Received: 15 June 2021 | Revised: 9 November 2021 | Accepted: 7 December 2021

DOI: 10.1111/hel.12866

Helicobacter WILEY

ORIGINAL ARTICLE

Prevalence estimates of *Helicobacter* species infection in pancreatic and biliary tract cancers

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Funding information
the Ministry of Education, Culture, Sports, Science and Technology, Grant/Award Number: 21H0318, 17H04127 and 26293145

Abstract

Background: *Helicobacter pylori* infection is a well-established risk factor for gastric cancer and has been linked to other gastrointestinal diseases, including pancreatic and biliary tract cancers; however, the relevance of enterohepatic non-*H. pylori* helicobacters to the pathophysiology of these diseases remains unclear.

Materials and Methods: We estimated the prevalence of two enterohepatic non-*H. pylori* helicobacters (*Helicobacter hepaticus* and *Helicobacter bilis)* in the framework of a hospital-based case-control study involving 121 patients with biliary tract cancer, pancreatic cancer, or other gastrointestinal diseases. Bile and blood samples were collected from the patients undergoing endoscopic retrograde cholangiopancreatography. The presence of *H. bilis*, *H. hepaticus*, and other *Helicobacter* spp. was examined using bacterial culture, PCR-based detection, and serological tests.

Results: Culture of *Helicobacter* spp. from biliary brush samples was unsuccessful. Approximately 13.0% (15/115) of the bile samples collected from patients with a variety of gastrointestinal cancers, including pancreatic and biliary tract cancers, tested positive for one of the enterohepatic non-*H. pylori* helicobacter species as determined by PCR. Specifically, *H. bilis* and *H. hepaticus* DNA were detected in 11 and 4 bile samples, respectively. Approximately 20%–40% of the patients tested positive for serum non-*H. pylori* helicobacter IgG antibodies. The seroprevalence of *H. bilis* and *H. hepaticus* in the patients without evidence of *H. pylori* infection appeared to be higher in the pancreatic cancer group than in the control group.

Conclusion: Our findings suggest a role for *Helicobacter* spp., especially *H. bilis* and *H. hepaticus*, in the etiology of pancreatic and biliary tract cancers.

KEYWORDS
biliary tract cancer, enterohepatic helicobacters, *Helicobacter bilis*, *Helicobacter hepaticus*, *Helicobacter pylori*, pancreatic cancer

Takako Osaki and Yingsong Lin have equal contribution.

1 | INTRODUCTION

In 2019, a total of 36,356 Japanese men and women died from pancreatic cancer, making it the fourth leading cause of cancer-related death.[1] For biliary tract cancer, both its incidence and mortality rates are higher in Japan than in other developed countries.[2] Common challenges for these two cancers include their largely unknown etiologies, a lack of screening methods in the general population, and a dismal 5-year survival rate. Therefore, identifying modifiable environmental risk factors and improving early detection are crucial for easing the burdens of these two cancers.

Mounting evidence suggests a role of infection in the etiology of gastrointestinal (GI) cancers, including gastric, pancreatic, and biliary tract cancers.[3] The discovery of *Helicobacter pylori*, a gram-negative bacterium colonizing the human stomach, has led to a paradigm shift in understanding the central role of *H. pylori* infection in the cascade of gastric carcinogenesis.[4] In addition to *H. pylori*, an increasing number of *Helicobacter* spp. have been isolated from the stomachs, intestinal tracts, and livers of mammals and birds, with at least 59 species being listed as of June 2021 according to the NCBI Taxonomy database.[5] Among them, *Helicobacter bilis* and *Helicobacter hepaticus* were detected in human bile samples and have been associated with the risks of chronic active hepatitis, gallstone formation, and biliary tract cancer.[6-8] However, the true prevalence of these enterohepatic non-*H. pylori* helicobacters in human populations and their roles in disease pathophysiology remain to be determined. Furthermore, attempts to culture *H. hepaticus* and *H. bilis* from human biospecimens have been unsuccessful[8] and have hampered the understanding of their pathogenicity.

Given the diverse outcomes associated with *H. pylori* infection, we hypothesized that enterohepatic non-*H. pylori* helicobacters are also involved in driving carcinogenesis in the pancreas and biliary tract. To address this hypothesis, we employed several approaches, such as bacterial culture, polymerase chain reaction (PCR)-based detection, and serological tests, to examine the prevalence of enterohepatic non-*H. pylori* helicobacters in a cohort of GI cancer patients. We focused on *H. bilis* and *H. hepaticus* because these two bacterial species are well characterized[9,10] in animals and have been linked to several GI diseases in humans. Additionally, we attempted to culture *H. bilis* and *H. hepaticus* from biliary brush samples in hopes to provide corroborating evidence on their roles in the pathogenesis of GI cancers.

2 | METHODS

2.1 | Clinical sample preparation

We performed this research in the framework of a multi-institutional hospital-based case-control study that focuses on genetic variations and bacterial infections in the etiologies of pancreatic and biliary tract cancers.[11] For the present study, a simple questionnaire was used to collect demographic and clinical data. Biospecimens, which included serum and bile samples, were collected from 35 patients with biliary tract cancer (extrahepatic bile duct, ampulla of Vater, and gallbladder), 59 patients with pancreatic cancer (pancreatic adenocarcinoma), and 27 control subjects. The control group comprised patients who were diagnosed with a variety of benign or malignant GI tract diseases, including cholelithiasis, gastric cancer, colon cancer, and suspected cancers. Bile samples were collected when the patients underwent endoscopic retrograde cholangiopancreatography (ERCP). For the patients with gastric or colon cancer, bile samples were collected because they had malignant biliary obstruction due to lymph node metastases. The collected bile samples were stored at −80°C until analysis. Biliary brush samples were additionally collected for bacterial culture.

2.2 | PCR-based assays for the detection of Helicobacter spp.

Polymerase chain reaction was performed to detect the presence of *Helicobacter* DNA. Briefly, DNA was extracted from the bile samples (500–800 μl) using a QIAamp DNA Blood Midi Kit (Qiagen; Hilden, Germany) and concentrated by ethanol precipitation. The precipitant was dissolved in 50 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). Then, 1–2 μl of the bile DNA preparation was added to a 20-μl (final volume) reaction mixture. Nested PCR was performed to amplify *Helicobacter* genus-specific 16S rRNA and *cdtB* genes. Sequencing primers are listed in Table S1, and genomic DNA from *H. bilis* ATCC 49314 strain, *H. hepaticus* ATCC 51449 strain, and *H. cinaedi* MRY08-1234 strain was used as positive controls. The full sequences of the PCR amplification products were subsequently subjected to direct sequencing. With the basic local alignment search tool (BLAST), we defined the presence of specific enterohepatic helicobacters of *H. bilis* and *H. hepaticus* as more than 97% sequence similarity between our sequencing results and known reference sequences and the confirmation of *H. bilis*, *H. hepaticus*, or *H. pylori* among the top matches.

2.3 | Bacterial culture and antigen preparation for whole-cell enzyme-linked immunosorbent assay (ELISA)

To prepare antigens, we incubated the *H. bilis* ATCC 49314 strain and *H. hepaticus* ATCC 51449 strain for 7 days using biphasic culture medium under microaerobic conditions (AnaeroPack Microbic, Mitsubishi Gas Chemical Company, Inc) with a H_2 gas generator (Sugiyamagen). Brain heart infusion (BHI) (Oxoid) agar with 7% sheep blood was used for the solid phase, and BHI with 5% fetal calf serum (FCS) was used for the liquid phase. Liquid cultures of the *H. bilis* ATCC 49314 strain and *H. hepaticus* ATCC 51449 strain were collected and centrifuged. In addition, the *H. pylori* TK1402 strain was cultured on BHI agar with 5% sheep blood for 3 days under microaerobic conditions and collected in 0.01 M phosphate-buffered saline (PBS) (pH 7.4). The harvested whole organisms were suspended

TABLE 1 Demographic characteristics of the study subjects

	Biliary tract cancer group[a]	Pancreatic cancer group	Control group[b]
Number	37	59	25
Men vs women	27 vs 10	28 vs 31	17 vs 8
Age (mean ± SD)	72.4±8.3	68.8±10.6	73.6±8.7
H. pylori seropositivity with the commercially available ELISA kit (Eiken E-plate)	8 (21.6%)	8 (13.6%)	10 (40.0%)
H. pylori positivity by the serological test employing whole-cell antigens	16 (43.2%)	19 (32.2%)	11 (44.0%)

[a]iagnoses included cancers of extrahepatic bile duct, ampulla of Vater, and gallbladder
[b]Diagnoses included cholelithiasis, gastric cancer, colon cancer, and suspected cancers.

in PBS and sonicated with an ultrasonic Sonifier 250 (Branson Ultrasonics Co.) for 5 min at 20 kHz. The sonicate was centrifuged at 15,000 *g* for 15 min, and the supernatant was filtered through a 0.45 μm pore size membrane (Merck Millipore). The supernatant served as antigens in whole-cell ELISA.

2.4 | Serological assays

Serum antibody reactivity to *H. bilis*, *H. hepaticus*, and *H. pylori* was measured by the whole-cell ELISA protocol developed in our laboratory. Microtitration plates were coated at 4°C for 18–42 h with whole-cell antigen (5 μg/ml). After being coated with each antigen at 4°C overnight, they were washed with PBS twice. Each antigen was blocked with PBS containing 1% skim milk (PBS-S) at 37°C for 1 h. After washing with PBS 3 times, each serum sample was added to the plates after a 500-fold dilution with PBS and incubated at 37°C for 1 h. After washing with PBS containing 0.05% Tween (PBS-T) 3 times, an anti-human IgG horseradish peroxidase-labeled antibody (I2136, Sigma Aldrich Merck) was added at a dilution of 1:10000 in PBS-S and reacted at 37°C for 1 h. After washing with PBS-T three times, the plates were treated with TMB substrate solution (3,3',5,5'-tetramethylbenzidine, BioFX® TMB). After incubation at room temperature for 5 min, the reaction was stopped by the addition of 2 N H_2SO_4. The optical density was measured at 450 nm using a microplate reader (model 550; Bio-Rad).

Additionally, for the diagnosis of *H. pylori* infection, serum anti-*H. pylori* IgG antibodies were measured using a commercially available ELISA kit (E-Plate "Eiken" *H. pylori* antibody; Eiken Chemical) at the SRL laboratory. Since an antibody tilter of 10 U/L was used as the cutoff threshold based on the commercial kit, this cutoff value was also used to determine seropositivity in the ELISAs employing whole-cell antigens (Figure S1A). The cutoff values for anti-*H. pylori* IgG antibodies were also applied to define seropositivity for anti-*H. bilis* antibodies and anti-*H. hepaticus* antibodies (Figure S1B,C).

Our study was conducted in accordance with the Declaration of Helsinki. All study subjects provided written informed consent, and the Ethics Committee at Aichi Medical University, Kyorin University, and all participating hospitals approved this study.

2.5 | Statistical analyses

Continuous variables are presented as the mean ± standard deviation, and categorical variables are presented as counts and percentages. Fisher's exact test was used to compare the proportion of subjects who tested positive for anti-*Helicobacter* spp. antibodies between the two groups. All tests were two-sided, and P values less than 0.05 were considered statistically significant.

3 | RESULTS

The demographic characteristics of the study subjects are shown in Table 1. The majority of patients in the biliary tract cancer group and the control group were male, while the male-to-female ratio in the pancreatic cancer group was almost 1:1. The mean patient age was 72.4 years in the biliary tract cancer group, 68.8 years in the pancreatic cancer group, and 73.6 years in the control group. Serological tests using whole-cell antigens yielded a higher seroprevalence of anti-*H. pylori* antibodies (32.2%–44.0%) than did serological tests using the commercial ELISA kit (13.6%–40.0%).

3.1 | Bacterial culture

We attempted to culture *H. bilis* and *H. hepaticus* using seven biliary brush samples obtained during ERCP, but none of our attempts were successful. Instead, we isolated *H. pylori* in two brush samples as well as bacteria other than *Helicobacter* spp., such as *Eikenella corrodens*, *Morganella morganii* (phylum Proteobacteria), *Lactobacillus plantarum*, and *Lactobacillus* sp. (phylum Firmicutes), in a subset of brush samples. Furthermore, a mixed population of Enterobacteriaceae and other bacteria was cultured on the medium.

3.2 | PCR-based detection of Helicobacter spp. from bile samples

Table 2 shows the PCR-based detection results of enterohepatic non-*H. pylori* helicobacters from the bile samples. Two biliary tract

cancer patients and 4 control subjects were excluded because of invalid results. Of the remaining 115 patients, we detected the presence of *H. bilis* 16S rRNA and/or *cdtB* gene in 11 patients and *H. hepaticus* 16S rRNA and *cdtB* genes in 4 patients.

Furthermore, *H. pylori* DNA was detected in 13 patients (4 with biliary tract cancer and 9 with pancreatic cancer). Among them, *H. bilis*-specific *cdtB* gene was also detected in 3 patients (1 with biliary tract cancer and 2 with pancreatic cancer), and *H. hepaticus*-specific *cdtB* gene was not detected in any patient.

In addition, *Helicobacter* spp. or Campylobacterales was detected in 7 patients (1 with biliary tract cancer, 3 with pancreatic cancer, and 3 controls) and *H. bilis*-specific *cdtB* gene was also detected 4 of 7 patients (1 with biliary tract cancer, and 3 with pancreatic cancer). *H. hepaticus*-specific *cdtB* gene was detected in 2 of 3 controls, whereas none of the *H. cinaedi*-specific *cdtB* gene were detected in all of the 115 patients.

Specifically, the prevalence of enterohepatic helicobacters of *H. bilis* and *H. hepaticus* was 11.4% (4/35) in the biliary tract cancer group, 11.9% (7/59) in the pancreatic cancer group, and 19.0% (4/21) in the control group. No significant differences in these prevalence estimates were noted among the three groups. In addition, in the biliary tract caner group, we observed no significant differences in prevalence estimates for either *H. bilis* or *H. hepaticus* by tumor location (data not shown).

3.3 | Seroprevalence of anti-*Helicobacter* spp. antibodies

The proportion of patients who tested positive for anti-*H. hepaticus* and anti-*H. bilis* antibodies were higher in the biliary tract and pancreatic cancer groups than in the control group (Table 3), although the differences were not statistically significant.

To remove the possible effects of cross-reactive antibody responses, we measured anti-*H. bilis* and anti-*H. hepaticus* IgG antibody titers in those patients without evidence of *H. pylori* infection by both the commercial ELISA kit and our own in-house serological tests (Table 4). No patients in the control group were found to be positive for either anti-*H. bilis* antibodies or anti-*H. hepaticus* antibodies. Compared with the control group, the pancreatic cancer group had a seemingly higher seropositivity of anti-*H. bilis* antibodies ($p = 0.046$).

Table 5 shows the number of subjects who tested positive by PCR-based detection as well as serological tests. Of the 6 PCR-confirmed positive samples for *H. bilis*, 4 were also positive for anti-*H. bilis* antibodies in pancreatic cancer patients.

4 | DISCUSSION

We explored the role of *Helicobacter* spp. in biliary tract, pancreatic, and other GI tract cancers by employing several approaches, such as bacterial culture, PCR-based detection of bacterial DNA, and serological tests. Despite the unsuccessful culture of *H. bilis* and *H. hepaticus*, we demonstrated the presence of *H. bilis* and *H. hepaticus* in approximately 13.0% (15/115) of the bile samples collected from patients with various GI cancers. In particular, pancreatic cancer patients tended to have a higher prevalence of *H. bilis* infection, an intriguing finding that deserves further study.

Complete differentiation of various *Helicobacter* spp. with the detection of 16S rRNA gene remains challenging. For example, *H. bilis* and *H. cinaedi* share high similarity in DNA sequences and exhibit a close phylogenetic relationship,[12] making it difficult to distinguish them solely based on 16S rRNA gene. Therefore, we performed a second PCR that may differentiate the *Helicobacter* spp. through the detection of *ctdB* gene, which encodes a subunit of cytolethal distending toxin (CDT). CDT is a bacterial virulence factor produced by Gram-negative pathogenic bacteria,[13,14] and it has been shown to play an important role in *H. hepaticus* infection in mice model.[15] CDT comprises three subunits (CdtA, CdtB, and CdtC), of which *cdtB* gene has been detected in the whole genome studies of enterohepatic non-*H. pylori* helicobacters. Using specific primer pairs, we were able to detect *cdtB* gene of the same species in most of the samples with the presence of 16S rRNA gene of *H. hepaticus* or *H. bilis*. Moreover, our results revealed that *cdtB* gene could be detected in the patients who were positive for *H. pylori* 16S rRNA gene, suggesting the advantage of using *cdtB* gene to detect enterohepatic non-*H. pylori* helicobacters present in patients infected with *H. pylori*. On the other

TABLE 2 PCR-based detection[a] of *Helicobacter* spp. DNA in the bile samples from 115 patients[b]

Helicobacter spp.	Biliary tract cancer group ($N = 35$)	Pancreatic cancer group ($N = 59$)	Control group ($N = 21$)
H. bilis 16S rRNA	2 (5.7%)	6 (10.2%)	3 (14.3%)
cdtB	1(2.9%)	4(6.8%)	1(4.8%)
H. hepaticus 16S rRNA	2 (5.7%)	1 (1.7%)	1 (4.8%)
cdtB	2 (5.7%)	1 (1.7%)	1 (4.8%)

[a]PCR amplification product was analyzed by direct sequencing. Sequence homology was determined with the basic local alignment search tool.

[b]*H. pylori* DNA was detected in 13 patients (4 and 9 with biliary tract cancer and pancreatic cancer, respectively) and other *Helicobacter* spp. or Campylobacterales DNA was detected in 5 bile samples: 1 in the biliary tract cancer group, 3 in the pancreatic cancer group, and 1 in the control group.

TABLE 3 Seroprevalence of *Helicobacter* spp. in the study subjects

	Biliary tract cancer group (*N* = 37)		Pancreatic cancer group (*N* = 59)		Control group (*N* = 25)	
	N	%	*N*	%	*N*	%
Positive for anti-*H. bilis* antibodies	13	35.1	24	40.7	8	32.0
Positive for anti-*H. hepaticus* antibodies	11	29.7	19	32.2	7	28.0
Positive for anti-*H. pylori* antibodies	16	43.2	20	33.9	11	44.0

Note: ELISA was performed using *Helicobacter* spp.-specific whole-cell antigens.

Cutoff values of seropositivity: 0.797 for anti-*H. bilis* antibodies, 0.598 for anti-*H. hepaticus* antibodies, and 0.984 for anti-*H. pylori* antibodies

None of the statistical tests were significant when comparing the proportions of study subjects in the pancreatic and biliary tract cancer groups with those in the control group positive for three *Helicobacter* spp.

TABLE 4 Seropositivity of *Helicobacter* spp. in patients who tested negative for *H. pylori* with the commercial serological test

	Biliary tract cancer group (*N* = 20)		Pancreatic cancer group (*N* = 37)[a]		Control group (*N* = 13)	
	N	%	*N*	%	*N*	%
Positive for anti-*H. bilis* antibodies	3[b]	15.0	10[b]	27.0	0	-
Positive for anti-*H. hepaticus* antibodies	2	10.0	6	16.2	0	-

Note: ELISA was performed using *Helicobacter* spp.-specific whole-cell antigens.

Cutoff values of seropositivity: 0.797 for anti-*H. bilis* antibodies, 0.598 for anti-*H. hepaticus* antibodies

All other between-group comparisons were statistically nonsignificant.

[a]$p = 0.046$ for the comparison of seropositivity for anti-*H. bilis* antibodies between the pancreatic cancer group and the control group.

[b]In the 13 cases that were negative for anti-*H. pylori* antibodies but positive for anti-*H. bilis* antibodies, 8 were also positive for anti-*H. hepaticus* antibodies.

hand, we failed to detect *H. cinaedi*-specific *cdtB* gene. One possible reason is that *cdtB* gene is not essential for bacterial survival, and thus, its sequence may not be highly conserved compared with 16S rRNA gene.

Isolating and growing individual bacterial species in pure culture can inform physiological properties and virulence potential, thus providing important insights into their contributions to disease etiology.[16] However, enterohepatic non-*H. pylori* helicobacters are notoriously difficult to culture. To date, none of the attempts to culture *H. bilis* or *H. hepaticus* from human biospecimens have been successful. We also failed to culture enterohepatic non-*H. pylori* helicobacters from the bile samples, although several types of *Helicobacter*-selective media and prolonged incubation times under microaerobic conditions were used. Several reasons may account for the unsuccessful culture.[17] Some slower-growing *Helicobacter* spp. with low abundance may be overlooked when using standard microbiological techniques. Another reason is that some fastidious species require complex growth conditions. In addition, growing bacteria may be inhibited by microbial competition and compounds produced by other bacteria.

With direct sequencing of the PCR amplification products, we demonstrated the presence of any enterohepatic non-*H. pylori* helicobacter in 13.0% (15/115) of the bile samples collected from patients with various GI disorders. Previous studies based on PCR have documented a wide variation in the prevalence of *Helicobacter* spp. in biliary tract samples.[18] While *H. bilis* was not detected in the biliary tract in a German study,[19] 87% (13/15) of the samples were PCR-positive in a Japanese study.[20] Of interest, the reported prevalence estimates were higher in countries with a high incidence of biliary tract cancer, such as Japan, than in other countries with a low incidence. The prevalence of PCR-confirmed enterohepatic non-*H. pylori* helicobacters observed in our study was lower than that in previous Japanese studies.[18] This discrepancy could be attributable to differences in sample size, the quality of biological specimens, and the choice of sequencing primers. Specifically, one possible reason is DNA template contamination, with potential sources coming from the host genome or bacteria other than enterohepatic non-*H. pylori* helicobacters.

Serological tests are widely used to estimate the prevalence of *H. pylori* infection in epidemiologic studies.[21] In contrast to well-developed serological tests targeting *H. pylori*, the antibody responses to other *Helicobacter* spp. are poorly understood, with no commercially available ELISA kits. Based on whole-cell ELISA, we found that approximately 30%–40% of the pancreatic and biliary tract cancer patients were seropositive for *H. bilis* or *H. hepaticus*, indicating past or present infection. Furthermore, given that the cross-reactivity of antibodies against *H. pylori* may have biased the prevalence estimates for *Helicobacter* spp., we estimated the seroprevalence for *H. bilis* and *H. hepaticus* among those who tested negative for *H. pylori* by both the commercial ELISA kit and whole-cell ELISA. Notably, the proportion of pancreatic cancer patients that were positive for anti-*H. bilis* antibodies appeared to be higher than that of the control subjects. However, two issues emerged in our study. One issue is the inconsistent results from the two ELISAs. A higher seroprevalence of *H. pylori* was observed in whole-cell ELISA than in the commercial ELISA kit. We consider that the differences in antigens used and the cross-reactivity to *H. hepaticus* or *H. bilis* may have contributed to the discrepant results. Another issue is the difficulty of differentiating two *Helicobacter* spp. based solely on serological tests. As shown in our study, in the 13 cases negative for anti-*H. pylori* antibodies but positive for anti-*H. bilis* antibodies, 8 were also positive for anti-*H. hepaticus* antibodies (Table 4). Further

TABLE 5 Number of study subjects who tested positive for *Helicobacter* spp. based on PCR[a] as well as serological tests

	Presence of *Helicobacter* spp. DNA based on PCR detection	Positive for serum anti-*H. bilis* IgG antibodies	Positive for serum anti-*H. hepaticus* IgG antibodies	Positive for serum anti-*H. pylori* IgG antibodies
Biliary tract cancer group				
H. bilis	2	0	0	2
H. hepaticus	2	1	1	2
H. pylori, *Helicobacter* sp., *or* Campylobacterales	5[b]	4	3	4
Pancreatic cancer group				
H. bilis	6	4	2	2
H. hepaticus	1	1	1	0
H. pylori, *Helicobacter* sp., or Campylobacterales	12[b]	6	5	6
Control group				
H. bilis	3	2	1	2
H. hepaticus	1	0	0	1
H. pylori, *Helicobacter* sp., or Campylobacterales	3[c]	0	0	1

[a]PCR amplification product of 16S rRNA gene was analyzed by direct sequencing. Sequence homology was determined with the basic local alignment search tool (BLAST).

[b]Presence of species-specific *cdtB* gene was also determined based on PCR. *H. bilis*-specific *cdtB* gene was detected in 2 of 5 biliary tract cancer cases, and 5 of 12 pancreatic cancer cases. Sequence homology was determined with BLAST.

[c]*H. hepaticus*-specific *cdtB* gene was detected in 2 of 3 controls. Sequence homology was determined with BLAST.

efforts are needed to optimize serological tests so that epidemiological studies could provide more accurate estimates of the risk for GI cancers associated with enterohepatic non-*H. pylori* helicobacters.

Consistent results from PCR-based and serological tests are thought to provide triangulation of evidence regarding persistent infection with enterohepatic non-*H. pylori* helicobacters. Although previous studies have shown the presence of non-*H. pylori* helicobacter DNAs, such as *H. bilis*, *H. hepaticus*, *H. rappini*, and *H. pullorum*, in patients with hepatobiliary or pancreatic diseases,[22,23] their causal roles in disease pathophysiology remain elusive. Our findings added to the evidence that enterohepatic non-*H. pylori* helicobacters might be more relevant in driving the carcinogenesis of biliary tract and pancreatic cancers, among other GI cancers. Similar to our results, a previous study showed a significantly higher prevalence of *H. bilis* in patients with bile duct and gallbladder cancers than in patients with gallstone and/or cholecystitis.[20] Mechanisms underlying the associations of *H. bilis* with hepatobiliary cancers have been incompletely understood. Experimental evidence suggested that *H. bilis* was involved in the formation of cholesterol gallstones and intrahepatic cholelithiasis, which are risk factors for hepatobiliary cancers.[6] In patients with hepatobiliary cancers, the biliary cell kinetics may be accelerated directly by *Helicobacter* spp..[24] Compared with hepatobiliary diseases, few studies have associated non-*H. pylori* helicobacters with pancreatic diseases.[22,25] One notable finding of our study was a seemingly higher proportion of pancreatic cancer patients who tested positive for *H. bilis*. Although there is no convincing evidence on the direct colonization of *H. bilis* in the pancreas, previous studies have reported a high prevalence of *Helicobacter* spp. ribosomal DNA, including *H. pylori* and *H. cineadi*, by PCR in paraffin-embedded pancreatic cancer tissue samples.[25] Given the observed associations, it is likely that enterohepatic non-*H. pylori* helicobacters colonize the pancreas through bacterial translocation, which is induced by either increased gut permeability and dysbiosis in the context of obesity and pancreatitis or environmental insults such as ERCP.[26] Further studies are needed to explore whether enterohepatic helicobacters play a causative role in the development of hepatobiliary and pancreatic diseases.

Our study does have several limitations. First, biospecimens from healthy control subjects were not available because invasive ERCP cannot be performed in those subjects. Thus, the prevalence of *Helicobacter* spp. in the normal biliary tract remains to be determined. Second, although culture-independent techniques, such as direct sequencing, were used to detect *Helicobacter* spp. DNA in bile samples from patients with GI cancers, the lack of confirmation by culture represents only the enterohepatic circulation of *Helicobacter* spp. DNA. Determining a causative role for enterohepatic non-*H. pylori* helicobacters needs evidence of triangulation, among which bacterial culture should constitute an important part. With a refocus on the role of culture in inferring causality,[27] further attempts are warranted to culture the "unculturable" enterohepatic non-*H. pylori* helicobacters. Third, while *H. pylori*-associated disease risk may be determined by the interactions between bacterial virulence and host susceptibility,[4] it remains unclear whether there exists a synergy between host and environmental factors for other enterohepatic non-*H. pylori* helicobacters. Further molecular epidemiologic studies are needed to explore their synergistic effects in driving tumorigenesis. Finally, the performance of our serological tests needs to be improved by using high-throughput antibody assays that employ *Helicobacter*-specific antigens.

In summary, our findings suggest a possible role of enterohepatic non-*H. pylori* helicobacters, especially *H. bilis* and *H. hepaticus*, in the etiology of pancreatic and biliary tract cancers. With mounting evidence on the role of diverse *Helicobacter* spp. in the pathogenesis of gastric and enterohepatic diseases, further studies are needed to address the interactions among bacteria, the host, and environmental factors that influence the host's susceptibility and the clinical outcome of infection.

ACKNOWLEDGMENT

This work was supported by Grants-in-Aid for Scientific Research (B) (21H0318, 17H04127, 26293145) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT). We are grateful to Haruhisa Nakao, an outstanding physician scientist, for his contributions to the recruitment of the study subjects. Unfortunately, he passed away on December 31, 2020. We thank Dr. Emiko Rimbara for providing us with valuable *H. cinaedi* genomic DNA.

CONFLICTS OF INTEREST

All other authors declare no conflicts of interest.

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How to cite this article: Osaki T, Lin Y, Sasahira N, et al. Prevalence estimates of *Helicobacter* species infection in pancreatic and biliary tract cancers. *Helicobacter*. 2022;27:e12866. doi:10.1111/hel.12866