Association of Helicobacter species with hepatitis C cirrhosis with or without hepatocellular carcinoma

M Rocha, P Avenaud, A Ménard, B Le Bail, C Balabaud, P Bioulac-Sage, D M de Magalhães Queiroz, F Méraud

Background and aims: Recent studies have suggested that bacterial coinfection with Helicobacter species in patients already infected with hepatitis C virus (HCV) could be involved in the development of cirrhosis and hepatocellular carcinoma (HCC). A retrospective cross sectional study was performed in order to explore the association between Helicobacter species and HCV associated liver diseases.

Methods: The presence of Helicobacter species was tested by polymerase chain reaction on liver samples from four groups of patients.

Results: Helicobacter 16S rDNA was found in only 4.2% of liver samples from control patients (n = 24) and in 3.5% of liver samples from patients with non-cirrhotic chronic hepatitis C (n = 29) while it was found in 68.0% of liver samples from patients with HCV positive cirrhosis without HCC (n = 25) as well as in 61.3% of cirrhotic liver samples from patients with HCV positive cirrhosis and HCC (n = 31). In addition, when the HCC tumour tissue was tested (n = 21), 90.5% of samples were positive. DNA from Helicobacter pylori and Helicobacter pullorum -like organisms was found.

Conclusions: There is an association between the presence of Helicobacter species DNA in the liver and hepatitis C cirrhosis, with or without HCC. Indeed, the presence of these bacteria could be the result of structural changes in the liver. Alternatively, Helicobacter species could be a co-risk factor in HCV chronic liver diseases. This result warrants prospective studies to determine the possible causal role of these bacteria in the progression of chronic hepatitis C.

Concerning HCV liver diseases, H pylori and H pullorum DNA were detected in the liver tissue of a large majority of HCC patients, suggesting that these bacteria are implicated in the progression of chronic hepatitis C to cirrhosis and to HCC. Recently, Dore et al confirmed these results while Fan et al found Helicobacter species in HBV positive HCC.

Based on these data, our aim was to develop new tools to study the prevalence of Helicobacter species in the liver of a large series of patients with HCV infection in order to evaluate the association between the presence of these bacteria in the liver and stage of disease.

MATERIALS AND METHODS

Patients and tissue sample analysis

A retrospective study was carried out on liver specimens collected from 109 patients (Bordeaux University Hospital) divided into four different groups according to liver pathology (table 1). Tissues not used for diagnostic purposes were immediately snap frozen in liquid nitrogen and kept frozen at −80°C until use, according to the safety and ethics rules followed by our university hospital.

The diagnosis of HCV infection was based on a third generation test (Ortho HCV 3.0 ELISA, Monolisa anti-HCV; Sanofi Diagnostics Pasteur Inc., France) and a positive HCV serum RNA (Cobas amplicor HCV 2.0; Roche Diagnostics, Branchburg, New Jersey, USA).

Pathological study

For all patients, conventional histology was performed on formalin fixed liver tissues. Sections were stained with haematoxylin eosin-safran, Masson’s trichromic stain, and reticulin stain.

Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HBV, hepatitis B virus; PCR, polymerase chain reaction.
HCV chronic hepatitis was scored using the METAVIR system.\textsuperscript{17} The diagnosis of HCC and other hepatic tumours was based on accepted criteria.\textsuperscript{18}

**Control strains used to determine polymerase chain reaction specificity**

A range of bacterial strains were used to test the specificity of different primers used in this study. These strains included a large panel of *Campylobacter* strains and other enteric bacteria commonly isolated from patients, as well as the following *Helicobacter* species: *H pylori* (ATCC 700392 and 700824), *H felis* (CCUG 28539 T), *H bilis* (ATCC 51630), *H hepaticus* (ATCC 51448), *H muridarum* (CCUG 49282), *H pullorum* (CCUG 33842 and CCUG 33839), and *F rappini* (CCUG 29176).

**DNA extraction**

DNA from frozen liver material (20–25 mg/specimen) was extracted using the QIAamp Kit (Qiagen Inc., Chatsworth, California, USA) according to the manufacturer's recommendations. DNA was stored at −20°C.

**PCR conditions**

Polymerase chain reaction (PCR) amplification was carried out in a final volume of 10 μl containing 1× buffer, 1.5 mM MgCl₂, 100 μM deoxynucleoside triphosphates, 0.5 μM each of the four primers, 0.4 U of Taq polymerase (Eurobio, Les Ulis, France), and 10 ng of DNA in a Perkin Elmer Cetus 9600 thermocycler under the conditions listed in table 2. PCR products were analysed on a 1–4% agarose gel or on a 12% polyacrylamide gel depending on amplicon size, and stained with ethidium bromide.

**Escherichia coli PCR amplification**

*Escherichia coli* malate dehydrogenase (mdh) gene was selected to develop an *E coli* species specific PCR. The design of the primer was based on the alignment of 205 mdh sequences from *E coli* as well as from closely related species such as *Salmonella typhimurium* and *Salmonella enteritica* (table 2). This PCR proved to be specific for *E coli*.

**Helicobacter genus and species specific PCR amplification**

*Helicobacter* genus specific primer pairs C97/C98\textsuperscript{20} and HS1/HS2\textsuperscript{12,21} were used to generate 16S rDNA amplicons of approximately 400 bp (table 2).

Samples generating a positive result with the *Helicobacter* genus specific PCR were subsequently analysed with six different sets of primers for detection of four of the species previously found in human liver—that is, *H bilis*, *H pullorum*, *H pylori*, and *F rappini* (table 2).

**Purification and cloning of PCR products for 16S rDNA and cdTB gene sequencing**

Different *H pylori* strains have been found in human gastric biopsies,\textsuperscript{22} and therefore the likelihood of mixed strains in hepatic biopsies cannot be excluded. Moreover, the presence of several copies of 16S and 23S rRNA genes in *H pylori* also indicates the possibility of heterozygosity.\textsuperscript{23} For these reasons, PCR products were cloned prior to sequencing. *Helicobacter* species 16S rDNA sequences were amplified with F2/R4-16S-CHPEC or C96/R4-16S-CHPEC primers, *H pylori* 23S rDNA sequences were amplified with HPY S/A primers, and *H pullorum cdTB* sequences were amplified with F4/R3-cdTB primers (table 2). PCR products were cloned into the pGEM-T easy vector System I (Promega, Madison, Wisconsin, USA), according to the manufacturer’s recommendations. Plasmids containing the expected amplicon were purified using the Qiagen Miniprep Kit (Qiagen) and sequencing was achieved on both strands with pGEM-T specific primers (F1-pGEMT: 5′-CGG CCA GTG AAT TGT AAT ACG-3′ and R1-pGEMT: 5′-ATG ACC ATG ATT ACG CCA AG-3′). For *Helicobacter* species 16S rDNA amplicons, internal sequences were determined with pseudo-universal eubacterial primers designed for this study (F1-16S-HCES: 5′-ACA CGG TCC AGA CTC CTA CG-3′; F4-16S-CHPEC: 5′-CAA GCG GTG GAG CAT GTG-3′;
R1-16S-CHPEC: 5'-AGCAGACTTTATGATGTGAC 16S rRNA (94°C; 30 sec, 60°C; 1 min, 72°C; 40 cycles) and R2-16S-CHPEC: 5'-AAG GCATGATGAC 16S rRNA (94°C; 30 sec, 60°C; 30 sec, 72°C; 40 cycles). 

RESULTS

Pathological findings (Table 1)

Control tissues from group I were considered strictly normal or subnormal (mild steatosis, mild non-specific lobular or portal inflammatory infiltrate); only one showed obstructive cholestasis which was considered a consequence of tumour compression. None of the cases showed chronic hepatitis, liver cell dysplasia, or fibrosis. In group II, liver biopsies showed typical features of chronic hepatitis C, with no fibrosis or portal fibrosis, with or without septa. In group III, patients presented with chronic hepatitis C of varying activity. Liver dysplasia, of the large or small cell type, was found in 16 cases (particularly abundant in four). Macronodules, of the macroregenerative or dysplastic type, were found in four liver samples. In group IV, hepatitis C activity, as well as dysplasia, was present in most livers and was either limited or extensive. All 31 HCC were of the classical type: unifocal in eight cases, bifocal in six, and multifocal in 17. The size of the largest tumoral nodule measured 14 cm. Macroglobulin and dysplastic nodules were frequently associated (n = 12).

In the 21 HCC cases where the tumour could be analysed for Helicobacter detection (non-necrotised HCC with tumour size >15 mm), well differentiated (grade 2, n = 8) or moderately differentiated HCC (grade 3, n = 13) with a classical trabecular (n = 11), trabeculoglandular (n = 8), or macrotrabeular and compact (n = 2) architecture was observed. 

Escherichia coli and Helicobacter genus detection (Fig 1)

Among the 130 specimens from 109 patients, none was positive for E coli. Helicobacter genus DNA was detected in 38 cases: 36 using H1/H2S primers and the same 36 plus two additional cases using C97/C98 primers. These positive cases were found mainly in group III (17 cases, 68%) and group IV (19/31 (61.3%) cases in cirrhotic liver; 19/21 (90.5%) cases in the tumour). In contrast, only one of 24 (4.2%) and one of 29 (3.5%) samples were positive in groups I and II, respectively, and the difference between groups I and II compared with groups III and IV was statistically significant (p<10^-4). Moreover, all 19 patients in group IV who were positive at the

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Table 2 Primers and polymerase chain reaction (PCR) conditions used for amplification of the different DNA targets

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Organism</th>
<th>Gene</th>
<th>Cycling conditions</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS-1</td>
<td>AACGATAGCTGCTATGCTACATGAGTAC</td>
<td>Helicobacter genus</td>
<td>16S RNA</td>
<td>(94°C; 30 sec, 60°C; 1 min 72°C; 40 cycles)</td>
<td>399</td>
<td>Germani</td>
</tr>
<tr>
<td>HS-2</td>
<td>GTGTAATCTGATGATGCTACATGAGTAC</td>
<td>Campylobacter genus</td>
<td>16S RNA</td>
<td>(94°C; 30 sec, 60°C; 1 min 72°C; 40 cycles)</td>
<td>398</td>
<td>Avenaud</td>
</tr>
<tr>
<td>C97</td>
<td>GCTATCGACCTGACCTGAGTAC</td>
<td>Helicobacter genus</td>
<td>16S RNA</td>
<td>(94°C; 30 sec, 54°C; 90 sec 72°C; 60 sec)</td>
<td>1456</td>
<td>Fox</td>
</tr>
<tr>
<td>C98</td>
<td>GATTATCGACCTGACCTGAGTAC</td>
<td>Helicobacter genus</td>
<td>16S RNA</td>
<td>(94°C; 30 sec, 60°C; 30 sec 72°C; 80 sec)</td>
<td>1374</td>
<td>This study</td>
</tr>
</tbody>
</table>

For all of these species were specific, with control strains listed in materials and methods. F4/R3-cdfB primers were used to determine the nucleotide sequence of H pullorum cdfB gene.
Three patients were positive using specific primers for *H pylori* positive for *Helicobacter*. Three samples were positive, in addition to the control strains. 

*cdt* remained undetected using the 16S rDNA PCR. F1- and R2- with primers for *H pullorum* the three patients positive for detected in liver specimens from these 35 patients and no *H pylori* glm was developed to detect *H pullorum Helicobacter canadensis*, another enterohepatic *H pullorum*. For these reasons a new PCR, designed on the *cdt* gene, was developed to detect *H pullorum* strains which possibly remained undetected using the 16S rDNA PCR. F1- and R2- *cdt*-pullorum specific primers were used; only the same three samples were positive, in addition to the control strains. The 35 remaining *Helicobacter* genus positive patients were positive for *H pylori* 23S rDNA and 22 were also positive for *H pylori* glm (61.1%). No *H pullorum* DNA sequence was detected in liver specimens from these 35 patients and no *H pylori* DNA sequence was detected in liver specimens from the three patients positive for *Helicobacter* DNA sequences.

Sequencing was performed on 16S rDNA amplicons of 1456 bp from patients C5 and C45 (positive for *H pylori* PCR) and of 1374 bp from patient C51 (positive for *H pylori* PCR) (table 4). The 16S rDNA sequences from biopsies C5 and C45 were 99% similar to the 16S rDNA of *H pullorum* for the entire length of the available sequence in GenBank. Moreover, these two sequences were different from each other as they had 10 mismatches. The 16S rDNA sequence from the C51 biopsy had 99% sequence homology with *H pylori* 16S rDNA. Another Blast program search was performed on a shorter fragment (398 bp) of C51 corresponding to the C97/98 amplicon usually used for such experiments. Interestingly, higher sequence homologies (99%) were found with 59 similar sequences of *H pylori* 16S rDNA, including previously reported sequences amplified from liver specimens from patients with HCC—that is, *Helicobacter* species “liver 3”, *Helicobacter* species “liver 2”, and *Helicobacter* species “liver 1”.24

Furthermore, the species specific amplicons of biopsies C5, C45, and C51 were also sequenced. The 675 bp *cdt* sequences from C5 and C45 biopsies were similar and were shown to have 98% homology with the only two *cdt* gene sequences of *H pullorum* available in GenBank (table 4), with several residue changes in the corresponding nucleotide and protein sequences. For the 23S rDNA sequence from biopsy C51, the higher sequence homologies (100%) were found with 17 similar sequences of *H pylori* 23S rDNA.

**DISCUSSION**

The discovery of the presence of *Helicobacter* species DNA in liver material from patients with liver disease has led to the challenging hypothesis that these bacteria may play a role in the evolution of hepatic lesions from chronic viral hepatitis to cirrhosis and HCC. Determinants of this evolution are not yet fully understood, including those occurring in HCV positive patients.

A similar evolution of stomach lesions from gastritis to atrophy, intestinal metaplasia, dysplasia, and adenocarcinoma has been linked to infection by *H pylori*.26 In an attempt to detect an association between *Helicobacter* species infection

<p>| Table 3 Identification at the species level of <em>Helicobacter</em> DNA present in liver material |
|-----------------------------------------|---------------------------------|---------------------------------|---------------------------------|</p>
<table>
<thead>
<tr>
<th>Histological group (n)</th>
<th><em>Helicobacter</em> genus</th>
<th><em>Helicobacter pylori</em></th>
<th><em>Helicobacter pullorum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>I-Control (24)</td>
<td>1</td>
<td>1</td>
<td>*</td>
</tr>
<tr>
<td>II-Hepatitis C virus (29)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>III-Cirrhosis C without HCC (25)</td>
<td>17</td>
<td>16</td>
<td>12*</td>
</tr>
<tr>
<td>IV-HCC and cirrhosis C</td>
<td>17††</td>
<td>16††</td>
<td>8*</td>
</tr>
<tr>
<td>Cirrhotic tissue (31)</td>
<td>19††</td>
<td>17††</td>
<td>10*</td>
</tr>
<tr>
<td>Tumoral tissue (21)</td>
<td>19††</td>
<td>17††</td>
<td>2††</td>
</tr>
</tbody>
</table>

HCC, hepatocellular carcinoma

*These cases were all positive using the 23S rDNA primers.

†Cases positive in cirrhotic liver tissue were also positive in the corresponding tumoral tissue.

††These cases were all positive using the 16S rDNA primers.

<p>| Table 4 Results of DNA sequencing on a subgroup of cases found positive for <em>Helicobacter pylori</em> or <em>Helicobacter pullorum</em> by polymerase chain reaction (PCR) |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|</p>
<table>
<thead>
<tr>
<th>Biopsy No</th>
<th>Group</th>
<th><em>H pylori</em></th>
<th><em>H pullorum</em></th>
<th>Sequence alignment results (Strain—Genbank accession number)</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5</td>
<td>III</td>
<td>23S rDNA</td>
<td>glmM</td>
<td>16S rDNA (universal)</td>
<td><em>H pylori</em> 23S rDNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UB 3659</td>
<td>L36147</td>
<td>/ NA</td>
<td>35818*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UB 151</td>
<td>L36144</td>
<td>/ NA</td>
<td>35818*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MC 238</td>
<td>U01329</td>
<td>2106</td>
<td>AF550406 /</td>
</tr>
<tr>
<td>C45</td>
<td>IV</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C51</td>
<td>IV†</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

NA, not amplified by the corresponding PCR. HPYS/A and cdtB for *H pylori* and *H pullorum* detection, respectively.

"H pullorum" taxon: 35818. "H pylori" taxon: 2106

For 16S rDNA, 23S rDNA, and cdtB sequences, amplified primer less sequences of 1414 or 1332, 222, and 675 bp were blasted and alignments were done using GenBank database. The 16S rDNA sequences for biopsies C5, C45, and C51 were submitted to GenBank and assigned accession numbers AY394474, AY394473 and AY394476, respectively. The cdtB sequences and the deduced protein sequences for biopsies C5 and C45 were assigned accession numbers AY394475 and AY394477, respectively. The 23S rRNA sequence for biopsies C51 was assigned accession number AY394478.
of the liver and liver pathology, we studied HCV positive patients. Our results showed an association: virtually all patients with HCC were *Helicobacter* species positive in their HCC tumour material and 61–68% of those with cirrhosis were positive in liver tissue compared with 4.5% and 3.2% of hepatitis patients and controls, respectively. The results were also highly consistent. For *Helicobacter* genus detection by PCR, tumour material was always positive when cirrhotic tissue was positive. The difference in age between patients with cirrhosis C and those with cirrhotic hepatitis C with HCC could explain the higher prevalence of *Helicobacter* in the group of patients with cirrhosis C. However, the control group, which was similar in size to this group, did not have a higher prevalence, indicating that in contrast with *Helicobacter* in the stomach, the prevalence in the liver does not increase with age.

These data are in agreement with several other studies which showed the presence of *Helicobacter* species in the liver of patients with and without non-cirrhotic HCC.13–16 Only two of these reports examined HCV positive patients. In the study of Ponzetto et al, 23 of 25 HCC HCV positive frozen liver specimens contained *Helicobacter* species DNA and sequencing of the 16S rDNA amplicons also revealed the presence of *H pylori* - and *H pullorum*-like organisms.17 More recently, a similar study was performed on formalin fixed paraffin embedded liver tissues.18 Despite the fact that DNA is not well conserved in such tissue, tests showed that six of 11 patients with HCV positive HCC contained *Helicobacter* species DNA in the liver, and three corresponded to *H pylori* as they contained vacA sequences.

Interestingly, and as previously reported in liver specimens from patients with HCC,13 16 the highest homology of our 398 bp 16S rDNA sequences was obtained with *H pylori* sequences previously reported in liver specimens from patients with non-cirrhotic HCC.12 This study is the first in which both tumour and cirrhotic liver tissue samples from patients with HCV positive HCC were tested. When comparing the results of cirrhotic liver tissue from patients with only cirrhosis (61.3%) with those with both cirrhosis and HCC (68%), a similar proportion of *Helicobacter* species positive specimens was obtained from patients with HCC.

However, at least one study did not find any *Helicobacter* species, raising the possibility that the results of other studies may be false positives.17 However, there are a number of arguments against this interpretation. Firstly, we did not find any specimens positive for *E coli*. This bacterium, which is one of the gut’s main inhabitants, would most likely be present in the liver as a contaminant, either via the portal circulation or by retrograde transfer from the duodenum. It is indeed an argument against the possibility of extrahepatic DNA sequestered in the liver via the portal circulation because in such cases *E coli* DNA would also be found. Secondly, we are confident that intra-laboratory contaminants cannot explain these results as we did not use nested PCR and, moreover, there was variability in the PCR products, as proven by sequencing. Finally, *Helicobacter* species cannot be considered as environmental contaminants. Therefore, we came to the same conclusion as Wadström et al19 that false negative results may have occurred in the study in which no *Helicobacter* species DNA were found in the liver.20 Indeed, the PCR positive control was not appropriate (gastric tissue) and not enough technical information on the PCR was given in the paper to determine whether there might have been another problem.

It may be argued that if a *Helicobacter* species is present in the liver, one should be able to culture it. Indeed, one case of positive culture was reported in a patient suffering from Wilson’s disease.20 The retrospective nature of our study, based on frozen material, made culture attempts difficult. In any case, it is most likely that these bacteria are in low quantities and in a special physiological state, rendering them difficult to culture under standard conditions. Even in the case of mouse liver disease due to *H hepaticus*, it has proved very difficult to culture the bacteria.21 Moreover, there are *Helicobacter* species such as *Helicobacter helmannii* which, despite their presence in high numbers (for example, in the pig stomach) do not grow on currently available media but only under very special conditions such as in the mouse stomach.22

Among the different *Helicobacter* species previously found in hepatobiliary diseases23 and tested by specific PCR in this study, neither *H bilis* nor *H rappini* was found but *H pullorum*-like DNA was detected in the liver of three patients. DNA from *H pylori*-like organisms was found in 35 of 38 *Helicobacter* genus positive patients but only 61.1% of these 35 cases were amplified by *glmM* PCR, suggesting that in the remaining cases either this gene is absent or nucleotide variations occur in this region impairing its detection by PCR. Another possibility is that an unknown *Helicobacter* species close to *H pylori* is present.

A limitation of our study is obviously its retrospective nature, which did not enable us (1) to determine whether *H pylori* was present in the stomach of these patients and (2) to gather all of the clinical and biological information needed for a more thorough analysis (for example, virus genotype, duration of viral infection). However, we believe that this study, which included a large series of HCC patients with specimens obtained both in tumour and cirrhotic tissue and for which a number of new tools were developed, offers a strong argument in favour of an association between *Helicobacter* species and both HCV cirrhosis and hepatitis C cirrhosis with HCC.

In conclusion, based on this study we have no argument for a causal association and it may well be that the presence of *Helicobacter* is the consequence of structural changes in the liver (namely, intrahepatic shunts) when cirrhosis occurs.31 12 However, the possibility that *Helicobacter* infection is a risk factor for the evolution towards cirrhosis remains and deserves further exploration, given that these bacteria can produce toxins which may interfere with hepatic cells. A prospective study, including bacterial culture from material obtained from the stomach in addition to liver tissue, is warranted to determine the *H pylori* status of the stomach in these patients.

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