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

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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.

There are extensive data indicating that chronic infection can lead to cancer in various organs. Parasites, such as schistosomes and liver flukes, and a bacterium, *Helicobacter pylori*, were classified as type I carcinogens by the International Agency for Research on Cancer in 1994.¹ The most compelling evidence comes from hepatocellular carcinomas (HCC) where chronic hepatitis B virus (HBV) infection is considered to be a direct aetiological factor and hepatitis C virus (HCV) infection a major risk factor for this disease.¹

Most HCC in developed countries are linked to chronic HCV infection, alcoholic cirrhosis, and sometimes HBV infection.² The liver fluke, *Opisthorchis viverrini*, is also a risk factor for the development of cholangiocarcinoma.³ While a small proportion of the general population is infected by HCV, chronic hepatitis occurs in up to 75% of cases; 20% will develop cirrhosis, and in 3–5% of patients, liver cirrhosis will evolve into HCC each year.⁴ The reasons for this evolution are not well understood and may involve co-risk factors due to HCV per se, such as host genetic factors and environmental factors. Current risk factors cannot fully explain this evolution.⁵

A bacterium, *Helicobacter hepaticus*, from the mouse gut flora colonises the bile canaliculi and liver in mice, induces chronic hepatitis, and its persistence leads to the development of a carcinoma.^{6–7} In this model, as well as in the human stomach infected by *H. pylori*, chronic inflammation induced by the pathogen is considered to be a key factor in carcinoma development.

In the past few years, the emergence of novel and diverse *Helicobacter* species associated with the pathogenesis of human enterohepatic diseases has been observed.^{8–11} *Helicobacter* species can also be present in the liver of HCV negative patients and have been associated with HCC development in the non-cirrhotic liver.^{12–13}

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.¹⁴ Recently, Dore *et al* confirmed these results¹⁵ while Fan *et al* found *Helicobacter* species in HBV positive HCC.¹⁶

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 haematein-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

Table 1 Pathological features in the four patient groups

	Group I	Group II	Group III	Group IV
No	24	29	25	31
Mean age (y)	53	40.1	53.8	57.8
Sex (M/F)	15/9	19/10	14/11	25/6
METAVIR score				
Fibrosis				
F0–F1	24	15	0	0
F2	0	12	0	0
F3	0	2	0	0
F4	0	0	25	31
Activity				
A0	23	0	0	0
A1	1	12	7	6
A2	0	15	12	19
A3	0	2	6	5
Dysplasia				
Absent	24	29	7	2
Present	0	0	16	28

Biopsy samples from control patients (group I) were taken from the non-tumoral part of hepatectomy specimens after resection for hepatic benign tumours (n = 12; that is, seven focal nodular hyperplasias, two cavernous haemangiomas, one liver cell adenoma, one angiomyolipoma, and one solitary fibrous tumour) or metastatic tumours (n = 12; that is, nine colorectal, one mammary, one oesophageal, and one ovarian).

Patients in group II presented with chronic active hepatitis C without cirrhosis. Three patients also had a moderately excessive alcohol intake (>20 g of alcohol/day for women and 40 g for men) and in one case serological sequelae of hepatitis B infection.

Group III consisted of samples from patients who underwent liver transplantation for terminal stage cirrhosis due to hepatitis C. Two patients also had a past history of excessive alcohol intake and four had sequelae of hepatitis B infection (6/25).

Patients in group IV presented with cirrhotic hepatitis C and hepatocellular carcinoma. Most samples from this last group were obtained after total (n = 28) or partial (n = 3) hepatectomy. In addition to HCV infection, there was also a medical history of excessive alcohol intake in six cases, past hepatitis B in two cases, and both risk factors were present in one case (9/31). In group IV, tumour material was only available in 21 cases.

HCV chronic hepatitis was scored using the METAVIR system.¹⁷ The diagnosis of HCC and other hepatic tumours was based on accepted criteria.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* (ATCC 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, with minor modifications.¹⁹ 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/C98²⁰ and HS1/HS2^{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,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.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';

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

Name	Sequence	Organism	Gene	Cycling conditions	Amplicon size (bp)	Reference
HS-1	AACGATGAAGCTTCTAGCTTGCTAG	<i>Helicobacter</i> genus	16S rRNA	(94°C; 30 sec, 60°C; 1 min 72°C;	399	Germani ²¹
HS-2	GTGCTTATTCGTAGATACCGTCAT	<i>Campylobacter</i> genus		30 sec)×40		Avenaud ¹²
C97	GCTATGACGGGTATCC	<i>Helicobacter</i> genus	16S rRNA	(94°C; 30 sec, 54°C; 90 sec 72°C;	398	Fox ²⁰
C98	GAITTTACCCCTACACCA			60 sec)×35		
F2-16S-CHEPC	ATCCTGGCTCAGAGTGAACG	Pseudo-universal	16S rRNA	(94°C; 30 sec, 60°C; 30 sec, 72°C;	1456	This study
R4-16S-CHEPC	CCTACGGTTACCTTGTACGAC			80 sec)×40		
C96	ACGGGTGAGTAACGCATAGG	<i>Helicobacter</i> genus	16S rRNA	(94°C; 30 sec, 60°C; 30 sec, 72°C;	1374	This study
R4-16S-CHEPC	CCTACGGTTACCTTGTACGAC			80 sec)×40		
F1- <i>mdh</i> -coli	TTGTGCAATGTGCTACGTT	<i>Escherichia coli</i> *	<i>mdh</i>	(94°C; 1 min, 60°C; 1 min, 72°C;	130	This study
R1- <i>mdh</i> -coli	ATACCTTCAGCGCGTTCT			1 min)×35		
No name	ATGAATGCTAGTGTGTGAG	<i>Helicobacter pullorum</i>	16S rRNA	(94°C; 30 sec, 60°C; 1 min, 72°C;	467	Stanley ²⁴
No name	GATTGGCTCCACTTCACA	<i>Helicobacter canadensis</i>		80 sec)×40		
HPY S	AGGTTAAGAGGATGCGTCAGTC	<i>Helicobacter pylori</i> *	23S rRNA	(94°C; 1 min, 55°C; 1 min, 72°C;	267	Ménard ³³
HPY A	CGCATGATATCCATTAGCAGT			1 min)×40		
F1- <i>glmM</i> -HP	AACGCCCTTTCTCTCAAGC	<i>Helicobacter pylori</i> *	<i>glmM</i>	(94°C; 30 sec, 60°C; 30 sec, 72°C;	130	This study
R1- <i>glmM</i> -HP	GCATGCATTCGCTCACAA			10 sec)×40		
F1- <i>cdtB</i> -pullorum	GTCCTTTGAGTGGATTGGATTCT	<i>Helicobacter pullorum</i> *	<i>cdtB</i>	(94°C; 30 sec, 60°C; 1 min, 72°C;	140	This study
F2- <i>cdtB</i> -pullorum	GCCAGCAAGTCTTTTGGAGTG			20 sec)×40	148	
R2- <i>cdtB</i> -pullorum	CACCTCCGGGTGCTTGTGTAT					
F4- <i>cdtB</i>	TCAGCTAGCACAGAAAATAATGG	Non-specific	<i>cdtB</i>	(94°C; 30 sec, 55°C; 60 sec, 72°C;	719	This study
		<i>Helicobacter pullorum</i>		30 sec)×40		
R3- <i>cdtB</i>	ACTAGCTGCTGCCAAAATCG					
F2- <i>cdtB</i> -bilis	CGAATCTATTATCCGGGCTTG	<i>Helicobacter bilis</i> *	<i>cdtB</i>	(94°C; 30 sec, 60°C; 1 min, 72°C;	151	This study
R2- <i>cdtB</i> -bilis	GCCAAAGCGAGTCTATCATTAG			30 sec)×40		
F1- <i>ureB</i> -rappini	GATGATTAGGCGACACAGC	<i>Helicobacter rappini</i> *	<i>ureB</i>	(94°C; 30 sec, 60°C; 30 sec, 72°C;	101	This study
R2- <i>ureB</i> -rappini	CCCCAGATTCTATCTGCTTACTC			10 sec)×40		

Cycling conditions: after four minutes of initial denaturation at 94°C, each reaction mixture was amplified for 35–40 cycles under the conditions indicated, depending on the PCR. After the last cycle, a final extension was continued for another seven minutes.

F, forward; R, reverse.

Partial Genbank available *cdtB* sequences of *H pullorum* (AF220065), *H bilis* (AF243077), and *H hepaticus* (AF163667 and AF243076) were aligned in order to design the most possible specific *H pullorum* and *H bilis* PCR on the *cdtB* gene. Forward F1- and F2-*cdtB*-pullorum were designed to be used with the R2-*cdtB*-pullorum reverse primer.

*All of these PCR were species specific, with control strains listed in materials and methods. F4/R3-*cdtB* primers were used to determine the nucleotide sequence of *H pullorum cdtB* gene.

R1-16S-HCES: 5'-CTT GCA CCC TCC GTA TTA CC-3'; and R2-16S-CHEPC: 5'-AAG GGC CAT GAT GAC TTG AC-3').

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,

Figure 1 Percentage of positive biopsies for *Helicobacter* genus by DNA detection. Groups I–IV correspond to control patients (n = 24), patients with chronic hepatitis C (n = 29), patients with post hepatitis C cirrhosis (n = 25), and patients with cirrhosis and HCC (n = 31), respectively. Concerning group IV, biopsies were obtained in cirrhotic tissue for all (Ci) and in tumoral (Tu) tissue for 21 patients. *Helicobacter* genus positivity and negativity was compared using Fisher's exact test. *Cases positive in cirrhotic liver tissue were also positive in corresponding tumoral tissue.

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. Macroregenerative 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 macrotrabecular 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 HS1/HS2 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

Table 3 Identification at the species level of *Helicobacter* DNA present in liver material

Histological group (n)	<i>Helicobacter</i> genus	<i>Helicobacter pylori</i>		<i>Helicobacter pullorum</i>	
		23S rDNA	glmM	16S rDNA	cdtB
I-Control (24)	1	1	1*	0	0
II-Hepatitis C virus (29)	1	1	1*	0	0
III-Cirrhosis C without HCC (25)	17	16	12*	1	1§
IV-HCC and cirrhosis C					
Cirrhotic tissue (31)	17†	16†	8*	1†	1§
Tumoral tissue (21)	19	17	10*	2	2§

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.

cirrhotic liver tissue level were also positive in the corresponding tumoral tissue.

As some of the patients, mainly from groups III and IV, had other aetiological factors, we examined if the presence of *Helicobacter* was associated with these factors, and found that it was evenly distributed.

Helicobacter species identification

Material from 38 *Helicobacter* genus positive patients was then tested with species specific primers (table 3). None reacted with primers for *cdtB* of *H. bilis* or for *ureB* of *F. rappini* by PCR. Three patients were positive using specific primers for 16S rDNA of *H. pullorum*.²⁴ However, this PCR did not react with our two *H. pullorum* control strains which can amplify *Helicobacter canadensis*, another enterohepatic *Helicobacter*.²⁵ For these reasons a new PCR, designed on the *cdtB* gene, was developed to detect *H. pullorum* strains which possibly remained undetected using the 16S rDNA PCR. F1- and R2-*cdtB*-*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* *glmM* (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 *H. pullorum* DNA sequences.

Sequencing was performed on 16S rDNA amplicons of 1456 bp from patients C5 and C45 (positive for *H. pullorum* 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”.¹²

Four other 16S rDNA C97/C98 amplicons of the 35 samples positive for *H. pylori* PCR were also sequenced for both DNA strands. Sequences obtained were shown to be similar to *H. pylori* 16S rRNA gene (data not shown).

Furthermore, the species specific amplicons of biopsies C5, C45, and C51 were also sequenced. The 675 bp *cdtB* sequences from C5 and C45 biopsies were similar and were shown to have 98% homology with the only two *cdtB* 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

Table 4 Results of DNA sequencing on a subgroup of cases found positive for *Helicobacter pylori* or *Helicobacter pullorum* by polymerase chain reaction (PCR)

Biopsy No	Group	PCR				Sequence alignment results (Strain—Genbank accession number)					Conclusion	
		<i>H. pylori</i>		<i>H. pullorum</i>								
		23S rDNA	<i>glmM</i>	16S rDNA	<i>cdtB</i>	16S rDNA (universal)	<i>H. pylori</i> 23S rDNA	<i>H. pullorum</i> <i>cdtB</i>				
C5	III	—	—	+	+	UB 3659	L36147	/	NA	35818*	AF220065	" <i>H. pullorum</i> "-like
C45	IV	—	—	+	+	UB 151	L36144	/	NA	35818*	AF220065	" <i>H. pullorum</i> "-like
C51	IV	+	+	—	—	MC 238	U01329	210§	AF550406	/	NA	" <i>H. pylori</i> "-like

NA, not amplified by the corresponding PCR: HPY5/A and *cdtB* for *H. pylori* and *H. pullorum* detection, respectively.**H. pullorum* taxon: 35818. §*H. pylori* taxon: 210.

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.³⁴ 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 rDNA 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 age 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.^{12–16} 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.¹⁴ More recently, a similar study was performed on formalin fixed paraffin embedded liver tissues.¹⁵ 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.²⁷ 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 al*²⁸ that false negative results may have occurred in the study in which no *Helicobacter* species DNA were found in the liver.²⁷ 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.¹⁰ 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.²⁹ Moreover, there are *Helicobacter* species such as *Helicobacter heilmannii* 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.³⁰

Among the different *Helicobacter* species previously found in hepatobiliary diseases²⁰ 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, 32} 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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REFERENCES

- 1 IARC. Schistosomes, liver flukes and *Helicobacter pylori*. In: IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon, 7–14 June 1994. *Monographs on the evaluation of carcinogenic risks to humans*. Lyon: IARC, 1994;61:1–241.
- 2 El-Serag HB. Hepatocellular carcinoma and hepatitis C in the United States. *Hepatology* 2002;36:S74–83.
- 3 Srivatanakul P. Epidemiology of liver cancer in Thailand. *Asian Pac J Cancer Prev* 2001;2:117–21.
- 4 Seeff LB. Natural history of chronic hepatitis C. *Hepatology* 2002;36:S35–46.
- 5 El-Serag HB. Hepatocellular carcinoma: an epidemiologic view. *J Clin Gastroenterol* 2002;35:S72–8.
- 6 Fox JG, Dewhirst FE, Tully JG, et al. *Helicobacter hepaticus* sp. nov., a microaerophilic bacterium isolated from livers and intestinal mucosal scrapings from mice. *J Clin Microbiol* 1994;32:1238–45.
- 7 Ward JM, Fox JG, Anver MR, et al. 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;86:1222–7.
- 8 Nilsson HO, Castedal M, Olsson R, et al. Detection of *Helicobacter* in the liver of patients with chronic cholestatic liver diseases. *J Physiol Pharmacol* 1999;50:875–82.
- 9 Nilsson HO, Taneera J, Castedal M, et al. Identification of *Helicobacter pylori* and other *Helicobacter* species by PCR, hybridization, and partial DNA sequencing in human liver samples from patients with primary sclerosing cholangitis or primary biliary cirrhosis. *J Clin Microbiol* 2000;38:1072–6.
- 10 Queiroz DMD, Santos A. Isolation of a *Helicobacter* strain from the human liver. *Gastroenterology* 2001;121:1023–4.
- 11 Solnick JV, Schauer DB. Emergence of diverse *Helicobacter* species in the pathogenesis of gastric and enterohepatic diseases. *Clin Microbiol Rev* 2001;14:59–97.
- 12 Avenaud P, Marais A, Monteiro L, et al. Detection of *Helicobacter* species in the liver of patients with and without primary liver carcinoma. *Cancer* 2000;89:1431–9.
- 13 Nilsson HO, Mulchandani R, Stenram U, et al. *Helicobacter* species identified in liver from patients with cholangiocarcinoma and hepatocellular carcinoma. *Gastroenterology* 2001;120:323–4.
- 14 Ponzetto A, Pellicano R, Leone N, et al. *Helicobacter* infection and cirrhosis in hepatitis C virus carriage: is it an innocent bystander or a troublemaker? *Med Hypotheses* 2000;54:275–7.
- 15 Dore MP, Realdi G, Mura D, et al. *Helicobacter* infection in patients with HCV-related chronic hepatitis, cirrhosis, and hepatocellular carcinoma. *Dig Dis Sci* 2002;47:1638–43.
- 16 Fan X-G, Peng Xi-N, Huang Y, et al. *Helicobacter* species ribosomal DNA recovered from the liver tissue of Chinese patients with primary hepatocellular carcinoma. *Clin Infect Dis* 2002;35:1555–7.
- 17 Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* 1996;24:289–93.
- 18 IARC. Pathology and genetics of tumours of the digestive system. In: *Tumors of the liver and intrahepatic bile ducts*. Lyon: IARC, 2000;61:157–217.
- 19 Monteiro L, Gras N, Vidal R, et al. Detection of *Helicobacter pylori* DNA in human feces by PCR: DNA stability and removal of inhibitors. *J Microbiol Methods* 2001;45:89–94.
- 20 Fox JG, Dewhirst FE, Shen ZL, et al. Hepatic *Helicobacter* species identified in bile and gallbladder tissue from Chileans with chronic cholecystitis. *Gastroenterology* 1998;114:755–63.
- 21 Germani Y, Dauga C, Duval P, et al. 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;148:315–26.
- 22 Oleastro M, Ménard A, Santos A, et al. 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.
- 23 Taylor DE, Eaton M, Chang N, et al. Construction of a *Helicobacter pylori* genome map and demonstration of diversity at the genome level. *J Bacteriol* 1992;174:6800–6.
- 24 Stanley J, Linton D, Burnens AP, et al. *Helicobacter pullorum* sp. nov. – genotype and phenotype of a new species isolated from poultry and human patients with gastroenteritis. *Microbiology* 1994;140:3441–9.
- 25 Fox JG, Chien CC, Dewhirst FE, et al. *Helicobacter canadensis* sp nov isolated from humans with diarrhea as an example of an emerging pathogen. *J Clin Microbiol* 2000;38:2546–9.
- 26 Correa P, Fox J, Fontham E, et al. *Helicobacter pylori* and gastric carcinoma. *Cancer* 1990;66:2569–74.
- 27 Coppola N, de Stefano G, Marrocco C, et al. Absence of *Helicobacter* spp in the liver of patients with primary or metastatic liver cancer. *Hepatology* 2002;36:1300–1.
- 28 Wadström T, Abu Al-Soud W, Ljungh A, et al. Absence of *Helicobacter* species in the liver of patients with primary or metastatic liver cancer. *Hepatology* 2003;38:532–3.
- 29 Fox JG, Li X, Yan L, et al. Chronic proliferative hepatitis in A/JCr mice associated with persistent *Helicobacter hepaticus* infection: A model of *Helicobacter*-induced carcinogenesis. *Infect Immun* 1996;64:1548–58.
- 30 Solnick JV, O'Rourke J, Lee A, et al. An uncultured gastric spiral organism is a newly identified *Helicobacter* in humans. *J Infect Dis* 1993;168:379–85.
- 31 Villeneuve JP, Dagenais M, Huet PM, et al. The hepatic microcirculation in the isolated perfused human liver. *Hepatology* 1996;23:24–31.
- 32 Hano H, Takasaki S. Three-dimensional observations on the alterations of lobular architecture in chronic hepatitis with special reference to its angioarchitecture for a better understanding of the formal pathogenesis of liver cirrhosis. *Virchows Arch* 2003;443:655–63.
- 33 Ménard A, Santos A, Mégraud F, et al. PCR-restriction fragment length polymorphism can also detect point mutation A2142C in the 23S rRNA gene, associated with *Helicobacter pylori* resistance to clarithromycin. *Antimicrob Agents Chemother* 2002;46:1156–7.
- 34 Altschul SF, Madden TL, Schaffer AA, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997;25:3389–402.