Helicobacter pylori: association with gall bladder disorders in Pakistan

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ABSTRACT

Helicobacter species colonise the biliary tract and therefore this study explores the relationship between of *Helicobacter pylori* and cholecystitis. Bile and gall bladder tissue samples were obtained from 144 patients who underwent cholecystectomy. Of these, 89 had chronic cholecystitis with cholelithiasis, 44 had gall bladder carcinoma and 11 had gall bladder polyps. Histopathology examination included special staining and immunohistochemistry (IHC), while *Helicobacter* species (*H. pylori, H. bilis* and *H. hepaticus*) were detected by the polymerase chain reaction (PCR). Sequencing and BLAST query of PCR products was undertaken and samples were considered to contain *H. pylori* if both PCR and IHC were positive. Immunohistochemistry for *H. pylori* was positive in 22 (25%) cases compared to five (9%) in the control group ($P=0.02$). Testing (PCR) for 16S rDNA was positive in 23 (26%) cases compared to six (11%) controls ($P=0.03$). Negative PCR results were obtained for *H. bilis* and *H. hepaticus*. Twenty-four (89%) were positive by both 16S rDNA PCR and IHC for *H. pylori* ($P<0.001$). Both PCR for 16S rDNA and IHC were positive in 21 (24%) cases compared to five (9%) controls ($P=0.03$). Sequencing of 16S rRNA and *glmM* PCR products were consistent with *H. pylori.* In conclusion, *H. pylori* DNA was demonstrated in cases of chronic cholecystitis and gall bladder carcinoma associated with cholelithiasis, but this association requires further study.

KEY WORDS: Bile.
Cholecystitis.
Cholelithiasis.
Gallbladder diseases.
Helicobacter pylori.

Introduction

Helicobacter species colonise the human gastrointestinal and hepatobiliary tract, usually resulting in chronic infection coupled with an inflammatory host response. This colonisation is associated with a variety of gastrointestinal and hepatobiliary diseases.[1] The enterohepatic helicobacters such as *Helicobacter hepaticus* and *H. bilis*, first recognised in laboratory rodents, have been considered a component of the normal flora. *H. hepaticus* colonises the lower gastrointestinal tract, including the caecum, colon and hepatobiliary system of mice and causes chronic active hepatitis and typhlocolitis in immunocompetent mice and can lead to liver carcinoma in male mice of susceptible strains.[2–5] *H. bilis* was also identified in inbred mice with chronic hepatitis.[6] Patients with chronic liver disease have been reported to have significantly higher levels of antibody to *H. bilis* and *H. hepaticus* compared to healthy subjects.[7]

These urease-producing bacteria may induce stone formation by hydrolysing urea into ammonia that increases the pH and favours precipitation of salts that lead to subsequent stone formation.[8,9] It has also been demonstrated that cholesterol gallstone-prone C57L/J mice rarely develop gallstones unless they are infected with certain cholelithogenic enterohepatic *Helicobacter* species.[10] In a previous study, all four urease-positive *Helicobacter* species (*H. hepaticus, H. bilis, H. pylori* and *H. mustelae*) were shown to be capable of precipitating calcium.[11] This suggests that urease-positive *Helicobacter* species that are able to survive in or colonise the bile ducts may induce the formation of gallstones, both directly via their urease activity or indirectly via the immune response.[11] *H. pylori* has also been shown to colonise areas of gastric metaplasia in the gall bladder, producing histological changes similar to those seen in the gastric mucosa.[12] Isolation of *H. pylori* DNA from gallstones further supports its presence in the gall bladder.[12]

Helicobacter species have been detected in patients with biliary tract cancer significantly more frequently than in controls.[13–15] There are reports that biliary *Helicobacter* colonisation is frequent in countries with a high incidence of gall bladder carcinoma, the highest incidence rates in this region being reported for women in Delhi, India (21.5/100,000), and in Karachi, Pakistan (13.8/100,000).[16] In a previous study, the incidence of gallstones was determined in cases of carcinoma of the gall bladder. In this series, 93% of patients had gallstones (85% were female, age range: 27–65 years).[17] A recent study in Karachi looked at the frequency of biliary infection associated with cholelithiasis. Results showed that a total of 36% had bile culture positive for *Escherichia coli* (17%) *Klebsiella* (9%), *Pseudomonas* (6%), *Staphylococcus aureus* (2%), *Salmonella* (1%) and *Bacteroides fragilis* (1%).[18] However, *H. pylori* also has a high prevalence in the local population.[19]

The prevalence of *H. pylori* in bile and the gall bladder has not been examined previously in patients with gall bladder disorders in Pakistan. Therefore, this study aims to

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investigate the frequency of *H. pylori* in gall bladder tissue and bile from patients who underwent cholecystectomy for cholelithiasis, and compare them with those having cholecystectomy for gall bladder polyps (GBP) and carcinoma (GBC).

Materials and Methods

One hundred and forty-four patients undergoing cholecystectomy were enrolled on the study between January 2008 and December 2009 (101 [70%] females, 43 [30%] males; mean age: 49±14 [range 18–84]). All presented with recurrent upper right abdominal pain. Eighty-nine patients who had cholecystectomy for cholelithiasis associated with chronic cholecystitis were included in the study group. The control group comprised 55 patients who underwent cholecystectomy, 11 for GBP and 44 for GBC. Of the GBC patients, 25% (11/44) also had gallstones. None of the GBP patients had stones. The study was approved by the institutional ethics review committee.

Histopathology examination included haematoxylin and eosin (H&E), Giemsa and Warthin-Starry staining and immunohistochemistry (IHC). Gall bladder tissue and bile were used to extract DNA for PCR of the *H. pylori* 16S ribosomal DNA (rDNA) and phosphoglucosamine mutase (*glmM*) gene. If positive, these were further evaluated for cytotoxin-associated gene (*cagA*) and vacuolating cytotoxin-A (*vacA*) gene alleles. Diagnosis of *H. pylori* infection was established when both PCR and IHC were positive. To detect *H. bilis* and *H. hepaticus*, specific primer sets for 16S ribosomal RNA (rRNA) sequences of *H. bilis* and *H. hepaticus* were used.

Immunohistochemistry staining for *H. pylori* was performed using streptavidin peroxidase (SP). Anti-*H. pylori* antibody (Dako) was diluted (1 in 10), and phosphate-buffered saline (PBS) was used as blank control, normal blood serum as a negative control, and sections of gastric mucosa containing *H. pylori* as the positive control.

DNA extraction

Extraction of DNA from bile and gall bladder tissue was performed as described previously.[20] Briefly, 450 µL bile was diluted with an equal volume of PBS (pH 8) and centrifuged at 14 000 x*g* for 20 min. The supernatant was discarded and

Table 1. Oligonucleotide primers used to amplify the 16S rRNA and *Helicobacter pylori*-specific genes.

Gene	Sequence (5' to 3')	Reaction conditions	Amplicon size (bp)	Position
16S rRNA	C97 5'-GCT ATG ACG GGT ATC C-3' C 98 5'-GAT TTT ACC CCT ACA CCA-3'	94˚C for 5 min 94˚C for 1 min 55˚C for 1 min 72˚C for 90 sec (35 cycles) 72˚C for 7 min	400	276–291 681–698
glmM	GGATAAGCTTTTAGGGGTGTTAGGGG GCTTACTTTCTAACACTAACGCGC	94˚C for 1 min 56˚C for 1 min 72˚C for 1 min (35 cycles) 72˚C for 5 min	296	
cagA D008 R008	 ATAATGCTAAATTAGACAACTTGAGCGA TTAGAATAATCAACAAACATCACGCCAT	94˚C for 5 min 94˚C for 1 min 55˚C for 1 min 72˚C for 1.5 min (35 cycles) 72˚C for 5 min	297	1751–2048
vacA M1 VA3-F VA3-R M2 VA4-F VA4-R S1a SS1-F VA1-R S1b SS3-F VA1-R	 GGTCAAAATGCGGTCATGG CCATTGGTACCTGTAGAAAC GGAGCCCCAGGAAACATTG CATAACTAGCGCCTTGCAC GTCAGCATCACACCGCAAC CTGCTTGAATGCGCCAAAC AGCGCCATACCGCAAGAG CTGCTTGAATGCGCCAAAC	95˚C for 5 min 95˚C for 1 min 52˚C for 1 min 72˚C for 1 min (35 cycles) 72˚C for 5 min	290 352 190 187	2741–3030 976–1327 866–1055
H. bilis F R	 CAGAACTGCATTTGAAACTAC AAGCTCTGGCAAGCCAGC	94˚C for 5 min 94˚C for 1 min 55˚C for 1 min 72˚C for 1 min (35 cycles) 72˚C for 5 min	418	
H. hepaticus F R	 GAAACTGTTACTCTG TCAAGCTCCCCGAAGGG	94˚C for 5 min 94˚C for 1 min 55˚C for 1 min 72˚C for 1 min (35 cycles) 72˚C for 5 min	405	

Table 2. Diagnostic yield of various tests in different groups.

		Cholecystitis with cholelithiasis (*n*=89)	Control group (*n*=55)	*P* value
Polymerase chain reaction				
16S rDNA	Positive	23 (26)	6 (11)	0.03
	Negative	66 (74)	49 (89)	
glmM	Positive	21 (24)	5 (9)	0.03
	Negative	68 (76)	50 (91)	
cagA	Positive	4 (5)	0 (0)	0.30
	Negative	85 (95)	55 (100)	
VacA s1am1	Positive	4 (5)	0 (0)	0.30
	Negative	85 (95)	55 (100)	
VacA s1am1	Positive	8 (9)	3 (6)	0.53
	Negative	81 (91)	52 (94)	
Immunohistochemistry	Positive	22 (25)	5 (9)	0.02
	Negative	67 (75)	50 (91)	
Criteria used for the confirmation of *H. pylori* infection				
16S rRNA PCR+ Immunohistochemistry	Positive	21 (24)	5 (9)	0.03
	Negative	68 (76)	50 (91)	
PCR *glmM*+ immunohistochemistry	Positive	21 (24)	5 (9)	0.03
	Negative	68 (76)	50 (91)	

Univariate analysis was performed by using the independent sample *t*-test, Pearson χ^2 test and Fisher Exact test were also used as appropriate. $P<0.05$ was considered statistically significant. Percentages shown in parentheses.

the pellet was mixed with 250 µL PBS and recentrifuged. Bile sample pellets were incubated with lysis buffer (50 mmol/L Tris HCl [pH 7.2], 1 mmol/L EDTA, 0.45% sodium dodecyl sulphate [SDS], 0.45% NP-40 and 100 mg/mL protease K) for 20 h at 56°C and extracted (x2) with phenol–chloroform (equal volumes). After another extraction with chloroform, the DNA was precipitated with absolute ethanol and dissolved in TE buffer (10 mmol/L Tris [pH 8] and 1 mmol/L EDTA).

Extraction of DNA from gall bladder tissue was performed as described previously.[21] Gall bladder tissue (50 mg) was homogenised to uniformity in 400 µL sterile water and centrifuged at 8000 x*g* for 10 min. Then, 400 µL lysis buffer (100 mmol/L NaCl, 10 mmol/L Tris-HCl [pH 8], 25 mmol/L EDTA, 0.5% SDS) and 10 µL proteinase K (10 mg/mL) were added. Incubation was carried out at 56°C for 20 h, and proteinase K was inactivated by boiling for 10 min. This was followed by phenol-chloroform extraction and ethanol precipitation. The resulting pellet was allowed to dissolve in 35 µL TE buffer (10 mmol/L Tris-HCl [pH 7.4] and 0.1 mmol/L EDTA [pH 8]) for 20 h at 37°C. Samples were stored at –20°C before PCR amplification was performed. DNA content and purity was determined by measuring absorbance at 260 nm and 280 nm using a spectrophotometer (Beckman DU-600, USA).

PCR amplification

Samples were amplified by *Helicobacter* genus-specific 16S rRNA primers. The forward (C97) and reverse (C98) primers amplified a product of approximately 400 bp.[21] Samples generating a positive result were subsequently analysed with eight different sets of primers. Two specific primer sets were used to detect *H. bilis* and *H. hepaticus* 16S rRNA sequences previously described.[22] The other six specific primers used were to look for the *glmM* and *vacA* alleles (e.g., *s1a, s1b, s1m1* and *s1m2*, and *cagA* genes),[23,24] which encode potential virulence factors in *H. pylori*. Nucleotide primer sequences and PCR conditions are shown in Table 1. The amplified products were analysed on 2% (w/v) agarose gel stained with ethidium bromide and observed under short-wavelength ultraviolet light.

Sequence analyses

Amplicons were purified from the agarose gel by a DNA extraction kit (Roche, Indianapolis, Indiana, USA). Three amplicons from the gall bladder were sequenced to verify that they were *H. pylori* 16S rRNA and *glmM* genes. Sequence analysis was performed (Macrogen, Seoul, South Korea) using primers for 16S rRNA and *glmM* genes. Sequence comparison was carried out using the BLAST program and the GenBank database.

Statistical analysis

The Statistical Package for Social Science (SPSS Release 16, standard version) was used for data analysis. Descriptive analysis was performed for demographic and clinical features. Results were presented as mean±SD for quantitative variables and number (percentage) for qualitative variables. Univariate analysis was performed using the independent sample *t*-test, and Pearson's χ^2 test and Fisher's Exact test were used where appropriate. $P<0.05$ was considered statistically significant. The kappa (κ) test was performed to assess agreement between two methods of testing.

Results

Histological examination for *H. pylori* proved inconclusive by H&E, Giemsa and Warthin-Starry staining. Immunohistochemistry demonstrated *H. pylori* in 25% (22/89) of the case specimens and in 9% (5/55) of the controls ($P=0.02$; Table 2).

Table 3a. Comparison of cholecystitis with cholelithiasis and gall bladder carcinoma without stones.

		Cholecystitis with cholelithiasis (*n*=89)	Gall bladder carcinoma (*n*=38)	*P* value
PCR				
16SrRNA	Positive	23 (26)	1 (3)	0.001
	Negative	66 (74)	37 (97)	
GlmM	Positive	21 (10)	0 (0)	0.201
	Negative	68 (90)	38 (100)	
Immunohistochemistry	Positive	22 (25)	0 (0)	<0.001
	Negative	67 (75)	38 (100)	

Univariate analysis was performed by using the independent sample *t*-test, Pearson χ^2 test and Fisher Exact test were also used as appropriate. $P<0.05$ was considered statistically significant. Percentages shown in parentheses.

Results of PCR amplification of 16S rDNA, *glmM, cagA* and *vacA* in the different groups are shown in Table 2. None of the samples showed PCR positivity for either *H. bilis* or *H. hepaticus. H. pylori* 16S rDNA PCR and IHC were both positive in 24% (21/89) of study cases compared to 9% (5/55) of the control group ($P=0.03$; Table 2). The *glmM* gene PCR and IHC were also both positive in 24% (21/89) of study group cases compared to 9% (5/55) of the control group ($P=0.03$; Table 2).

There was significant correlation between PCR for *H. pylori* 16S rDNA and IHC, with 20/23 in the study group positive by both techniques ($\kappa=85\%$, $P<0.001$). Both PCR for 16S rDNA and IHC were positive in 21(24%) cases of cholecystitis associated with gallstones, in no cases of gall bladder carcinoma without cholelithiasis ($P<0.001$) and in 5/6 (83%) cases of gall bladder carcinoma with cholelithiasis ($P=0.005$; Tables 3a–c).

Three 16S rRNA and *glmM* gene PCR products were selected at random for sequencing. The 16S rRNA gene sequences HM596599–HM596601 and *glmM* gene sequences GQ334379–GQ334382 produced significant alignments with NC 008086.1 *H. pylori* HPAG1, NC 000915.1 *H. pylori* 26695, NC 000921.1 *H. pylori* J99 and NC 008229.1.

Table 3b. Comparison of cholecystitis with cholelithiasis and gall bladder carcinoma with stones.

		Cholecystitis with cholelithiasis (*n*=89)	Gall bladder carcinoma with stones (*n*=6)	*P* value
PCR				
16S rRNA	Positive	23 (26)	5 (83)	0.008
	Negative	66 (74)	1 (17)	
glmM	Positive	21 (10)	5 (83)	0.005
	Negative	68 (90)	1 (17)	
Immunohistochemistry	Positive	22 (25)	5 (83)	0.007
	Negative	67 (75)	1 (17)	

Univariate analysis was performed by using the independent sample *t*-test, Pearson χ^2 test and Fisher Exact test were also used as appropriate. $P<0.05$ was considered statistically significant. Percentages shown in parentheses.

Table 3c. Comparison of cholecystitis with cholelithiasis and gall bladder polyp.

		Cholecystitis with cholelithiasis (*n*=89)	Gall bladder polyp (*n*=11)	*P* value
PCR				
16S rRNA	Positive	23 (26)	0 (0)	0.06
	Negative	66 (74)	11 (100)	
glmM	Positive	21 (24)	0 (0)	0.11
	Negative	68 (74)	11 (100)	
Immunohistochemistry	Positive	22 (23)	0 (0)	0.17
	Negative	67 (77)	11 (100)	

Univariate analysis was performed by using the independent sample *t*-test, Pearson χ^2 test and Fisher Exact test were also used as appropriate. $P<0.05$ was considered statistically significant. Percentages shown in parentheses.

Discussion

This study demonstrated that *H. pylori* DNA was positive in 24% in a study group comprising patients undergoing cholecystectomy for cholelithiasis associated with chronic cholecystitis by PCR and IHC ($P=0.03$ and $P=0.03$, respectively). However, it was not demonstrated in gall bladder carcinoma without stones or in cases of gall bladder polyps.

It is interesting that H&E, Giemsa and Warthin-Starry stains were inconclusive while IHC results correlated with PCR findings. This could be explained by the absence of areas of intestinal metaplasia in the gall bladder mucosa, as described previously.[12] It is also known that after treatment for chronic active gastritis, *H pylori* can change shape and decrease in number, making identification and differentiation from extracellular debris or mucin difficult. In such cases, IHC improves the rate of identification, even when histological examination and cultures were negative.[25,26]

This study did not attempt to culture *H. pylori* from the bile or the gall bladder as poor culture yield is known to follow storage of these specimens. Direct culture of clinical samples would increase the probability of *in vitro H. pylori* isolation and identification.

In a recent study, Chen *et al.* demonstrated *H. pylori* in the gall bladder of patients with chronic cholecystitis,[27] in whom the incidence of bile reflux was significantly higher. *H. pylori*-like bacteria were found in 14% of gall bladders using the Warthin-Starry silver stain and in 7% using IHC.[27] They also demonstrated that in patients with cholecystitis associated with cholelithiasis there was a high incidence of gastric *H. pylori* infection. Similar results were reported by Caldwell *et al.* who studied *H. pylori* infection rate and gastritis in patients with chronic calculus cholecystitis before and after cholecystectomy and found that the duodenogastric reflux increased after cholecystectomy.[28]

Bile regurgitation appears to play a role in selecting *H. pylori* that are resistant to bile salts, and, while aggravating any injury to the gastric mucosa, they are able to enter the gall bladder from the duodenum.[14] Also, low bile pH has been shown to permit survival of *H. pylori*.[29]

Biliary infection, cystic and common bile duct obstruction and acute cholecystitis have been shown to decrease bile pH, therefore increasing the likelihood of infection with *Helicobacter* species in these diseases.[30,31] The implications of these studies are that *H. pylori* can resist the effect of bile salts and survive under basic conditions.

It is known that increased incidence of hepatobiliary disease is associated with chronic carriage of *Salmonella typhi* and also a high risk of hepatobiliary cancer.[32] Whether or not *H. pylori* has a similar relationship in patients with chronic cholecystitis can only be ascertained by a large prospective study of hepatobiliary disease using appropriate tests for *H. pylori* diagnosis. Thus, currently it is only possible to conclude that *H. pylori* is present in the bile and gall bladder of Pakistani patients with chronic cholecystitis associated with cholelithiasis and gall bladder carcinoma associated with cholelithiasis, and is involved in the immunopathogenesis of gall bladder disorders in some cases. □

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