

H. pylori

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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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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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 ($P =$

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 enterohepatic 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 amplicor HCV 2.0, Roche Diagnostics, Neuilly sur Seine, France) in serum, and elevated alanine aminotransferase (ALT) levels for more than 6 mo. Exclusion criteria were age under 18 years, co-infection with hepatitis B virus (HBV) or human immunodeficiency virus (HIV).

The following data were collected at the time of enrolment of patients with chronic HCV infection: age, gender, route of HCV transmission, duration of HCV infection, alcohol intake, aspartate aminotransferase (AST), ALT, gamma-glutamyl transpeptidase (GGT), HCV genotype and viral load.

All patients seronegative for HCV and undergoing hepatic resection for benign tumors or metastatic tumors during the study period, were eligible and served as controls. All patients gave their informed consent and the study protocol conformed to the 1975 declaration of Helsinki and was approved by the Ethics Committee of our institution.

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 hematein-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-G 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 ¹³C-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^[12].

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[®] 7000 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[®] 7000 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 HS1/HS2^[8,18] 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 beilmannii*, *Helicobacter fennelliae*, *Flexispira rappini*, *Helicobacter bilis*, *Helicobacter cinaedi*, *Helicobacter winghamensis*, *Helicobacter pullorum*, and *Helicobacter bizozzeronii* 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-Helico-TQM: 5'-CCGTGTCT-CAGTTCAGTGTGT-3' and S1-Helico-TQM: 5'-GAT-CAGCCTATGTCCTATCAGCTTGT-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-TCACCCTCTCAGGCC-GGATACCC-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 Prologo (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. sbilis*, *H. spullorum*, *H. pylori* and *F. rappini*^[12]. For *H. sbilis* and *H. spullorum* primers amplifying a 151-bp product and a 140-bp product on the *cdtB* 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^[20], 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^[12]. *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^[23].

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, GGT 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

Table 1 Characteristics of the 79 patients at the time of inclusion

Characteristics	Group 1 (Hepatitis)	Group 2 (Cirrhosis)	Group 3 (Controls)
Patients (n)	41	12	26
Age (yr)	50 ± 13	60 ± 11 ^a	49 ± 12
Gender (M/F)	14/27	5/7	13/13
Route of transmission, n (%)			
Transfusion	10 (24%)	5 (42%)	-
IVDU	11 (27%)	3 (25%)	-
Other or unknown	20 (49%)	4 (33%)	-
Duration of infection (yr)	19 ± 9	23 ± 5	-
Alcohol intake (g/d)	12 ± 23	12 ± 14	-
AST (xULN)	1.5 ± 1.1	3.7 ± 2.4 ^b	-
ALT (xULN)	1.7 ± 1.3	3.7 ± 2.8 ^b	-
GGT (xULN)	1.4 ± 0.7	2.0 ± 1.9	-
HCV genotype, n (%)			
1	24 (58%)	8 (67%)	-
2/3	11 (27%)	4 (33%)	-
Other	6 (15%)	0 (0%)	-
HCV viral load (IU/mL)	827 889 ± 123 741	923 026 ± 181 866	-
Histological lesions (Metavir)			
Activity, n (%)			
A0-1	16 (39%)	0 (0%)	-
A2-3	25 (61%)	12 (100%) ^a	-
Fibrosis, n (%)			
F0-1	16 (39%)	-	-
F2	23 (56%)	-	-
F3	2 (5%)	-	-
F4	-	12 (100%)	-

IVDU: Intravenous drug use; ULN: Upper limit of normal value; ^a*P* < 0.05, ^b*P* < 0.01 vs hepatitis group.

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

Table 2 Factors associated with *Helicobacter* DNA presence in the liver

Factor	<i>Helicobacter</i> DNA		<i>P</i>
	Present	Absent	
Patients (n)	12	41	
Age (yr)	46 ± 13	54 ± 13	0.05
Gender (M/F)	5/7	12/29	NS
Route of transmission			
Transfusion	3 (25%)	12 (29%)	
IVDU	3 (25%)	11 (27%)	NS
Other or unknown	6 (50%)	18 (44%)	
Duration of infection (yr) (n = 36)	18 ± 7	21 ± 8	NS
Alcohol intake (g/d)	5 ± 8	14 ± 24	NS
AST (xULN)	2.2 ± 2.1	1.9 ± 1.5	NS
ALT (xULN)	2.7 ± 2.9	2.0 ± 1.5	NS
GGT (xULN)	1.3 ± 0.5	1.6 ± 1.2	NS
HCV genotype			
1	9 (75%)	23 (56%)	
2/3	2 (17%)	13 (24%)	NS
Other	1 (8%)	5 (20%)	
HCV viral load (IU/mL)	663 824 ± 569 933	972 157 ± 1 388 853	NS
Histological lesions (Metavir score)			
Activity			
A0-1	4 (33%)	12 (29%)	
A2-3	8 (67%)	29 (71%)	NS
Fibrosis			
F0-2	7 (58%)	32 (78%)	
F3-4	5 (42%)	9 (22%)	NS

IVDU: Intravenous drug use; ULN: Upper limit of normal value; NS: Not significant.

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. bilis* 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.*^[29] 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.	<i>H. pylori</i> infection		<i>H. pylori</i> PCR				<i>H. pullorum</i> PCR	Conclusion
		Serology	UBT	23S rDNA	<i>ureA</i>	26 kDa	<i>glmM</i>	<i>cdtB</i>	
Group 1 (Hepatitis)	1	-	-	-	-	-	-	-	Not identified
	2	-	-	-	-	+	-	+	" <i>H. pullorum</i> "-like ¹
	3	-	-	+	+	+	-	-	" <i>H. pylori</i> "-like ¹
	4	+	+	+	+	-	-	-	" <i>H. pylori</i> "-like
	5	+	+	+	+	+	-	-	" <i>H. pylori</i> "-like ¹
	6	-	-	-	-	-	-	-	Not identified
	7	-	-	+	-	-	-	-	" <i>H. pylori</i> "-like ¹
Group 2 (Cirrhosis)	8	-	-	+	+	+	-	-	" <i>H. pylori</i> "-like ¹
	9	-	-	+	+	+	+	-	" <i>H. pylori</i> "-like ¹
	10	ND	-	+	+	+	+	-	" <i>H. pylori</i> "-like
	11	-	-	-	-	+	-	-	" <i>H. pylori</i> "-like
	12	+	-	-	-	+	-	+	" <i>H. pullorum</i> "-like
Group 3 (Controls)	13	-	-	-	-	+	-	-	" <i>H. pylori</i> "-like
	14	-	-	-	-	+	-	-	" <i>H. pylori</i> "-like ¹
	15	+	ND	-	+	+	-	-	" <i>H. pylori</i> "-like
	16	-	-	+	+	+	-	-	" <i>H. pylori</i> "-like

¹Also 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.

harboured one of these 2 polymorphisms and 2 sequences did not present these polymorphisms.

DISCUSSION

In a previous retrospective cross-sectional study^[12], we showed that DNA from *H. pylori*- and *H. spullorum*-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

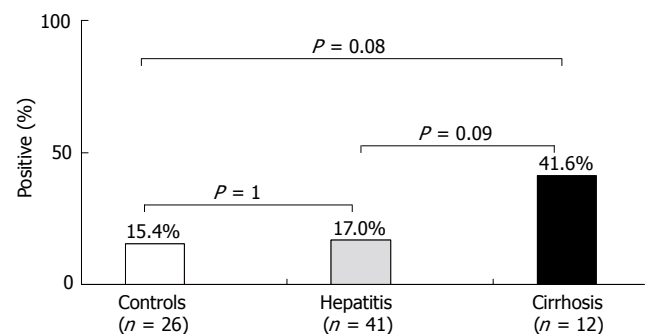


Figure 1 Percentage of positive biopsies for *Helicobacter* genus by DNA detection. Group 1 corresponds to hepatitis ($n = 41$), group 2 corresponds to cirrhosis ($n = 12$), and group 3 corresponds to controls ($n = 26$). *Helicobacter* genus positivity and negativity was compared using Fisher's exact test.

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^[24]. Furthermore, Oliveira *et al* only found *H. pylori* in two studies on ulcerative colitis^[25] and Crohn's disease patients^[26]. 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%^[15,27], 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. bizsonzeronii* could not be differentiated on the basis of the 16S rRNA gene sequence^[28].

There are a number of genes which are theoretically specific for *H pylori*, eg *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*^[29]. Indeed, this polymorphism was already present in the sequences first described by Avenaud *et al*^[8] and Ponzetto *et al*^[11], and was present in most of our cases (5/7). It is important to note that in the study of Verhoef *et al*^[29], *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^[10]. 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^[30]. 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. spullorum*-like organisms in the liver is much more likely as has been confirmed by *H. spullorum* specific *cdtB* 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^[31]. *H. pullorum* isolates were initially recovered from the cecal content of broiler chickens and from the liver and intestinal content of laying hens with vibriotic hepatitis, suggesting that this bacterium can infect the liver and that poultry may serve as the source of human infection^[31]. *H. pullorum* has also been cultured from immunocompetent patients with gastroenteritis and one HIV-infected patient^[31]. 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^[32]. There is clearly a potential for zoonotic food-borne 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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