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Helicobacter spp. Infection and Chronic Liver Diseases

Zakażenie *Helicobacter* spp. a przewlekłe choroby wątroby

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Abstract

Background. Studies on spiral-sharped bacteria of the genus *Helicobacter* have focused mainly on *Helicobacter pylori*. However, in the last few years a great number of novel *Helicobacter* species have been isolated from animals and humans.
Objectives. The aim of the study was to determine the correlation between infection by *Helicobacter* spp. such as *H. pylori*, *H. hepaticus*, and *H. bilis* and pathological hepatic changes in patients with chronic liver diseases.
Material and Methods. The study included 56 patients aged 20–60 years diagnosed for various chronic liver diseases, e.g. chronic viral hepatitis types B or C, co-infection of HBV and HCV, autoimmune hepatitis, hemochromatosis, Wilson's disease, and non-alcoholic fatty liver disease (NAFLD). Thick-needle hepatic biopsy specimens and serum samples from each patient were analyzed. The presence of *Helicobacter* spp. in the biopsy specimens was determined by culture on solid media and polymerase chain reaction (PCR). The level of anti-*H. pylori* IgG antibodies in the patients' sera was detected by ELISA.
Results. Examination of the hepatic biopsies for *Helicobacter* spp. infection by different culture methods was negative for all samples. The *Helicobacter ureB* gene was identified by PCR in 7 of the 56 biopsies (12.5%). Among these patients, 5 were diagnosed for hepatitis C or B, 1 for hemochromatosis, and 1 for NAFLD with no identified viral infection. Anti-*H. pylori* IgG antibodies in the serum samples were detected in 52% of the examined subjects and in all 7 patients with positive PCR results.
Conclusions. The coexistence of *H. pylori* infection and chronic liver disease, especially viral hepatitis, might be possible in humans. Further studies are needed to clarify the relationship between *H. pylori*, *H. hepaticus*, and *H. bilis* infection and pathological hepatic changes in humans. So far, a detrimental effect of *Helicobacter* species on the liver could not be confirmed or excluded. There was no correlation between the presence of DNA detected by PCR assay of liver samples and the presence of specific antibodies in the patients' sera (**Adv Clin Exp Med 2007, 16, 4, 537–542**).

Key words: *Helicobacter* spp., chronic liver diseases.

Streszczenie

Wprowadzenie. Badania nad drobnoustrojami z rodzaju *Helicobacter* koncentrowały się do tej pory głównie na zakażeniach wywołanych przez *H. pylori*. Jednocześnie donoszono o nowych gatunkach tych spiralnych bakterii u zwierząt i ludzi.
Cel pracy. Ustalenie zależności między obecnością zakażenia drobnoustrojami z rodzaju *Helicobacter: H. pylori, H. hepaticus i H. bilis* a patogenezą zmian patologicznych w wątrobie u pacjentów z przewlekłymi chorobami tego narządu.
Materiał i metody. Badaniami objęto 56 pacjentów obojga płci w wieku 20–60 lat, diagnozowanych i leczonych w Katedrze i Klinice Chorób Zakaźnych, Chorób Wątroby i Nabytych Niedoborów Odpornościowych AM we Wrocławiu z powodu przewlekłych chorób wątroby, takich jak: wirusowe zapalenie wątroby typu B lub C, koinfekcja HBV i HCV, autoimmunologiczne zapalenie wątroby, choroba Wilsona, hemochromatoza, cholestaza, pierwotna marskość żółciowa (PBC) oraz niealkoholowa choroba stłuszczeniowa wątroby (NAFLD – *non alcoholic fatty liver disease*). Przedmiotem badań było 56 bioptatów pobranych z wątroby (za pomocą biopsji gruboigłowej) oraz 56 próbek surowicy. Badania diagnostyczne wykonano w Katedrze i Zakładzie Mikrobiologii AM we Wrocławiu.

Wyniki. Z materiału biopsyjnego, pobranego od chorych z przewlekłymi chorobami wątroby, nie udało się wyizolować i wyhodować drobnoustrojów z rodzaju *Helicobacter.* Metodą PCR zidentyfikowano gen *ure H. pylori* w 7/56 bioptatach (n = 7), tj. u 12,5% pacjentów. U 5 spośród tych pacjentów wykazano zakażenie HBV lub HCV (n = 5), jeden pacjent był chory na hemochromatozę (n = 1), u jednego chorego stwierdzono marskość ze współistniejącym stłuszczeniem wątroby i ujemny wynik na obecność zakażenia wirusami hepatotropowymi (n = 1). Przeciwciała klasy IgG anty-*H. pylori* stwierdzono u 52% zbadanych chorych, przy czym wśród pacjentów z dodatnim wynikiem badania PCR w kierunku zakażenia *H. pylori* przeciwciała wykryto u 7/7 badanych (100%).
Wnioski. Zakażenie *Helicobacter pylori* być może współistnieje z przewlekłymi chorobami wątroby u ludzi, a zwłaszcza z zapaleniem wątroby na tle zakażenia wirusami hepatotropowymi. Związek między zakażeniem *H. pylori, H. hepaticus, H. bilis* a chorobami wątroby u człowieka wymaga dalszych badań, ponieważ dotychczasowe obserwacje nie pozwoliły ani na potwierdzenie, ani na wykluczenie szkodliwego oddziaływania *Helicobacter* spp. na wątrobę (**Adv Clin Exp Med 2007, 16, 4, 537–542**).

Słowa kluczowe: *Helicobacter* spp., przewlekłe choroby wątroby.

Research to date on Gram-negative bacilli of the genus *Helicobacter* has mainly concentrated on *H. pylori* infection. However, novel species of these spiral-shaped bacteria in animals and humans have recently been reported. They are mainly localized in the gastrointestinal (*H. heilmannii*, *H. cinaedi*, *H. fenneliae*, *H. pullorum*, *H. westmaedii*, *H. canadensis*, and *H. rappini*) and biliary tracts (*H. bilis*) and their coexistence with members of the intestinal flora creates difficulties in their isolation by routine culture and microscopic methods [1, 2]. The increasing number of *Helicobacter* spp. isolated from animals and research based on animal models seem to confirm their significant role in the pathogeneses of human diseases. Because of their ability to survive in bile, enterohepatic *Helicobacter* species (EHS) may colonize the biliary tract and liver and induce the development of inflammatory changes and malignancies in these organs. *H. hepaticus* and *H. bilis* have been found in mice and *H. cinaedi* was isolated from the colon, enteric lymph nodes, and liver of Rhesus monkeys [3–5]. *H. canis* was reported in dogs and cats with chronic hepatitis and "*H. rappini*" in sheep with cholecystitis [6, 7].

H. hepaticus is the best known enterohepatic *Helicobacter* sp. It is known to cause colitis, chronic hepatitis, and primary hepatocellular carcinoma in immunocompromised mice. It is a spiral-shaped, motile bacterium, 1.5–5 μm long and 0.2–0.3 μm wide, with bipolar flagella. The most important virulence factor of *H. hepaticus*, as in *H. pullorum*, is cytolethal distending toxin (CDT). *H. hepaticus* is a difficult bacterium to culture in routine laboratory conditions. It requires very specific media for growth and a microaerophilic atmosphere. The mechanism by which *H. hepaticus* and other EHS move from the intestine to the liver and biliary tract are poorly understood. Based on experiments in animal models, direct migration from the lumen of the intestine to the biliary tract or penetration into macrophages and further spread by lymphatic and blood vessels have been suggested [8].

The role of *H. hepaticus* and other species of the EHS group in the pathogeneses of human diseases has not yet been determined. Studies on liver diseases, especially those in which the basis for pathological changes is unknown or those in which the progression of the inflammatory process is different in different patients, suggest the coexistence of another etiologic, perhaps infectious, agent playing a role in the pathogeneses of these diseases. The potential significance of the organisms of the genus *Helicobacter* should therefore not be underestimated. The aim of the study was to determine the relationship between infection by such *Helicobacter* spp. as *H. pylori*, *H. hepaticus*, and *H. bilis* and the pathogenesis of pathological hepatic changes in patients with chronic liver diseases.

Material and Methods

The study involved 56 patients of both sexes, 20 to 60 years of age, diagnosed and hospitalized at the Department of Infectious Diseases, Hepatology, and Acquired Immunodeficiency Syndromes, Wrocław Silesian Piasts University of Medicine in Wrocław, for various chronic liver diseases. The diagnoses of different etiologies, such as chronic viral hepatitis types C or B, co-infection of HBV and HCV, autoimmune hepatitis, Wilson disease, hemochromatosis, cholestasis, primary biliary cirrhosis (PBC), and non-alcoholic fatty liver disease (NAFLD), were based on disease history, biochemical and viral findings, and liver histopathology. The study protocol was approved by the institutional ethics committee. Informed consent was obtained from all of the patients before enrolling in the study.

Serum samples and thick-needle liver biopsy samples were taken from each patient and analyzed. Two liver samples were taken from each patient by thick-needle liver biopsy: one was placed in Brain Heart Infusion (BHI) (Biome-

rieux) for culturing and one in a sterile empty tube for polymerase chain reaction (PCR) testing. The liver biopsy samples were immediately stored at –70°C until DNA was isolated.

Helicobacter Culture

The liver biopsy samples were suspended in BHI within 2–3 hours after the biopsy and taken to the Department of Microbiology, Silesian Piasts University of Medicine in Wrocław. The samples were homogenized, stained by the Gram method, and cultured. Three types of agar medium were used in this study:

– Columbia agar (Difco) supplemented with 7% hemolysed horse blood,

– Columbia agar (Difco) supplemented with 7% hemolysed horse blood and a selective supplement (Dent) containing vancomycin (5 mg/l), trimethoprim (2.5 mg/l), cefsulodin (2.5 mg/l), and amphotericin B (2.5 mg/l). These two types of medium were used for culturing *H. pylori*. The cultures were incubated at 37°C for 3–5 days under microaerobic conditions (10% CO_2, 5% O_2, 85% N_2),

– Brucella agar (Becton Dickinson) supplemented with 5% hemolysed horse blood, inactivated horse serum, 1% IsoVitalex (Becton Dickinson), 1% (v/v) hemin, and activated charcoal to culture *H. hepaticus*, *H. pullorum*, and *H. bilis* as described by Ananieva and al. [9]. The cultures were incubated under different temperature conditions: at 37°C, 35°C, or 42°C for 7–14 days under anaerobic and microaerobic conditions (5% O_2, 5% H_2, 5% CO_2, 85% N_2).

Polymerase Chain Reaction (PCR)

Genomic DNA from the 56 liver biopsies was extracted using the Genomic DNA Prep Plus A&A Biotechnology kit according to the protocol supplied by the manufacturer. Briefly, the biopsy samples were ground and centrifuged for 5 min at 10,000 × *g*. The pellet was resuspended in 300 µl of extraction buffer (10 mM Tris-HCl, pH 8.5, and 0.5% Tween 20) and proteinase K (final concentration: 0.5 mg/ml). The mixture was incubated at 50°C for one hour, after which the enzyme was inactivated by boiling for 10 min. The DNA was extracted and preserved at –20°C until amplification was performed.

Amplification with *H. pylori ure B* gene was performed by the PCR-*H. pylori* diagnostic test made by "DNA-Gdańsk II" according to the manufacturer's protocol. All primers were synthesized at "DNA Gdańsk" (Poland) and their sequences are trade secrets of that company. The amplification was carried out in a total volume of 50 µl, containing 2 µl DNA, 42.5 µl PCR buffer, 5.0 µl dNTPs, 0.2 µmol/l primers specific to the urease gene, and 0.5 µl DNA polymerase DELTA 2. The amplification conditions were optimized and the reaction mixture was amplified for 35 cycles as follows: initial denaturation for 3 min at 94°C, denaturation for 30 s at 94°C**,** amplification for 30 s at 69°C, annealing for 30 s at 72°C, and final extension for 2 min at 72°C**.** The amplification product size was 262 bp, typical of *H. pylori*. To avoid PCR contamination, the PCR mixtures were prepared in a dedicated area used only for PCR and the PCR products were opened in a laminar flow hood separated from the PCR preparation area. The amplification was performed in a PTC 200 thermocycler (MJ Research Inc., Syngen). The 262-bp PCR products were analyzed with 2% agarose (Sigma) gels containing ethidium bromide and visualized with UV light. *H. pylori* DNA was used as a positive control and distilled water as a negative control in the experiment.

Detection of IgG Anti-*H. pylori* Antibodies by ELISA

The presence of serum anti-*H. pylori* antibodies was determined in all the examined patients. The 56 serum samples from the patients were stored at –20°C and analyzed by ELISA by the method described by Gościniak [10]. The optical density was read at a wavelength of 450 nm and compared with a blind probe using a Dynatech MR 500 ELISA reader.

Results

The cultures of the 56 hepatic biopsies for *H. pylori* and other *Helicobacter* spp. using specific culture media for very fastidious organisms under different temperature and air conditions were negative for all samples. *Helicobacter ureB* gene was identified by PCR in 7 of the 56 biopsies (12.5% of the patients). Among these, 5 patients were diagnosed with hepatitis C or B, 1 with hemochromatosis, and 1 with NAFLD with no identified viral infection. Anti-*H. pylori* IgG antibodies were detected in 52% of the serum samples of the examined subjects and in all the 7 patients with positive PCR results (Table 1).

Table 1. Presence of *Helicobacter* sp. urease gene and anti-*H. pylori* antibodies in patients with different liver diseases

Tabela 1. Obecność genu ureazy *Helicobacter* sp. i przeciwciał anty-*H. pylori* u pacjentów z różnymi chorobami wątroby

Diagnosis (Rozpoznanie)	Number of patients (Liczba chorych) (n = 56) %	*Helicobacter urease* gene detected by PCR (Gen ureazy *Helicobacter* oznaczany za pomocą PCR)		Anti-*H. pylori* antibodies (Przeciwciała anty-*H. pylori*)	
		positive (dodatni)	negative (ujemny)	positive (dodatni)	negative (ujemny)
HCV	19	3	16	10	9
HBV	15	2	13	9	4
HCV, HBV	3	0	3	1	2
Non-alcoholic fatty liver disease (Niealkoholowe stłuszczenie wątroby)	7	1	6	3	4
Other* (Inne)*	12	1	11	6	8
Total (Razem)	56 (100%)	7 (12.5%)	49 (87.5%)	29 (52%)	27 (48%)

n – number of patients, HCV – hepatitis C, HBV – hepatitis B.
* Other diagnoses – cholestasis, Wilson disease, hemochromatosis, primary bilary cirrhosis (PBC), lymphgranuloma, autoimmunological hepatitis, hepatotoxicity.

n – liczba pacjentów, HCV – zapalenie wątroby typu C, HBV – zapalenie wątroby typu B.
* Inne rozpoznania: cholestaza, choroba Wilsona, hemochromatoza, pierwotna marskość wątroby (PBC), ziarnica złośliwa, autoimmunologiczne zapalenie wątroby, toksyczne uszkodzenie wątroby.

Discussion

In the present study, *Helicobacter* spp. infection was not determined by culture methods in the 56 examined patients. No organism of the genus *Helicobacter* was isolated and cultured though different culture media prepared for fastidious *Helicobacters* and a wide range of temperature (37°C, 35°C, 42°C) and characteristic atmosphere conditions (5% O_2, 5% H_2, 5% CO_2, 85% N_2) were used. This seems to confirm observations by other authors about unsuccessful culturing of *Helicobacter* spp. from clinical specimens using standard culture methods. These organisms grow very slowly, have very fastidious growth requirements, and, though provided with supplements and proper growth conditions, successful culture is rare. The single case of a *Helicobacter*-positive culture known in the literature concerns a *H. pylori* strain isolated from the liver of a 23-year-old woman with cirrhosis due to Wilson's disease [11].

The pioneering study by Fox et al. [12] indicated the presence of *Helicobater* spp. DNA in bile samples and resected gallbladder tissue in 46 Chilean patients with chronic cholecystitis. Cultures from frozen samples were negative although DNA sequence analysis showed the presence of *Helicobacter* DNA in 13 of 23 examined bile samples and in 9 of 23 gallbladder tissues. In five specimens *H. bilis* was detected, in two *H. rappini*, and in one sample *H. pullorum*. Ponzetto et al. [13] indicated that *H. pylori* and *H. pullorum* may play a role in the pathogenesis and progression of hepatocirrhosis in patients with viral hepatitis and primary hepatocellular carcinoma. The presence of DNA was detected by PCR in 11 of 12 biopsies taken from patients with primary biliary cirrhosis and in 9 of 12 biopsies from patients with primary sclerosing cholangitis [14]. Avenaud et al. [15] confirmed by PCR genetic material of *Helicobacter* spp. in liver and gallbladder tissue taken from patients with primary hepatocarcinoma and cholestatic carcinoma. The presence of *Helicobacter* spp. in liver and biliary tract diseases was also detected using molecular methods by other investigators [16–19].

In the event of culture failure, polymerase chain reaction (PCR) and serological methods were performed in the present study for further investigations to determine *Helicobacter* spp. infection in liver samples and to characterize the *H. pylori* status of the patients. IgG anti-*H. pylori* antibodies were detected in 52% of the serum samples. The low rate of antibodies in the examined patients, compared with other reports [20, 21], is a consequence of the young age, from 20–40 years, of the patients of the present study,

and the range of infected person in this age group in the Polish population is 50% and increases with age [22].

Urease gene characteristic for *Helicobacter* spp. was detected in 7 liver biopsies (12.5%) in the present study by using PCR, which confirms the results of other investigators [23, 24]. However, some authors reported higher rates of isolated *Helicobacter* spp. DNA in liver samples by using more specific and sensitive molecular methods [14, 25, 26]. The fact that specific anti-*H. pylori* antibodies were detected in all patients with positive PCR results deserves attention. Moreover, these were mainly patients with hepatitis C or B. A high prevalence of anti-*H. pylori* antibodies in hepatitis C-infected patients was reported by Nilsson et al. [27].

The culture difficulties, the invasive procedures used to obtain the clinical material, and the lack of possibility to apply more sensitive molecular methods to detect genetic material of *Helicobacter* spp. in the examined samples were the main reasons why a positive correlation between the presence of *H. pylori* or other *Helicobacter* spp. infection and chronic liver diseases could not be evaluated with certainty. However, these results, especially the determination of the presence of *Helicobacter* spp. in liver biopsies from patients with various liver diseases, indicate that there is a necessity to make further attempts to determine the role of *Helicobacter* bacteria in the pathogeneses of chronic liver diseases in humans.

The authors conclude that co-existence of *H. pylori* infection and chronic liver diseases, especially viral hepatitis in humans, might be possible. Further studies are needed to clarify the relationship between *H. pylori*, *H. hepaticus*, and *H. bilis* infection and pathological hepatic changes in humans. So far, the detrimental effect of *Helicobacter* species on the liver could be neither confirmed nor excluded. No correlation was found between the presence of DNA detected by PCR assay of liver sample specimens and the presence of specific antibodies in patients' sera.

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