**Relationship between the severity of hepatitis C virus-related liver disease and the presence of *Helicobacter* species in the liver: A prospective study**

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**Abstract**

**AIM:** To determine the presence of *Helicobacter* species DNA in the liver of chronic hepatitis C (CHC) patients with and without cirrhosis as compared to controls, and to identify the bacterial species involved.

**METHODS:** Seventy-nine consecutive patients (HBV and HIV negative) with a liver sample obtained after liver biopsy or hepatic resection were studied: 41 with CHC without cirrhosis, 12 with CHC and cirrhosis, and 26 controls (HCV negative). Polymerase chain reactions (PCRs) targeting *Helicobacter* 16S rDNA and species-specific were performed on DNA extracted from the liver. A gastric infection with *H pylori* was determined by serology and confirmed by 13C-urea breath test.

**RESULTS:** Overall, *Helicobacter* 16S rDNA was found in 16 patients (20.2%). Although positive cases tended to be higher in CHC patients with cirrhosis (41.6%) than in those without cirrhosis (17.0%) or in controls (15.4%), the difference was not statistically significant ($P = 0.08$). *H pylori*-like DNA was identified in 12 cases and *H. pullorum* DNA in 2, while 2 cases remained unidentified. Gastric infection with *H pylori* was found in only 2 of these patients.

**CONCLUSION:** Our results do not confirm the association of *Helicobacter* species DNA in the liver of CHC patients with advanced liver disease. The lack of correlation between positive *H pylori* serology and the presence of *H pylori*-like DNA in the liver may indicate the presence of a variant of this species.

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**Key words:** Hepatitis C virus; Hepatitis; Cirrhosis; *Helicobacter*

INTRODUCTION

Chronic hepatitis C virus (HCV) infection is a major public health problem with over 170 million people infected worldwide. It is the leading cause of chronic liver disease and the main indication for liver transplantation in the Western world[1]. Approximately 80% of patients with acute infection develop chronic hepatitis. Chronic hepatitis C is associated with a wide spectrum of liver histological lesions ranging from mild chronic hepatitis to cirrhosis and hepatocellular carcinoma (HCC)[2]. The course of HCV-related hepatic disease varies markedly from one patient to another. Several factors including age at exposure, duration of infection, alcohol intake, male gender and more recently steatosis have been shown to be associated with fibrosis progression[3-6]. However, even in the absence of these factors, disease progression may be observed in some...
patients, suggesting the role of other factors which remain to be identified. Host genetic factors or environmental factors, such as a bacterial coinfection, could be involved.

Several Helicobacter species colonize the liver of animals and induce hepatitis[7]. In the past few years, the emergence of new Helicobacter species associated with the pathogenesis of human enterohelicobacterial diseases has been observed[8-10]. H pylori and Helicobacter pullorum (H. pullorum) DNA have been detected in the liver tissue of patients with chronic hepatitis C and HCC, suggesting that these bacteria could be implicated in the progression of chronic hepatitis C to cirrhosis and HCC[11]. In addition, we have shown recently in a cross-sectional retrospective study, a significant association between the presence of Helicobacter species DNA in the liver and HCV-related cirrhosis with and without HCC[12]. Given the limitation of retrospective studies, the aim of this new study was to prospectively determine the prevalence of Helicobacter infection of the liver in HCV-infected patients with and without cirrhosis as compared to controls.

MATERIALS AND METHODS

Study population
All patients with chronic hepatitis C undergoing a percutaneous liver biopsy at the Hepatology Clinics of Bordeaux University between March 2002 and December 2003 were eligible. Chronic hepatitis C was defined by detection of HCV antibodies using a third generation test (Ortho HCV 3.0 ELISA Monolisa anti-HCV, BioRad, Marne la Coquette, France), detectable HCV RNA (Cobas ampicollic HCV 2.0, Roche Diagnostics, Neuilly sur Seine, France) in serum, and elevated alanine aminotransferase (ALT) levels as compared to controls.

Processing of liver tissues
Fresh liver tissues obtained either from needle biopsies or from surgical specimens during usual diagnostic or therapeutic procedures, were immediately cut into three parts with three different conditioning protocols: formalin fixation for routine histology (at least 1 cm for needle biopsies), freezing in liquid nitrogen-cooled isopentane followed by storage at -80°C before molecular biology, and immersion in a specific culture medium for culture characterization of the bacterium.

Pathological study
For all patients, conventional liver histology was performed on formalin-fixed liver tissues. Sections were stained with hematoxylin-eosin-safran, Masson’s trichromic stain and reticulin stain. In patients with chronic hepatitis C, liver fibrosis and necroinflammatory activity were evaluated semi-quantitatively according to the METAVIR scoring system[13]. Fibrosis was staged on a 0-4 scale as follows: F0 = no fibrosis; F1 = portal fibrosis without septa; F2 = portal fibrosis and few septa; F3 = numerous septa without cirrhosis; F4 = cirrhosis. Activity was graded as follows: A0 = none; A1 = mild; A2 = moderate; A3 = severe. The diagnosis of hepatocellular carcinoma was based on usual criteria[14].

H pylori infection diagnosis
Serum samples from all patients were tested for anti H pylori IgG antibodies, using the commercially available kit Pyloriset EIA-III (Orion Diagnostica, Espoo, Finland) and the Western blot assay HELICO BLOT 2.1 (Genelabs Diagnostics®, Singapore). When antibodies were present, gastric infection with H pylori was searched by 14C-urea breath test (UBT) or by culture on gastric biopsy specimens obtained during upper gastrointestinal endoscopy when indicated for diagnosis of esophageal varices in patients with cirrhosis. UBT was performed after overnight fasting, 75 mg of urea was ingested after citric acid. Air samples were obtained before and 30 min after urea ingestion and analyzed by isotope ratio mass spectrometry. Serology and UBT have 95% sensitivity and specificity[15].

Helicobacter culture from liver tissue
Standard Helicobacter culture methods were used including Wilkins Chalgren agar with antibiotics[16] and chocolate agar without antibiotics. The plates with fresh liver tissues were incubated at 37°C for 4 d, one set in a microaerobic atmosphere and another in an anaerobic atmosphere.

To maximize the culture of Helicobacter from the biopsies, a flask of confluent murine hepatic (CCL 9.1) cells was systematically inoculated with liver samples and observed during a week for a color change or opacification of the medium, or for a cytopathic effect. The medium was also observed microscopically before discarded.

DNA extraction from liver tissues
DNA from frozen liver material (20 to 25 mg/specimen) was extracted by using the QIAamp kit (Qiagen Inc., Chatsworth, CA) as previously described[13].

PCR conditions
Standard PCR amplifications were carried out as previously reported and PCR products were analyzed on a 1%-4% agarose gel, depending on the amplicon size, and stained with ethidium bromide[12].

Two real time PCRs were performed using the TaqMan® or SYBR® Green chemistries. The TaqMan real time PCR amplification and hybridization reactions were carried out in a final volume of 10 µL containing 5 µL of TaqMan® Universal PCR Master Mix, 0.3 µmol/L of each primer, 0.2 µmol/L of the labeled probe Helico-spp-16S and 1 µL
of purified DNA in a ABI PRISM® 7900 thermocycler (Applied Biosystems, Foster City, CA). DNA was amplified using the following cycling parameters: heating at 95°C for 10 min followed by 40 cycles of a two-stage temperature profile of 95°C for 15 s and 60°C for 1 min. The SYBR® Green real time PCR amplification and melting curve analysis were carried out in a final volume of 10 µL containing 5 µL of SYBR® Green PCR Master Mix, 0.3 µmol/L of each primer and 1 µL of purified DNA in a ABI PRISM® 7900 thermocycler (Applied Biosystems). DNA was amplified using the following cycling parameters: heating at 50°C for 2 min, then at 95°C for 10 min followed by 40 cycles of a two-stage temperature profile of 95°C for 15 s and 60°C for 1 min. Then a dissociation of the amplicon from 60°C to 95°C was followed during 20 min.

Escherichia coli PCR amplification
A PCR targeting a 130-bp sequence of the malate dehydrogenase (mdh) gene of E. coli was applied to the liver tissue as previously described[12].

Helicobacter genus- and species-specific PCR amplification
Helicobacter genus-specific primer pairs C97/C98[17] and H51/H52[18] were used to generate 16S rDNA amplicons of approximately 400 bp. Moreover, a new PCR targeting the Helicobacter genus 16S rRNA gene was developed using the TaqMan chemistry. Sequences of different Helicobacter species such as H pylori, Helicobacter heilmannii, Helicobacter fennelliae, Flexiplora rappini, Helicobacter bilis, Helicobacter cinaedi, Helicobacter winghamensis, Helicobacter pullorum, and Helicobacter bizzozeronii were aligned. Closely related 16S rRNA genes from various bacteria, such as Campylobacter coli, Campylobacter jejuni, Campylobacter fetus, Wolinella succinogenes, were also included and a search was carried out to identify conserved regions specific to the Helicobacter genus. As a result, a set of primers (AS2-Helicoc-TQM: 5′-CCGTGTCTCAGTTCCAGTGTGT-3′ and S1-Helicoc-TQM: 5′-GATCAGCTATGTCCTATCAGCTTGT-3′) were designed to amplify a 106-bp sequence in Helicobacter species. After the specificity of the PCR was ensured, a probe was also designed (Helico-spp-16S FAM-TCACCCTCTCAGGCCGGATACC-TAMRA). TaqMan probes were synthesized with the FAM reporter dye covalently linked to the 5′P ends and the TAMRA quencher dye at the 3′P ends which were phosphorylated to prevent probe extension. Primers and probes used for the TaqMan assays synthesized by Proligo (Paris, France) were designed using a Primer Express software package PE-ABI (Applied Biosystems).

Samples generating a positive result with the Helicobacter genus-specific PCR were subsequently analyzed with seven different sets of primers for the detection of four species previously found in human liver, i.e. H. salis, H. spilorum, H pylori and F. rappini[22]. For H. salis and H. spilorum primers amplifying a 151-bp product and a 140-bp product on the cdbB gene were used, respectively[12]. For F. rappini, primers amplifying a 101-bp product on the ureB gene were used[12]. For H pylori, four real time PCRs were performed for detecting a 267-bp product on the 23S rRNA gene[19], a 146-bp product on the glmM (ureC) gene[22], a 231-bp product on the ureA gene[21], and a 303-bp product on the 26 kDa specific antigen gene[22].

Purification and cloning of PCR products for 16S rDNA sequencing
Given the possibility of the presence of different strains in the same sample, PCR products were cloned prior to sequencing. Helicobacter species 16S rDNA samples were amplified, cloned and sequenced as previously reported[23]. Helicobacter genus-specific and species-specific amplified primer-less sequences were compared to the GenBank database with the Blast program at the National Center for Biotechnology Information Computer server[29].

Statistical analysis
Descriptive statistics are provided as means ± SD. One way analysis of variance (ANOVA) or the Mann-Whitney test was used when necessary for statistical comparison of quantitative data, and the chi-square test or Fisher’s exact test for qualitative data. P < 0.05 was considered statistically significant.

RESULTS
Characteristics of the study population
A total of 79 patients with a liver sample obtained after percutaneous liver biopsy or hepatic resection were included. The median length of the needle biopsy specimens was 1.4 cm (mean 1.35 cm). The characteristics of the patients are shown in Table 1.

Fifty-three patients who had chronic hepatitis C were divided into two groups: group 1 consisting of patients without cirrhosis (n = 41), group 2 consisting of patients with cirrhosis (n = 12). One patient in group 2 also had HCC. As expected, patients with cirrhosis (group 2) were significantly older than those without cirrhosis (group 1) (60 ± 11 years vs 50 ± 13 years, respectively; P < 0.03) and had significantly higher AST levels (3.7 ± 2.4 upper limit of normal value vs 1.5 ± 1.1 upper limit of normal value, respectively; P = 0.001) and ALT levels (3.7 ± 2.8 ULN vs 1.7 ± 1.3 ULN, respectively; P < 0.003). Conversely, they did not differ in gender distribution, route of HCV transmission, duration of infection, alcohol intake, GGTL levels, HCV genotype and viral load.

Group 3 consisted of HCV negative control patients (n = 26) in whom liver tissues were taken from the non-tumoral part of hepatectomy specimens after resection for hepatic benign tumors (n = 11; i.e. 4 focal nodular hyperplasias, 2 hydatid cysts, 1 cavernous hemangioma, 1 liver cell adenoma, 1 biliary cyst, 1 cystadenoma, 1 abscess) or metastatic tumors (n = 15; i.e. all 15 of colorectal origin).

Helicobacter culture
Despite our efforts, it was impossible to grow any Helicobacter strain from the liver, either on the plate media or in the tissue culture flasks.

Helicobacter genus detection by PCR
Overall, Helicobacter genus DNA was detected in the liver of 16 patients (20.2%). These positive cases were found in
7/41 (17.0%) patients of group 1, 5/12 (41.6%) patients of group 2, and 4/26 (15.4%) patients of group 3 (Figure 1). Although positive cases tended to be more frequent in patients with cirrhosis than in those without cirrhosis or in controls, the difference did not reach statistical significance (P = 0.08). Among the 79 liver specimens studied, none was positive by PCR for E. coli, a frequent colonizer of the gut.

A number of factors were tested for their association with the presence of Helicobacter DNA in the liver of 53 patients with chronic hepatitis C (with or without cirrhosis). None of these factors was associated with the presence of Helicobacter (Table 2). Positive patients were significantly younger than negative patients (46 ± 13 years vs 54 ± 13 years, respectively; P = 0.05).

**Helicobacter pylori serology and urea breath test**

Overall, anti H pylori IgG antibodies were detected in 20/76 (26.3%) cases. These positive cases were found in 12/41 (29.2%) patients of group 1, 3/11 (27.0%) patients of group 2 and 5/24 (20.8%) patients of group 3. As shown in Table 3, among the 16 patients positive for Helicobacter DNA in the liver, anti H pylori IgG antibodies were detected in 2/7 (28.5%) patients of group 1, 1/4 (25.0%) patients of group 2 and 1/4 (25.0%) patients of group 3. Serology was not performed in one positive patient. All the results obtained by ELISA were also confirmed using an immunoblot specific for H pylori. Gastric infection was confirmed by UBT in the 2 positive patients of group 1 but not in the positive patient of group 2. UBT was not performed in one positive control patient.

**Identification of Helicobacter species**

Material from 16 Helicobacter genus positive patients was tested with species-specific primers (Table 3). Neither H. billii nor H. rappini was found (data not shown). H pylori-like organisms were identified in 12 (75%) cases and H. spullorum-like organisms were identified in 2 cases (12.5%), 2 samples remained unidentified. H pylori-like organisms were present in 4/7 (57%) patients of group 1, 4/5 (80.0%) patients of group 2, and 4/4 (100%) patients of group 3. These results were confirmed by sequencing the 16S rRNA gene in the 7 cases where it was carried out. Among the positive samples by sequencing or by H pylori specific PCR, none of them reacted with the highly conserved primer designed to amplify the specific vacA gene of H pylori and variable results were obtained with other H pylori specific targets, such as ureA, glmM. Among the 7 samples for which the near complete 16S rRNA gene was sequenced, the 2 nucleotide polymorphisms in position 92 and 130 which were found to be associated with Helicobacter 16S rDNA sequences from the liver described by Verhoef et al. were detected in 5 instances: 3 sequences harboured the 2 polymorphisms simultaneously, 2 sequences...
Table 3 Results of *H pylori* serology, urea breath test (UBT) and species identification in the liver, for the 16 patients positive for *Helicobacter* genus-specific PCR in the liver

| Groups        | Patient No. | H pylori infection Serology | 23S rDNA | H pylori PCR ureA (kDa) | glmM | H. pullorum PCR cdtB | Conclusion          |
|---------------|-------------|----------------------------|----------|------------------------|------|----------------------|---------------------|
| Group 1       | 1           | -                          | -        | -                      | -    | -                    | Not identified       |
| (Hepatitis)   | 2           | -                          | -        | +                      | -    | +                    | "H. pullorum"-like1 |
|               | 3           | -                          | -        | +                      | +    | -                    | "H pylori"-like     |
|               | 4           | +                          | +        | +                      | -    | +                    | "H pylori"-like     |
|               | 5           | +                          | +        | +                      | -    | +                    | "H pylori"-like     |
|               | 6           | -                          | -        | -                      | -    | -                    | Not identified       |
|               | 7           | -                          | -        | +                      | -    | -                    | "H pylori"-like     |
| Group 2       | 8           | -                          | +        | +                      | -    | -                    | "H pylori"-like     |
| (Cirrhosis)   | 9           | -                          | +        | +                      | +    | -                    | "H pylori"-like     |
|               | 10          | ND                         | -        | +                      | +    | -                    | "H pylori"-like     |
|               | 11          | -                          | -        | -                      | -    | +                    | "H pylori"-like     |
|               | 12          | +                          | -        | +                      | -    | -                    | "H. pullorum"-like  |
| Group 3       | 13          | -                          | -        | -                      | -    | +                    | "H pylori"-like     |
| (Controls)    | 14          | -                          | -        | -                      | -    | -                    | "H pylori"-like     |
|               | 15          | +                          | ND       | -                      | +    | +                    | "H pylori"-like     |
|               | 16          | -                          | -        | +                      | +    | -                    | "H pylori"-like     |

1 Also confirmed by 16S rDNA sequence; ND: Not determined. The 16S rRNA amplified primer-less sequences of approximate 1370 bp for biopsies 2, 3, 5, 7, 8, 9 and 14 were submitted to GenBank and assigned accession numbers DQ062210 to DQ062216, respectively.

DISCUSSION

In a previous retrospective cross-sectional study,[12] we showed that DNA from *H pylori* and *H. pullorum*-like organisms was present in the liver of patients with hepatitis C cirrhosis with or without HCC, suggesting that *Helicobacter* species could be a co-risk factor for progression of HCV chronic liver diseases. However, a limitation of this preliminary study was its retrospective nature which did not allow us to determine whether *H pylori* was present in the stomach of these patients and to gather all of the clinical and biological information on HCV genotype and duration of infection, etc, which is needed for a more accurate analysis.

In this prospective study, we were able to better characterize the presence of *Helicobacter* species in the liver of a group of consecutive patients with HCV infection with or without cirrhosis, using both the tools which were developed in the retrospective study as well as new tools.

The results of the present study only partially confirm those obtained previously. Although the prevalence of *Helicobacter* DNA tended to be higher in the liver samples from patients with hepatitis C cirrhosis than in those from HCV-infected patients without cirrhosis or from controls, the difference did not reach statistical significance (*P* = 0.09 and *P* = 0.08, respectively). However, we cannot exclude the fact that given the limited number of patients with HCV-induced cirrhosis, this result may be due to a lack of statistical power. The negative results obtained with regard to the detection of *E. coli* DNA in liver material confirm that *H pylori* DNA detected is not the result of a non specific transport or failure in elimination by a non functional liver. Indeed, since *E. coli* is constantly present in the human intestine, if a non specific bacterial translocation occurred, we could find *E. coli* DNA in the liver.

This study did not enable us to solve the issue of identifying specific *Helicobacter* species involved since no positive culture occurred on the media usually used for *Helicobacter* culture. Indeed, in another study bacteria closely related to *H pylori* morphologically were visualized in the liver of 6 out of 20 patients with HCC[25]. Furthermore, Oliveira et al only found *H pylori* in two studies on ulcerative colitis[25] and Crohn’s disease patients[20]. For the latter, *H pylori* was more frequent than in the intestinal mucosa of the control group. These two studies were however carried out in a country of high *H pylori* prevalence, Brazil.

In the current study, we also had the possibility to perform *H pylori* serology on most patients. Interestingly, among the 15 patients (out of 16) positive for *Helicobacter* DNA in the liver, only 4 (26.6%) had *H pylori* antibodies.
Given this surprising result, immunoblot was performed and the results were confirmed.

Unfortunately, the protocol did not include a UBT when Helicobacter DNA was present in the liver, but only when H. pylori serology was positive. Indeed, 2 out of 3 positive serology cases were confirmed by UBT. As the sensitivity of our serological kit is 95%, it is surprising that such a high number of patients (11/15) did not produce H. pylori antibodies, which casts a doubt on the identification of the species present. Two potential explanations can be given: (1) H. pylori is truly present in the liver, but not in the stomach, and at an insufficient load to stimulate an immune response; and (2) another Helicobacter species which is closely related to H. pylori but does not cross-react with H. pylori antigens, is present in the liver. These patients did not receive H. pylori eradication therapy, which could explain the eradication of the bacteria. The possibility of immunodeficiency in HCV positive patients, especially with cirrhosis can also be considered as an explanation of the lack of serological response but we have not found arguments for this hypothesis.

The 1370 bp sequence of the 16S rRNA gene of six patients with H. pylori DNA shows 99% homology with H. pylori, but it is known that 16S rDNA sequences do not have a good discriminatory power in the Helicobacter genus. A recent taxonomic analysis showed for example that H. felis, H. salomonis and H. bizzozeronii could not be differentiated on the basis of the 16S rRNA gene sequence [29].

There are a number of genes which are theoretically specific for H. pylori, e.g. ureA, glmM, and vacA. Although they were looked for in this study, they were not uniformly present. The absence of the H. pylori specific vacA detected by a PCR targeting a short sequence is particularly surprising. Furthermore, a striking feature in the 16S rDNA sequences obtained from liver material is the presence of 2 nucleotide polymorphisms at the positions 92 and 130 as described by Verhoef et al. [30]. Indeed, this polymorphism was already present in the sequences first described by Avenaud et al. [31] and Ponzetto et al. [32], and was present in most of our cases (5/7). It is important to note that in the study of Verhoef et al. [30], H. pylori was grown from the stomach of 3 out of 9 (33%) patients with this Helicobacter 16S rDNA polymorphism in the liver, and the corresponding gastric strains also had this polymorphism and were confirmed to be H. pylori. It is therefore most likely that this species corresponds to a variant of H. pylori with different properties including bile resistance and the ability to colonize the liver. This polymorphism is also present in the only strain grown from liver material [31]. The study of strains obtained from the same patients several years apart has shown a greater diversity than in other bacterial species studied due to high rates of mutations and recombinations [33]. This observation has led to the concept of quasi species which could correspond to the situation found in the liver.

Despite the doubt concerning the reality of the presence of H. pylori in the liver (absence of culture and negative H. pylori specific PCR), the presence of H. pullorum-like organisms in the liver is much more likely as has been confirmed by H. pullorum specific cdA/B PCR and by sequencing of the 16S rRNA gene. The identification of H. pullorum is easy, given that this bacterium has been designated as a separate species on the basis of 16S rRNA gene sequencing [34]. H. pullorum isolates were initially recovered from the cecal content of broiler chickens and from the liver and intestinal content of laying hens with vibriotoxic hepatitis, suggesting that this bacterium can infect the liver and that poultry may serve as the source of human infection [35]. H. pullorum has also been cultured from immunodeficient patients with gastroenteritis and one HIV-infected patient [36]. One individual, in addition to having diarrhea, developed elevated liver enzyme levels and hepatomegaly which, although not proven, may have been induced by H. pullorum invasion of the liver in a manner similar to the organism’s ability to cause hepatitis in chickens [37]. There is clearly a potential for zoonotic foodborne transmission of H. pullorum to humans, as is known to occur with Campylobacter species.

In conclusion, Helicobacter DNA can be present in the liver of patients with liver disease, with a tendency to have a higher prevalence in those with cirrhosis. Although H. pullorum appears to be regularly found at a low rate, the exact nature of the main Helicobacter species present is still uncertain. It is most likely a variant of H. pylori which has acquired specific properties. Further studies exploring both the liver and stomach of patients with liver diseases need to be carried out to unravel the mystery of this intriguing association.

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REFERENCES

1. Lauer GM, Walker BD. Hepatitis C virus infection. N Engl J Med 2001; 345: 41-52
2. Marcellin P, Asselah T, Boyer N. Fibrosis and disease progression in hepatitis C. Hepatology 2002; 36: S47-S56
3. Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAIVIR, CLINIVIR, and DOSVIRC groups. Liver 1997; 349: 825-832
4. Poynard T, Ratziu V, Charlotte F, Goodman Z, McHutchison J, Albrecht J. Rates and risk factors of liver fibrosis progression in patients with chronic hepatitis c. J Hepatol 2001; 34: 730-739
5. Roudot-Thoraval F, Bastie A, Pawlotsky JM, Dhuemenaux D. Epidemiological factors affecting the severity of hepatitis C virus-related liver disease: a French survey of 6,664 patients. The Study Group for the Prevalence and the Epidemiology of Hepatitis C Virus. Hepatology 1997; 26: 485-490
6. Castéra L, Hézode C, Roudot-Thoraval F, Bastie A, Zafrazi ES, Pawlotsky JM, Dhuemenaux D. Worsening of steatosis is an independent factor of fibrosis progression in untreated patients with chronic hepatitis C and paired liver biopsies. Gut 2003; 52: 298-292
7. Ward JM, Fox IG, Anwer MR, Haines DC, George CV, Collins MJ Jr, Gorelick PL, Nagashima K, Gonda MA, Gilden RV. 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: 1222-1227
8. Avenaud P, Marais A, Monteiro L, Le Bail B, Bioulac Sage P, Balabaud C, Megraud F. Detection of Helicobacter species in the liver of patients with and without primary liver carcinoma. Cancer 2000; 89: 1431-1439
9. Solnick Jv, Schauer DB. Emergence of diverse Helicobacter

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species in the pathogenesis of gastric and enterohepatic diseases. Clin Microbiol Rev 2001; 14: 59-97
10 de Magalhães Queiroz DM, Santos A. Isolation of a Helicobacter species from the human liver. Gastroenterology 2001; 121: 1023-1024
11 Ponzetto A, Pellicano R, Leone N, Cutufia MA, Turrini F, Grgioni WF, D'Errico A, Motterpen P, Rizzetto M, Silengo L. Helicobacter infection and cirrhosis in hepatitis C virus carriage: is it an innocent bystander or a troublemaker? Med Hypotheses 2000; 54: 277-277
12 Rocha M, Avenaud P, Menard A, Le Bail B, Balabaud C, Boulac-Sage P, de Magalhães Queiroz DM, Mégraud F. Association of Helicobacter species with hepatitis C cirrhosis with or without hepatocellular carcinoma. Gut 2005; 54: 396-401
13 Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The META VIR Cooperation Study Group. Hepatology 1996; 24: 289-293
14 IARC. Tumors of the liver and intrahepatic bile ducts. In: Pathology and genetics of tumours of the digestive system. Lyon, 2000: 157-217
15 Monteiro L, de Mascarel A, Sarraetsqueta AM, Bergey B, Barberis C, Talty B, Roux D, Schouler L, Goldfain D, Lamouliatte H, Mégraud F. Diagnosis of Helicobacter pylori infection: noninvasive methods compared to invasive methods and evaluation of two new tests. Am J Gastroenterol 2001; 96: 353-358
16 Mégraud F, Lehn N, Lind T, Bayerdörffer E, O'Morain C, Spiller R, Unge P, van Zanten SV, Wrangstadh M, Burman CF. Antimicrobial susceptibility testing of Helicobacter pylori in a large multicenter trial: the MACH 2 study. Antimicrob Agents Chemotherapy 1999; 43: 2747-2752
17 Fox JG, Dewhirst FE, Shen Z, Feng Y, Taylor NS, Paster BJ, Ericson RL, Lau CN, Correa P, Araya JC, Roa I. Hepatic Helicobacter species identified in bile and gallbladder tissue from Chilenes with chronic cholecystitis. Gastroenterology 1998; 114: 755-763
18 Germani Y, Dauga C, Duval P, Huerre M, Levy M, Plaloux G, Sansonetti P, Grémont PA. Strategy for the detection of Helicobacter species by amplification of 16S rRNA genes and identification of H. felis in a human gastric biopsy. Res Microbiol 1997; 148: 315-326
19 Oleastro M, Menard A, Santos A, Lamouliatte H, Monteiro L, Barthélémy P, Mégraud F. Real-time PCR assay for rapid and accurate detection of point mutations conferring resistance to clarithromycin in Helicobacter pylori. J Clin Microbiol 2003; 41: 397-402
20 Boonjakaukul JK, Syvanen M, Suryaprasad A, Bowlus CL, Solnick JV. Transcription profile of Helicobacter pylori in the human stomach reflects its physiology in vivo. J Infect Dis 2004; 190: 946-956
21 He Q, Wang JP, Osato M, Lachman LB. Real-time quantitative PCR for detection of Helicobacter pylori. J Clin Microbiol 2002; 40: 3720-3728
22 Mikula M, Dzwoniec A, Jaguszyn-Krynicka K, Ostrowski J. Quantitative detection for low levels of Helicobacter pylori infection in experimentally infected mice by real-time PCR. J Microbiol Methods 2003; 55: 351-359
23 Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997; 25: 3389-3402
24 Huang Y, Fan XG, Wang ZM, Zhou JH, Tian XF, Li N. Identification of helicobacter species in human liver samples from patients with primary hepatocellular carcinoma. J Clin Pathol 2004; 57: 1273-1277
25 Oliveira AG, das Graças Pimenta Sanna M, Rocha GA, Rocha AM, Santos A, Dani R, Marinho FP, Moreira LS, de Lourdes Abreu Ferrari M, Moura SB, Castro LP, Queiroz DM. Helicobacter species in the intestinal mucosa of patients with ulcerative colitis. J Clin Microbiol 2004; 42: 384-386
26 Oliveira AG, Rocha GA, Rocha AM, Sanna Md, Moura SB, Dani R, Marinho FP, Moreira LS, Ferrari Mde L, Castro LP, Queiroz DM. Isolation of Helicobacter pylori from the intestinal mucosa of patients with Crohn's disease. Helicobacter 2006; 11: 2-9
27 Feldman RA, Deeks JJ, Evans SJ. Multi-laboratory comparison of eight commercially available Helicobacter pylori serology kits. Helicobacter pylori Serology Study Group. Eur J Clin Microbiol Infect Dis 1995; 14: 428-433
28 O’Rourke JL, Solnick JV, Neilan BA, Seidel K, Hayter R, Hansen LM, Lee A. Description of ‘Candidatus Helicobacter helimannii’ based on DNA sequence analysis of 16S rRNA and uracile colitis. Int J Syst Evol Microbiol 2004; 54: 2203-2211
29 Verhoef C, Pot RG, de Man RA, Zondervan PE, Kuipers EJ, IJzermans JN, Kusters JG. Detection of identical Helicobacter DNA in the stomach and in the non-cirrhotic liver of patients with hepatocellular carcinoma. Eur J Gastroenterol Hepatol 2003; 15: 1171-1174
30 Kuipers EJ, Israel DA, Kusters JG, Gerrits MM, Weel J, van Der Ende A, van Der Hulst RW, Wirth HP, Hooik-Nikanne J, Thompson SA, Blaser MJ. Quasispecies development of Helicobacter pylori observed in paired isolates obtained years apart from the same host. J Infect Dis 2000; 181: 273-282
31 Stanley J, Linton D, Burnens AP, Dewhirst FE, On SL, Porter A, Owen RJ, Costas M. Helicobacter pullorum sp. nov. - genotype and phenotype of a new species isolated from poultry and from human patients with gastroenteritis. Microbiology 1994; 140 (Pt 12): 3441-3449
32 Burnens AP, Stanley J, Morgenstern R, Nicolet J. Gastroenteritis associated with Helicobacter pullorum. Lancet 1994; 344: 1569-1570