samples were detected. *Helicobacter* spp. were detected more frequently in patients with extra- hepatic cholestasis (60%) and in patients with chronic cholestatic liver diseases (25%) than in patients with other chronic liver diseases (2.6%), $p = 0.002$ and $p = 0.06$, respectively.

Conclusions. *Helicobacter* spp. were found more frequently in liver samples of patients with chronic cholestatic liver diseases and extrahepatic cholestasis. It was observed only in *Helicobacter pylori*-seropositive patients. Though the pathological role of *Helicobacter* spp. in liver diseases remains unconfirmed, cholestasis in some cases may facilitate the extra gastric spread of these bacteria.

Key words: chronic liver disease, *Helicobacter*

INTRODUCTION

Discovery of *Helicobacter pylori* (*H. pylori*) and further elucidation of its pathogenetic role dramatically changed the understanding and treatment of chronic gastritis and peptic ulcer disease (1, 2). Some data suggested that *H. pylori* infection could be related not only to gastro-duodenal pathologies, but also to the extra-gastric conditions, such as ischemic heart disease (3, 4), vascular, immunological disorders (5, 6). Five bile-tolerant *Helicobacter* spp. associated with liver disorders of animals are known: *H. pullorum* in poultry, *H. hepaticus* and *H. bilis* in mice, *H. cholecysticus* in hamster, and *H. canis* in dogs (7). Recently, some *Helicobacter* spp. associated with the pathogenesis of gastric (1, 8–10) and extra-digestive manifestations (12, 13), have been detected in the liver of persons suffering from cholestatic diseases and hepatocellular carcinoma (HCC) arising from non-cirrhotic liver (7, 14). A new infectious agent, *H. hepaticus*, which causes chronic active hepatitis and associated liver tumors in A/J Cr mice, has been described by Ward et al. (1). *H. hepaticus* is currently the best studied of the enterohepatic *Helicobacter* spp. and has many features common with *H. pylori*: both persistently infect their hosts and cause chronic inflammation which can progress to carcinoma (15). *H. fennelliae* and *H. cinaedi* DNA from enterohepatic *Helicobacter* spp. has

Correspondence to: Vitalija Petrenkienė, Gastroenterological Department, Kaunas Nedical University Clinic, Eivenių 2, LT-50009, Kaunas, Lithuania. E-mail: vitalijap@takas.lt

been found in patients with hepatobiliary diseases as well, but the causal role of the bacteria in human liver disease has not yet been established (16, 17). The discovery of these *Helicobacter* spp., in conjunction with a number of clinical observations, suggested a possible relationship between *Helicobacter* infection and liver diseases.

Therefore we carried out a study to detect *Helicobacter* spp. in human hepatic tissue and to establish its possible relationship with different liver diseases.

PATIENTS AND METHODS

Liver biopsy samples from 54 consecutive patients (mean age 47.0 ± 7.0 years) with various liver diseases were analyzed. Patients with elevated aminotransferases (ALT or/and AST > 1.5 time to normal), without acute viral hepatitis and without contraindications to liver biopsy were included into the study. Liver biopsy specimens were collected from patients with chronic hepatitis B or C (n = 14), fatty liver (n = 9), steatohepatitis (n = 9), viral liver cirrhosis (n = 6), primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) (n = = 4), extra hepatic cholestasis (due to choledocholithiasis, biliary stricture, pancreatitis) (n = 5), other chronic liver diseases (hemochromatosis, drug-related hepatitis, granulomatous hepatitis, autoimmune hepatitis) (n = 7).

Liver biopsy was performed with a standard Menghini 1.6 mm needle. Paraffin-embedded liver biopsy samples were tested by polymerase chain reaction (PCR) for presence of genomic 16S ribosomal RNA (16S rRNA) of *Helicobacter* spp., using specific primers and the gene encoding 26 kDa surface protein. Immunoglobulin G (IgG) antibodies against *H. pylori* in sera were detected by enzyme-linked immunosorbent assay (ELISA).

H. pylori enzyme immunoassay (IgG-EIA)

The antigen used in the EIA is an acid glycine extract of strain CCUG 17874. Sera were analyzed in duplicates, and a third well, lacking antigen, was used as a control for non-specific reactivity. Maxisorp immunoplates (F96, NUNC, Denmark) were coated (100 µl per well) with a protein concentration of 5 µg per ml. An alkaline phosphatase-labelled antihuman Ig G antibodies (Sigma, St. Louis, USA) was used as a second antibody. After repeated washings, 100 µl of a substrate solution per well, containing p-nitrophenyl phosphate (Sigma), diethanolamine and $MgCl_2$ was added. The EIA test results are expressed as corrected mean absorbance values (A 405 nm) in percent of a reference standard (human gamma globulin, Pharmacia & Upjohn, Stockholm, Sweden). A relative antibody activity (RAA value) greater than 35 and less than 25 units was defined as positive and negative, respectively; the values between these were regarded as low positive values (borderlines). The cut-off values are based on EIA and immunoblot analyses of serum samples from patients with a positive and a negative gastric cultures for *H. pylori,* healthy blood donors, and children.

H. pylori immunoblot (Immunoglobulin G)

The patient serum samples that showed positive and borderline values in *H. pylori* EIA were further analyzed by immunoblotting for detection of specific protein bands of *H. pylori*. An antigen prepared from *H. pylori* reference strain was used for sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) with a 5–20% gradient gel (Exel Gel, Pharmacia Biotech AB, Uppsala, Sweden) and 130 µg of 5% gel of the antigen was run along with a low molecular weight standard. After overnight separation the proteins from each gel were transferred to a PVDF membrane (Millipore, Intertech, Bedeford, Massachusetts, USA) and stained with amido black. The membranes were blocked, then dried, cut into strips and used for immunoblotting.

PCR method

DNA extraction was performed using QIA amp DNA mini kits (Germany). DNA was purified using QIA amp kits up to 50 kb in size with fragments of approximately 20–30 kb predominating.

PCR amplification. 2.5 U/ml of rTth (MBI Fermentas, Vilnius, Lithuania), 25 mM $MgCl_2$ were used; 5 µl of an extracted sample was added to the PCR reaction mixture. All primers were purchased from Scandinavian Gene Synthesis (Köping, Sweden). The PCR was performed in a Perkin Elmer thermocycler (Gene Amp PCR system 2400). The amplified products were analyzed with 1.5% (wt/vol) agarose (Bio-Rad Laboratories) gels, and the sizes of the PCR products were estimated by comparison with 100-bp DNA size markers (MBI Fermentas). In each amplification event, a corresponding *Helicobacter DNA* extract was used as a positive control. For the *Helicobacter* PCR, *H. pylori* DNA was used as a positive control. Double-distilled water was used as the negative control (Fig. 1).

PCR for the genus Helicobacter. Samples were amplified with the *Helicobacter* genus-specific 16S rRNA primers. The forward (C 97) and the reverse (C 98) primer amplified a product of approximately 400 bp. Amplification consisted of initial denaturation at 94 °C for 1 min, primer annealing at 63 °C

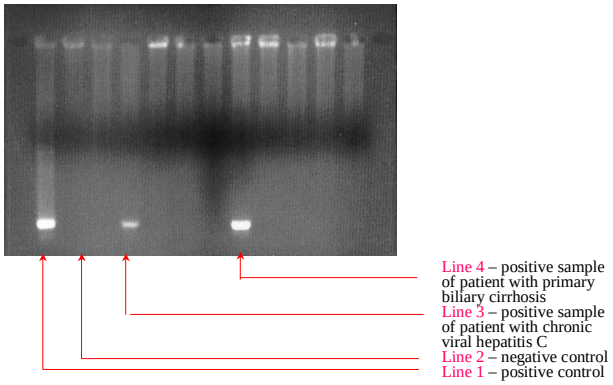

Fig. 1. Analysis of *Helicobacter* spp. revealed by PCR in liver samples of patients with chronic liver diseases

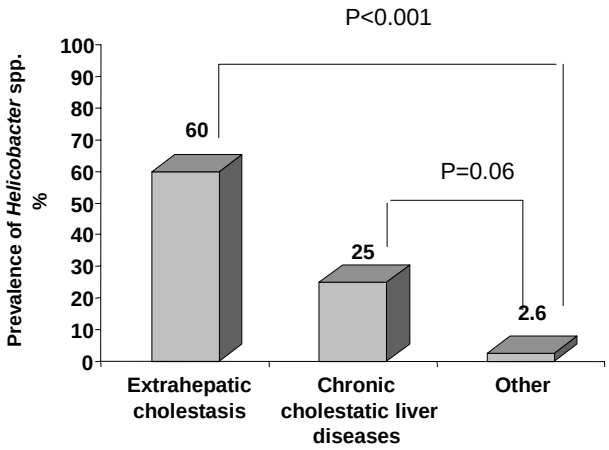

Fig. 2. Prevalence of *Helicobater* spp. by PCR in liver sample tissue of patients referred for liver biopsy

for 30 s, and extension at 72 °C for 30 s. The samples were amplified for 35 cycles, with a final extension step at 72 °C for 7 min.

RESULTS

Forty seven (87%) of the patients were *H. pylori*-seropositive by ELISA. In 5 (10.6%) of *H. pylori*-seropositive patients *Helicobacter* spp. was detected in liver biopsy samples. *Helicobacter* spp. in liver biopsy specimens was detected more frequently in patients with extrahepatic cholestasis due to choledocholithiasis or pancreatitis (3/5, or 60%) and in patients with chronic cholestatic liver diseases (PBC or PSC) in 1/4 (25%) than in patients with other chronic liver diseases (1/38, or 2.6%), p = 0.002 and p = 0.06, respectively (Fig. 2).

There were no significant differences in the functional liver test (ALT, AST, bilirubin, prothrombin) values among *H. pylori*-negative patients, *H. pylori*-positive patients with *Helicobacter* spp. in liver specimens and *H. pylori*-positive patients without *Helicobacter* spp. in liver specimens. Although the mean value of alkaline phosphatase (ALP) in PCR and ELISA-positive patients was numerically higher than in other groups of patients (Table), the difference was statistically insignificant.

DISCUSSION

The possibility that *Helicobacter* spp. could infect the biliary tract and the liver of humans has been reported by several authors in different settings. Fox et al. have (15) shown the presence of *Helicobacter* spp. in the bile of Chileans with chronic cholecystitis. Avenaud and co-workers have demonstrated by PCR the presence of genomic sequences of *Helicobacter* spp. in the liver of 8 patients with HCC without primary diagnosis of cirrhosis, a further analysis by sequencing showed that these species were *H. pylori* and *H. felis* (16). Whether *Helicobacter* spp. could act as a cofactor in the progression towards cirrhosis and carcinogenesis in humans with viral hepatitis is still questionable (18).

A high prevalence of antibodies to *H. pylori* in patients with liver diseases, presence of *Helicobacter* spp. in liver tissue samples of patients with cholestatic liver diseases have suggested an association between *Helicobacter* and liver diseases (19–22).

Table. **Results of functional liver tests in patients referred for liver biopsy**

Group of patients	ALT (U/L) ± SD	AST (U/L) ± SD	ALP (U/L) ± SD	Bilirubin (μmol/l) ± SD	Prothrombin (INR) ± SD
ELISA – neg. N = 7	178.1 ± 118.6	106.3 ± 110.3	299.6 ± 301.2	29.1 ± 38.6	1.07 ± 0.31
ELISA - pos., PCR - neg. N = 42	97.7 ± 119.4	95.6 ± 109.2	154.1 ± 291.7	39.2 ± 42.9	1.05 ± 0.36
ELISA - pos., PCR - pos.. N = 5	139.4 ± 114.6	85,4 ± 111.6	429.4 ± 467.2	20.1 ± 36.4	1.18 ± 0.30
P value	>0.05	>0.05	>0.05	>0.05	>0.05

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; PCR, polymerase chain reaction.

These observations prompted us to explore a possible association between *Helicobacter* spp. and chronic liver diseases. In our study, *Helicobacter* spp. in liver tissue were found only in *H. pylori*-seropositive patients. *Helicobacter* spp. were detected with a higher frequency in the liver samples of patients with intrahepatic cholestasis (25%) due to PBC or PSC and extrahepatic cholestasis (60%) than in patients with other chronic liver diseases (2.6%). These findings may support the hypothesis that the cholestasis may be related with a better transmission of *Helicobacter* spp. to the liver, but the exact way remains unclear.

In conclusion, although *Helicobacter* spp. was more frequently detected in patients with cholestasis, the role of the infection remains unclear, and further studies are needed to disclose the relationship of *Helicobacter* spp. with liver and biliary diseases.

Received 4 February 2004
Accepted 27 May 2004

References

1. Ward JM, Fox JG, Anver MR et al. Chronic active hepatitis and associated liver tumors in mice caused by a persistent bacterial infection with a novel *Helicobacter* species. J Natl Cancer Inst 1994; 86: 1222–7.
2. Blaser MJ, Perez-Perez GI, Kleanthous H et al. Infection with *Helicobacter pylori* strains possessing *cag*A is associated with an increased risk of developing adenocarcinoma of the stomach. Cancer Res 1995; 56: 2111–5.
3. Murray L, Bamford K, O"Reilly D, Mc Crum E, Evans A. *Helicobacter pylori* infection: relation with cardiovascular risk factors, ischemic heart disease and social class. Br Heart J 1995; 74: 497–501.
4. Rathbone B, Martin D, Stephen J, Thomson JR, Samani Nj. *Helicobacter pylori* seropositivity in subjects with acute myocardial infarction. Heart 1996; 76: 308–11.
5. Gasbarrini A, Franceschi F, Gasbarrini G, Pola P. Extra intestinal pathology associated with *Helicobacter* infection. Eur J Gastroenterol Hepatol 1997; 9: 231–3.
6. Gasbarrini A, Serricchio M, Tondi P, Gasbarrini G, Pola P. Association of *Helicobacter pylori* infection with primary Raynaud phenomenon. Lancet 1996; 348: 966–7.
7. Nilsson H, Taneera J, Castedal M, Glatz E, Olsson R, Wadstrom T. Identification of *Helicobacter pylori* and *Helicobacter species* by PCR, hybridization and partial DNA sequencing in human liver samples from patients with primary sclerosing cholangitis or primary biliary cirrhosis. J Clin Microbiol 2000; 38: 1072–6.
8. Bulent K, Murat A, Esin A, Fatih K, Murat H, Hakan H et al. Association of CagA and VacA presence with ulcer and non-ulcer dyspepsia in a Turkish population. World J Gastroenterol 2003; 9: 1580–3.
9. Palmas F, Pellicano R, Massimetti E, Berrutti M, Fagoonee S, Rizzetto M. Eradication *of Helicobacter pylori infection* with proton pump inhibitor-based triple therapy. A randomized study. Panminerva Med 2002; 44: 145–7.
10. Testino G, Cornaggia M, De Iaco F. *Helicobacter pylori* influence on gastric acid secretion in duodenal ulcer patients diagnosed for the first time. Panminerva Med 2002; 44: 19–22.
11. Li S, Lu AP, Zhang L, Li YD. Anti-*Helicobacter pylori* immunoglobulin G (IgG) and IgA antibody responses and the value of clinical presentations in diagnosis of *H. pylori* infection in patients with precancerous lesions. World J Gastroenterol 2003; 9: 755–8.
12. Roussos A, Philippou N, Gourgoulianis KI. *Helicobacter pylori* infection and respiratory diseases: a review. World Gastroenterol 2003; 9: 5–8.
13. Yakoob J, Jafri W, Abid S. *Helicobacter pylori* infection and micronutrient deficiencies. World J Gastroenterol 2003; 9: 2137–9.
14. Avenaud P, Marais A, Monteiro L, Lebail B, Bioulac Sage P, Balabaud C, et al. Detection of *Helicobacter* species in the liver of patients with and without primary liver carcinoma. Cancer 2000; 89: 1431–9.
15. Suerbaum S, Michetti P. *Helicobacter pylori* infection. *N Engl J Med* 2002; 347: 1175–86.
16. Solnick JV, Schauer DB. Emergence of diverse *Helicobacter* species in the pathogenesis of gastric and enterohepatic diseases. *Clin Microbiol Rev* 2001; 14: 59–97.
17. Fox JG, Dewhirst FE, Shen Z, Feng Y, Taylor NS, Paster BJ et al. Hepatic *Helicobacter* species identified in bile and gallbladder tissue from Chileans with chronic cholecystitis. Ga*stroenterology* 1998; 114: 755–63.
18. Fagoonee S, Pellicano R, Rizzetto M, Ponzetto A. The journey from hepatitis to hepatocellular carcinoma: bridging role of *Helicobacter* species. Panminerva Med 2001; 43: 279–82.
19. Calvet X, Navarro M, Gil M, Mas P, Rivero E, Sanfeliu I et al**.** Seroprevalence and epidemiology of *Helicobacter pylori* infection in patients with cirrhosis. J Hepatol 1997; 26: 1249–54.
20. Nilsson I, Lindgren S, Eriksson S, Wadström T. Analysis of antibodies to *Helicobacter hepaticus* in sera from patients with chronic liver disease. Gut 1998; 43 (Suppl. 2): A117–8.
21. Lin TT, Yeh CT, Wu CS, Liaw YF. Detection and partial sequences analysis of *Helicobacter pylori* DNA in the bile samples. Dig Dis Sci 1995; 40: 2214–9.
22. Siringo S, Vaira D, Menegatti M, Piscaglia F, Sofia S, Gaetani M et al. High prevalence of *Helicobacter pylori* in liver cirrhosis. Dig Dis Sci 1997; 42: 2024–30.

**Vitalija Petrenkienė, Astra Vitkauskienė,
Laimas Jonaitis, Limas Kupčinskas, Torkel Wadström**

HELICOBACTER GENTIES BAKTERIJŲ NUSTATYMAS KEPENŲ AUDINYJE

S a n t r a u k a

Įvadas. *Helicobacter hepaticus* sukelia pelėms lėtinį hepatitą ir hepatoceliulinę karcinomą. Pastaruoju metu įvairių *Helicobacter* genties bakterijų buvo rasta tarp pacientų,

sergančių cholestazinėmis kepenų ligomis ir hepatoceliuline karcinoma. Tačiau *Helicobacter* genties bakterijų reikšmė žmonių kepenų ir tulžies latakų ligoms lieka neaiški.

Tikslas. Nustatyti *Helicobacter* genties bakterijas kepenų audinyje pacientams, sergantiems įvairiomis kepenų ligomis.

Pacientai ir metodai. Penkiasdešimt keturiems pacientams, sergantiems įvairiomis kepenų ligomis (14 – lėtiniu virusiniu hepatitu, 9 – kepenų steatoze, 9 – steatohepatitu, 6 – virusine kepenų ciroze, 4 – pirmine biliarine ciroze ir pirminiu sklerozuojančiu cholangitu, 5 – ekstrahepatine cholestaze ir 7 – kitomis lėtinėmis kepenų ligomis), polimerazės grandininės reakcijos metodu, naudojant rūšiai specifinius pradmenis, kepenų pavyzdžiuose nustatytos *Helicobacter* genties bakterijos, pacientų serume ištirti *Helicobacter pylori* antikūnai.

Rezultatai. Keturiasdešimt septynių (87%) pacientų serume buvo rasti antikūnai prieš *Helicobacter pylori*, iš jų 5 (10,6%) pacientų kepenyse buvo nustatytos *Helicobacter* genties bakterijos. *Helicobacter* genties bakterijos dažniau nustatytos pacientams, kuriems diagnozuota ekstrahepatinė cholestazė (60%) ir kurie sirgo lėtinėmis cholestazinėmis kepenų ligomis (25%), negu pacientams, sergantiems kitomis lėtinėmis kepenų ligomis (2,6%); atitinkamai $p = 0,002$ ir $p = 0,06$.

Išvados. *Helicobacter* genties bakterijos kepenų audinyje dažniau randamos tarp *Helicobacter pylori* infekuotų ligonių, kuriems diagnozuota ekstrahepatinė arba intrahepatinė cholestazė. Nors *Helicobacter* genties bakterijų reikšmė kepenų ligoms lieka neaiški, kai kuriais atvejais cholestazė gali sąlygoti ekstragastrinį šių bakterijų pasklidimą.