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Molecular detection of Helicobacter species in the bile and histopathological evaluation of gall bladders of patients with calculus cholycystitis in Duhok province, Kurdistan region of Iraq

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Abstract

Background and objective: The presence of bile-resistant Helicobacter spp. in bile and gallbladder tissues has been proposed as a cause of gallbladder disease. This study aimed to assess the histopathological events in calculus cholecystitis and investigate the presence of the DNA of Helicobacter spp. in the bile.

Methods: Forty patients who underwent cholecystectomy for calculus cholecystitis were evaluated for the presence of Helicobacter spp. by polymerase chain reaction methods and gallbladder tissue was evaluated histopathologically. Gall bladders were fixed in formalin and subjected to histopathological investigation while bile sample was used for the extraction of DNA.

Results: The histopathological findings revealed that twenty-seven (67.5%) samples showed mild chronic inflammation, one (2.5 %) sample with acute inflammation, four (10 %) samples with acute on chronic inflammation, eight (20 %) samples have normal histological findings. The molecular investigation revealed that twenty one (52 %) samples out of the forty showed the presence of genomic DNA after being subjected to DNA extraction and that fourteen (66.7 %) samples out of the twenty one showed amplification bands of 300 bp after PCR amplification indicating the presence of Helicobacter spp. within the samples. But no sample revealed the presence of *H. pylori* DNA when PCR was applied.

Conclusion: Helicobacter spp. are considered as a determining factor for the development of gall bladder inflammation.

Keywords: Helicobacter spp.; Gallbladder; Bile; PCR; 16 S rRNA; HPU1; HPU2.

Introduction

The genus Helicobacter consists of over twenty recognized species, with many species awaiting formal recognition.[1] Members of the genus Helicobacter are all microaerophilic organisms and in almost are catalase and oxidase positive, and many but not all species are also ureases positive. Helicobacter species can be classified into two major groups, the gastric Helicobacter species and the entero-hepatic (non-gastric) Helicobacter species. Both groups show a high level of organ specificity, such that gastric helicobacters, in general, are not usually found in liver or intestine, and vice versa.[2] Beside the well-known *H. pylori*, several Helicobacter spp. are thought to be associated with various chronic digestive diseases.[3] In recent years, Helicobacter DNAs have been isolated from human bile and hepatobiliary tissue by polymerase chain reaction (PCR) assays.[4] Biliary lithiasis is one of the most common conditions requiring surgical intervention. Although the pathogenesis behind which remains unclear, chronic infection is already proposed as a potential risk factor.[5] A possible outcome of colonization by Helicobacter spp. is a chronic inflammation in the gallbladder tissue. This inflammation may alter the secretion of acid by gallbladder mucosa and eventually the acidification of the content[6] culminating

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in the reduction in the solubility of calcium salts in gallbladder bile and increase the risk of their precipitation in the lumen.[7] Interestingly, with continuous isolation of *H. pylori*, *H. bilis*, *H. hepaticus* and other Helicobacter spp. in bile, biliary tract tissue and stone specimens,[4] a hypothetical question was raised about the importance of these microorganisms in the development of biliary lithiasis. In fact, data are controversial in establishing an association between the colonization of Helicobacter spp. in biliary tract and gallbladder stone formation. Some indicated strong positive correlation while others pointed to totally negative results.[8] This study aimed to determine the histopathological findings in calculus cholecystitis and investigate the presence of Helicobacter spp. DNA in the bile of these patients.

Methods

Bile and gallbladder specimens were obtained after cholycystechtomy from forty patients. Bile samples were stored at -20^{o}C. The work on the gallbladder specimens was divided into two parts:

1.Histopathological evaluation:

Three tissue sections were taken from each gallbladder; from the fundus, from the body and the neck.[9] Tissue samples were processed in the usual way for paraffin-embedded blocks, then sectioned and slides were stained by hematoxylin & eosin.[10] Then stained histological sections were evaluated for the presence of inflammation and the types of inflammatory cells. Patients were classified accordingly into four groups:

a. Those with no remarkable tissue changes.

b. Those with acute inflammation when the predominant inflammatory cells are neutrophils.

c. Those with chronic inflammation when the predominant inflammatory cells are lymphocytes and plasma cells.

d. Those with acute over chronic inflammation when there is a mixed inflammatory cells infiltrate.

2. Molecular study: The bile of each individual patient was taken for the extraction of bacterial DNA and subsequent polymerase chain reaction (PCR) for which two sets of primers were selected (Table 1). The work with bile involved two steps:

a. Extraction of DNA

DNA was extracted by phenol-chloroform extraction method. In total, five hundred ml of each bile sample was washed 2-3 times with phosphate buffer saline (PBS) until its pH was adjusted to 7.4–7.6. Then incubated with 1000 ml lysis buffer [Tris–HCl, 50 mM (pH 8.0); EDTA, 100 mM; NaCl, 100 mM; SDS, 1%] and 0.5 mg/ml proteinase K followed by 2 hrs incubation at 56 ^{o}C. Samples were kept at 37 ^{o}C overnight and DNA was extracted twice with phenol-chloroform as follows; one volume of Tris-saturated phenol and one volume of chloroform were mixed followed by centrifugation at 10,000 rpm for

Table 1: Primers sequences and product sizes of amplicons for the genes selected in this study.

Primer	Sequence	PCR program	Gene	Product size (bp)
C97	F 5´GCTATGACGGGTATCC 3´	94^{o} (1 min), 55 ^{o} (30), 72 ^{o} (1 min), 40 cycle	16 Sr RNA	300 [16]
C98	R 5´GATTTTACCCCTACACCA3´			
HPU1	F 5´GCCAATGGTAAATTAGTT3´	94^{o} (1 min), 50 ^{o} (30), 72 ^{o} (1 min), 40 cycle	HPUA	400 [16]
HPU2	R 5´CTCCTTAATTGTTTTTAC3´			

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5 min at room temperature, then the upper aqueous phase was transferred to a fresh tube and an equal volume of chloroform was added, this step is repeated twice. The upper aqueous phase was transferred to a fresh tube and 1/10 the volume of 3 M sodium acetate was added. Furthermore, an equal volume of isopropanol was added to precipitate DNA. The mixture was then incubated for 30 min at -20°C and centrifuged at 10.000 rpm for 10 minutes; the supernatant was discarded and the pellet rinsed with 70 % cold ethanol. Finally, the pellet was air-dried and dissolved in 300 ml of TE buffer.[11]

b. Agarose gel electrophoresis

Agrose gel (1.0 %) was prepared according to previous work and electrophoresis was adopted to confirm the presence DNA.[12]

c. PCR Amplification

In the present study, two sets of primers were used C97, C98 primers were specific for the amplification of 16 S rRNA of Helicobacter spp. the reaction conditions were 94°C (1minute), 55°C (30 seconds),72°C (1minute) while HPU1, HPU2 primers were utilized for the amplification of urease gene A specific for *H. pylori* with reaction conditions of 94°C (1minute), 50°C (30 seconds), 72°C (1minute); the later conditions were manipulated several times to reach an optimum results (Table 1). Uniplex PCR assay was performed for each of the two primers individually. The lyophilized master mix was reconstituted by adding 16 µl D.W., 2 µl Primers (forward and reverse), and 2 µl DNA template (50 ng/ml).

Results

Examination of the histopathological sections revealed no remarkable changes in eight samples; one sample showed features of acute inflammation, twenty seven cases exhibited features of chronic inflammation and four showed evidence of acute on chronic inflammation (Table 2). Additional findings included cholesterolosis in four patients and antral metaplasia in one patient of those with chronic cholecystitis. The results of DNA extraction from bile showed that twenty one sample showed evidence for the presence of bacterial genomic DNA and one sample showed a faint band resembling DNA thus it was considered query and generally, positive samples constituted 55% (Figure 1).

Table 2: Histopathological findings of gall bladders.

Histopathological findings	No. of cases	(%)
Acute cholecystitis	1	(2.5)
Chronic cholycystitis	27	(67.5)
Acute on chronic cholycystitis	4	(10.0)
Normal ball bladder tissue	8	(20.0)
Total	40	(100)

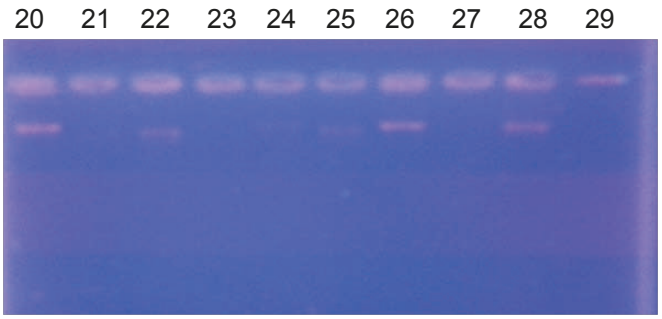

Figure 1: Bacterial Genomic DNA. Samples no. 20, 22, 25, 26, and 28 showed genomic DNA while other samples considered negative.

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The results revealed that out of the twenty two bile samples (the original twenty one and the one that was query in showing genomic DNA band) which showed the presence of bacterial genomic DNA, fourteen samples (63 %) were positive for the presence of Helicobacter spp DNA as it was evident by amplification bands of 300 bp (Figure 2) while all samples did not show amplification bands reflecting *H. pylori* specific sequences thus they were considered negative for the presence of *H. pylori* DNA.

Discussion

The presence of Helicobacter spp. DNA in the epithelial tissue of the gallbladder of individuals with cholelithiasis and cholecystitis had been discussed by several authors. Until now, more than 25 species of Helicobacter have been found, and these microorganisms have been confirmed to be causative agents of various diseases of the stomach, intestine, and the liver in human and mammals.[13] The results of current study revealed a higher figure for the presence of Helicobacter DNA in bile compared to the studies of Ghazal et al.,[14] and Lee et al.,[15] who reported values of 18% and 25%, respectively. However, our results came in line with those of other researchers[11,16] as high percentages of 42.9% and 96.7%, respectively were recorded for the presence of Helicobacter DNA for patients with different hepatobiliary diseases. Furthermore, it has been declared that 65% bile sample contained DNA of Helicobacter spp. which came in accordance to the results of the current study.[17] It was confirmed that intestinal Helicobacter spp. are bile-resistant, a property that may confer protection against the deleterious effects of bile in vivo and allow them to adapt better to the hepatobiliary surroundings.[11] Interestingly, the first and largestNorth American investigation addressing the issue of Helicobacter in bile revealed that Helicobacter DNA hadn't been detected in all samples involved. This suggests that Helicobacter is not a significant cause of biliary problems in general and this observation came in line with that of the current study as far as *H. pylori* DNA is looked for but when Helicobacter genus DNA is generally considered, the results of the two studied are contradictory; the discrepancy of results can be argued as it is possible that the organisms preferentially live in the bile canaliculi, as the biochemical nature of bile in this location is distinct from that in the common

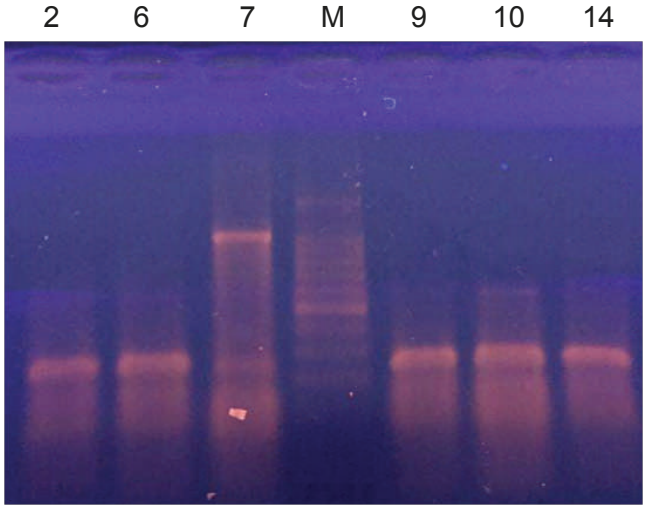

Figure 2: PCR amplification for Helicobacter spp with primers C97 and C98. Samples 2,6, 7, 9, 10, 14 showed the amplification product bands of 300 bp. The letter M in lane 4 showed the 100 bp molecular marker.

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bile duct. The other possible elucidation for this difference is that there is different infection prevalence in different geographical locations.[18] It was stated that helicobacter infection by itself might have no important role in the formation of gallstones. However, the participation of infection as a cofactor for the development of stones cannot be ruled out.[19] Moreover, no Helicobacter spp. were detected in the bile of German patients suggesting that there may be racial and demographic differences in the etiology of gallstones.[20] It can also be confirmed that the causes for observed inconsistencies in the detection rate of Helicobacter among different studies are obscure, but differences in the sensitivity of PCR and geographical variations in human exposure to Helicobacter spp. are two possible explanations.[14] Chronic inflammation of the gallbladder may occur as a possible outcome for the colonization of Helicobacter spp. This inflammation may interfere with acid secretion by gallbladder mucosa acid and hence acidification of the contents,[21] thereby, decreasing the solubility of calcium salts in bile and increasing the potential of their precipitation in the lumen.[7] Some investigators suggest that different Helicobacter species may be found in the human biliary tract but that the number of microorganisms can change according to the species, with multiple intestinal Helicobacter spp. being detected in higher numbers than *H. pylori*.[11] Indeed, the relationship between Helicobacter infection and cholelithiasis remains controversial. Some researchers have supported a cause-and-effect theory while some others even were not able to prove the existence of these bacteria in biliary specimens.[22] Also, there is a lack of strong evidence to determine the possible entry routes of Helicobacter sp. to the hepatobiliary tree including either the ascending duodenum infection or the portal system circulation pathway.[23]

Conclusion

In conclusion, the results of the present study revealed an important association between gallstones and the presence of Helicobacter DNA in the bile. Further studies are needed to determine whether Helicobacter spp. is a causative agent of biliary diseases or a cofactor.

Conflicts of interest

The authors report no conflicts of interest.

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