Serum antibodies to enterohepatic \textit{Helicobacter} spp. in patients with chronic liver diseases and in a population with high prevalence of \textit{H. pylori} infection

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Abstract

\textbf{Background.} Enteric \textit{Helicobacter} species might be a risk factor for chronic liver and biliary tract diseases.

\textbf{Aims.} To analyse serum antibody levels to three enteric \textit{Helicobacter} species in patients with various biliary tract and chronic liver diseases and compare results with corresponding parameters for an adult population group, known to have a high prevalence of \textit{Helicobacter pylori} infection, and with healthy blood donors, to explore a possible association of enteric \textit{Helicobacter} with chronic liver diseases.

\textbf{Subjects.} Sera of 90 patients with various chronic liver diseases, 121 Estonian adult persons and 68 blood donors were analysed.

\textbf{Methods.} Sera, previously tested for \textit{H. pylori} were analysed for IgG to \textit{Helicobacter hepaticus}, \textit{Helicobacter bilis} and \textit{Helicobacter pullorum}. ELISA was initially used for screening and exclusion of negative cases. Sera with positive ELISA results were further analysed by immunoblot. To remove cross-reactive antibodies between \textit{H. pylori} and the enteric species, sera were pre-absorbed with lysed \textit{H. pylori} cells.

\textbf{Results.} Liver patients showed a significantly higher seroprevalence to \textit{H. hepaticus} and \textit{H. bilis}, compared with the adult population group (\(p = 0.0001\) and 0.04, respectively), and to \textit{H. hepaticus}, compared with blood donors (\(p = 0.01\)). Patients with autoimmune hepatitis showed no significant antibody reactivity to the enteric \textit{Helicobacter} spp. in contrast to patients with other chronic liver diseases.

\textbf{Conclusion.} Patients with chronic liver diseases, except autoimmune hepatitis patients, showed increased antibody levels to \textit{H. bilis/H. hepaticus} compared with the population and blood donors indicating a possible role of enteric \textit{Helicobacter} in the natural course of chronic liver diseases. Immunoblot seems to be a promising method for serodiagnosis of infections with these fastidious pathogens.

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1. Introduction

Infectious agents may initiate chronic diseases with known autoimmune features [1]. Recent studies of enteric \textit{Helicobacter} indicate that infections with these microorganisms may be a risk factor for chronic liver and biliary tract diseases such as chronic cholecystitis, primary sclerosing cholangitis (PSC), primary biliary cirrhosis (PBC) and hepatocellular carcinoma [1–9]. Patients with chronic biliary inflammation can develop hepatobiliary tract malignancy (i.e. cholangiocarcinoma) and show an increased proliferation of the biliary tract epithelium cells in association with presence of \textit{Helicobacter} DNA [10]. \textit{Helicobacter} spp. DNA was detected by
PCR and sequence analysis in liver samples from patients with some chronic hepatic diseases and hepatocellular carcinoma [5,11–13], however, the role of these bacteria in human liver diseases has not yet been proved.

*H. hepaticus* was isolated from liver, caecal specimens and colonic mucosa of mice with chronic active hepatitis by Ward et al. [14] and is best studied among the enterohel- 
cobacter spp. The complete genome of *H. hepaticus* was published recently and displays very high homology with some enteric Campylobacter species [15]. *H. hepaticus* produces a cytotoxophil distending toxin (CDT) which causes progressive cell enlargement associated with cell cycle arrest, DNA mutations and cell death [16]. *H. hepaticus* infection may lead to autoimmune response to human heat shock pro-
temins (Hsp) [17].

*H. bilis* was identified in inbred mice with chronic hep-
atitis [18]. Sequencing of PCR-amplified 16S rRNA gene fragments of gallbladder samples showed that *H. bilis* infects the human gallbladder and causes chronic cholecystitis [5]. *H. pullorum* was isolated from caeca of normal chicken and from the liver and intestinal contents of chickens with hepatis. *H. pullorum* was also isolated from humans with gastroenteritis and analysed by biochemical tests, DNA hybridisation and 16s rRNA sequencing [19].

Real-time quantitative PCR is a highly sensitive method to detect DNA of *Helicobacter* located in the biliary tract and liver [11,12]. Serology has been developed to diagnose the non-gastric species of *Helicobacter*, however, serology tests might have limitations due to cross-reactivity between the closely related *Helicobacter* and Campylobacter species [20,21] and optimisations are looked for [3,22].

The seroprevalence of non-gastric enteric *Helicobacter* spp. infections in adults and children is poorly investigated. Increased levels of serum antibodies to *H. hepaticus* and *H. bilis* in patients with chronic liver diseases (CLDs) compared with a population group and blood donors were reported by Ananieva et al. [23].

In the present study, levels of serum antibodies to *H. hepaticus*, *H. bilis* and *H. pullorum* were assessed by immunoblot in the sera from patients with different CLDs. In addition, sera from an adult population with high prevalence of *H. pylori* infection, from a group of children and from blood donors were included to evaluate the significance of anti-
body responses to three enteric *Helicobacter* spp. in patients with CLDs.

2. Material and method

A total of 329 serum samples were investigated. Ninety patients with biliary tract and CLDs (38 male, 52 female, mean age 54.52 ± 14.16) with the following diagnoses were studied: alcoholic liver disease (n = 18), represented by patients with alcoholic hepatitis (n = 4) and patients with alco-
holic cirrhosis (n = 14); liver cirrhosis (n = 12) (the course of cirrhosis was chronic C hepatitis in four cases, chronic B hepatitis in one case, in two cases cirrhosis was due to autoimmune hepatitis (AIH) and in five cases the cause was unknown); chronic C hepatitis (n = 6), chronic B hepatitis (n = 7), PBC (n = 13), AIH (n = 9) and common bile duct stones represented by 25 patients. All patients were diagnosed and treated in the Division of Gastroenterology, Department of Internal Diseases, Tartu University. The diagnosis was based on disease history, biochemical and immunological findings and liver histology. A cohort consisting of 121 per-
sons from an adult Estonian population (52 male, 69 female, mean age 52.11 ± 15.19) with known and previously docu-
mented high prevalence of *H. pylori* infection [24] and 50 consecutive paediatric patients (18 male, 32 female, mean age 7.46 ± 3.96) hospitalised during 5 days in the Children’s Clinic of Tartu University with different diagnoses. Serum samples from 68 blood donors (21 male, 47 female, mean age 37.02 ± 11.18) were randomly collected at the Blood 
Centre of the University Hospital of Tartu; all tested nega-
tive for hepatitis B and C viruses. All serum samples were kept frozen at –20 °C until analysis.

2.1. Enteric *Helicobacter* ELISA

Acid glycine-extracted cell surface proteins of *H. pullo-
rum* (CCUG 33838), *H. bilis* (CCUG 38995) and *H. hep-
aticus* (CCUG 33637) were used as the coating antigens and 100 μl/well (5 μg protein/ml) were added in duplicate (Maxisorp Immunoplates, NUNC, Roskilde, Denmark) and incubated for 16 h at +8 °C. The plates were blocked for 1.5 h at 22 °C with 2% bovine serum albumin in PBS-T (phosphate-
buffered saline with 0.05% Tween-20). Sera, diluted 1:800, were applied and the plates were incubated for 60 min at 37 °C. As a reference standard, human gammaglobulin (Phar-
macia & Upjohn, Stockholm, Sweden) was used. A pool of 10 sera, negative by ELISA for *H. pylori*, *H. hepaticus*, *H. bilis* and *H. pullorum* was included as a negative control and a polyclonal rabbit antiserum to each of the four *Helicobacter* species was applied as a positive control.

An alkaline phosphatase-conjugated anti-human IgG anti-
body (dilution 1:2000) and anti-rabbit IgG antibody (dilu-
tion 1:500) (DakoCytomation A5, Glostrup, Denmark) were used as the secondary antibodies. Bound antibodies were visualised by adding a substrate buffer containing 1 mg p-
nitrophenyl phosphate (Sigma, St. Louis, MO, USA) per millilitre in a diethanolamine buffer, pH 9.8.

Absorbance was measured with a dual wavelength mode with 405 nm as the primary filter and 620 nm as the refer-
ence filter after 40 min incubation. Results are expressed as corrected mean absorbance values in percentages of the re-
ference standard as the relative antibody activity (RAA). The cut-off values for seropositivity to *H. hepaticus* and *H. bilis* (RAA ≥ 28) and for *H. pullorum* (RAA ≥ 25) were based on results obtained by testing 20 *H. pylori* negative blood donor sera (mean absorbance values plus two standard deviations). All sera were pre-absorbed (described below) before ELISA analysis. Interassay variation for ELISA with the *H. hepaticus*
antigen was 9.6%, with *H. bilis* 8.9% and with *H. pullorum* 8%. Sera showing increased antibody levels by ELISA as well as some negative sera were selected for immunoblot analysis.

2.2. *H. pylori* ELISA

The sera were initially tested for IgG antibodies to cell surface proteins of *H. pylori* (strain CCUG 17874), using an ELISA method published previously. The same cut-off value for *H. pylori* (RAA > 25) was followed [25]. Interassay variation for ELISA with *H. pylori* was 13.7%.

2.3. Absorption procedure

To minimise cross-reactivity between *H. pylori* and the other three *Helicobacter* species an absorption step was performed before the ELISA and immunoblot analyses. To 1 ml of lysed cells (A500 of 1.5) of *H. pylori*, 10 μl of serum were added and incubated for 2.5 h at 22 °C during constant shaking. Cells were removed by centrifugation at 12 000 × g for 15 min and supernatants kept for serology [22]. To test the effectiveness of the absorption of *H. pylori* antibodies, a *H. pylori* ELISA was performed [24]. All sera except two became negative, with a significant decrease of antibody levels (p < 0.00001). Additionally, we have performed the pre- and post-absorption ELISA for enterohepatic *Helicobacter* spp. antigens, which revealed a statistically significant decrease in antibody titre for *H. pullorum* before and after absorption with *H. pylori* whole cell lysate (p = 0.000001, according to the Wilcoxon test) (Fig. 1).

2.4. SDS–PAGE and immunoblot assay

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblot were performed as reported previously [26]. Acid glycine-extracted cell surface proteins of *H. hepaticus*, *H. bilis* and *H. pullorum* (125 μg/gel) were separated using SDS–PAGE in a 5–20% gradient gel with a 5% stacking gel at a constant voltage of 200 V for 6 h (Protein II Cell Vertical Electrophoresis equipment, Bio-Rad, Richmond, CA, USA). Separated proteins were transferred electrophoretically to a PVDF membrane (Micron Separations Inc., Westborough, MA, USA) using a Hoefer semidry electro-blotter (SemiPhor, Amersham Biosciences, Little Chalfont, UK). Transfer time was 90 min at a constant current of 1 mA/cm². The relative molecular masses (Mr) of separated proteins were estimated by using precision marker proteins including proteins ranging from 14.4 to 97.4 kDa (Bio-Rad). A polyclonal rabbit antiserum to each of the *Helicobacter* species [23] was included as the calibrator. Strips were incubated with pre-absorbed sera diluted 1:100. For *H. hepaticus*, reactivity to at least two of the proteins with molecular masses of 76, 30 and 21 kDa proteins, for *H. bilis* to the 20 and 22 kDa proteins and for *H. pullorum* to the 48, 45, 37, 20 and 16 kDa proteins appeared to have high specificity [3].

Furthermore, cross-reactivity between the *Helicobacter* species was evaluated by immunoblot experiments using SDS–PAGE electrophoresis simultaneously with acid glycine-extracted cell surface proteins of *H. pylori*, *H. hepaticus*, *H. bilis* and *H. pullorum* (125 μg/gel). Four patient sera were selected, each with high antibody ELISA titres to one of the *Helicobacter* species. Each of these sera was absorbed with the whole cell lysate of *H. pylori*, *H. pullorum*, *H. bilis* and *H. hepaticus*, meaning that four absorptions of the same serum were performed separately. The serum was then incubated with four strips containing separated proteins of each *Helicobacter* species (Fig. 2).

2.5. Statistical analysis

The data were presented as the positive or negative test results for antibodies against *H. hepaticus*, *H. bilis* and *H. pullorum* proteins. Differences in the prevalence of the antibodies between the groups were tested using χ²-test. The difference for RAA values in the pre- and post-absorption ELISA was evaluated using Wilcoxon test. *p* values < 0.05 were considered significant.

2.6. Ethics

This study was approved by the Ethics Committee of the University of Tartu.

3. Results

3.1. Analysis of cross-reactivity between the *Helicobacter* species

Immunoblots were performed with strips containing separated proteins of the four *Helicobacter* species. With the *H. pylori* antigen, pre-absorbed sera (positive for *H. pullorum*, *H. bilis* and *H. hepaticus*) showed a strong/moderate
High rate of ELISA-positive reactivity was found with the three enteric Helicobacter species for all tested samples, especially for the sample of adult population sample (41–52%), which was significantly higher than for all other studied groups (p < 0.05). The patients with biliary tract and CLDs had a significantly higher serorelevance to H. bilis compared with the blood donors (p = 0.02) (Fig. 3). According to ELISA, the patients with PBC showed the highest seroreactivity to the bile-tolerant Helicobacter spp. and a significant difference in the response to H. hepaticus compared with the patients with AIH (6/13 versus 0/9, p = 0.02). None of the nine patients with AIH responded to any of the bile-tolerant Helicobacter spp. Paediatric patients had a significantly lower serorelevance of IgG antibodies to all bile-tolerant Helicobacter spp. as well as to H. pylori (p < 0.01).

When the immunoblot method was used, antibody reactivity to the specific antigens of the enteric Helicobacter species reduced the number of ELISA-positive sera in all studied groups, especially in the group of adult population (44% for H. hepaticus, 41% for H. bilis, 52% for H. pullorum by ELISA versus 5%, 8% and 14%, by immunoblot, respectively, p < 0.0001).

Visual analysis of the immunoblot strips showed overall high reactivity to the protein bands with Ms of 58–62 kDa, corresponding in size to Helicobacter Hsps, which indicates non-specific ELISA results. As antibodies to Hsps are considered highly cross-reactive, we therefore decided to use only the immunoblot results for comparison different groups (Table 1).

The immunoblot results obtained with the sera of the patients with CLDs showed a significantly higher seroreactivity to H. hepaticus and H. bilis, compared with the results obtained with the sera from the adult population (p = 0.0001, 0.04), and to H. hepaticus compared with the results of the blood donors (p = 0.01). None of the blood donors was pos-

<table>
<thead>
<tr>
<th>Study group</th>
<th>H. hepaticus IB positive (%)</th>
<th>H. bilis IB positive (%)</th>
<th>H. pullorum IB positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult population</td>
<td>68 (1/5)</td>
<td>56 (3/2)</td>
<td>120 (9/4)</td>
</tr>
<tr>
<td>Liver patients</td>
<td>13/10 (0.7)</td>
<td>7/12 (0.58)</td>
<td>3/17 (0.18)</td>
</tr>
<tr>
<td>Blood donors</td>
<td>0/16 (0)</td>
<td>0/9 (0)</td>
<td>3/17 (0.18)</td>
</tr>
</tbody>
</table>

Statistically significant difference: *a > b (p = 0.0001); aa > ab (p = 0.01); bb > b (p = 0.04).
irive to \( H.\) hepaticus and \( H.\) bilis, and 3 out of 17 sera were positive to \( H.\) pullorum. No significant difference in seropositivity to the enteric Helicobacter species was noted within the group of patients with various CLDs according to the immunoblot results (\( p > 0.05\)). Five sera negative by ELISA became positive by immunoblot.

4. Discussion

Immunoblot analyses with the interpretation of antibody reactivity to specific antigens of the enteric Helicobacter species reduced the number of ELISA-positive sera in all studied groups, especially in the group of adult population, indicating a high rate of cross-reactivity between proteins shared by these Helicobacter species, e.g. Hsps, urease and flagella proteins. Careful analysis of reactivity to individual proteins of the four species seems necessary and cross-reactive antigens can be identified. Hsps are highly conserved immunogenic proteins, which appeared to be poorly absorbed by the method used in the present study [2,27]. Thus, they should be excluded if one aims to reveal species-specific immune reactions.

Many Helicobacter species are very difficult to culture and serological diagnosis of enterobacterial Helicobacter infections has the advantage of being uncomplicated and cost-efficient compared with sampling of liver and gall bladder biopsies for histology and PCR, that could be accompanied with a high risk for bleeding.

Increased antibody responses to \( H.\) hepaticus and \( H.\) bilis in patients with CLDs in comparison with blood donors and the adult population, are in concordance with results of our previous investigation where antibody responses to \( H.\) hepaticus and \( H.\) bilis remained high following an absorption procedure [23]. \( H.\) hepaticus has not yet been detected in human samples and significant reactivity to this species in the liver patients might reflect cross-reactivity to antigens of other Helicobacter species not analysed in this study [12,28,29].

Among the patients with CLDs, predominantly patients with PBC displayed significantly higher antibody reactivity to \( H.\) hepaticus compared with the patients with AIP. A study by Nilsson et al. [2] found that 8% of patients with CLDs showed increased serum antibody levels to \( H.\) hepaticus as measured by ELISA after an absorption step.

In another Swedish study of human liver samples using PCR, hybridisation and partial DNA sequencing, none of the samples from patients with PSC or PBC contained DNA of \( H.\) bilis, \( H.\) pullorum or \( H.\) hepaticus [11]. Boomkens et al. [30] reported no significant difference in the incidence of Helicobacter spp.-specific DNA either in PBC or PSC (29%) or the control group (34%). Rudi et al. [31] did not detect Helicobacter in bile samples of German patients with chronic cholecystitis by PCR. The authors explained this fact by regional differences in the distribution of bile-resistant Helicobacter species.

Thus, we can support a possible association between Helicobacter species and human chronic liver and biliary tract diseases [2,3,9,11,23]. However, antibody responses to these three enteric Helicobacter species showed no clear-cut relation to specific diagnostic group of patients with CLDs, which might be explained by the small number of investigated sera from each group.

In a study by Tolia et al. [32], Helicobacter DNA was detected in liver tissues from 65% of children with some CLD and from 40% of normal controls. Our study revealed low serum antibody responses to enteric Helicobacter in the Estonian paediatric patients, possibly because of differences in the study populations, who lived in different sanitary conditions, which might have influenced their microbial status. Moreover, in our study the group of paediatric patients consisted of children without diagnosed liver diseases and we used other methods.

5. Conclusions

Presence of cross-reacting protein antigens between \( H.\) pylori and the enteric Helicobacter species evaluated in this study showed that it is difficult to design an ELISA test with high specificity if not purified specific proteins of the respective species are identified and used. The immunoblot assay for defining antibody responses to such proteins seem to be preferable to establish serodiagnosis of infections with these emerging ‘new pathogens’. Patients with CLDs showed significantly higher serum antibody levels to \( H.\) hepaticus and \( H.\) bilis compared with the adult population and blood donors. This important finding supports the opinion of a possible role of enteric Helicobacter spp. in the natural course of CLDs.

Conflict of interest statement

None declared.

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