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IDENTIFICATION OF HELICOBACTER SP. IN BILE AND LAPAROSCOPIC RESECTED GALL BLADDER TISSUE FROM EGYPTIANS WITH CHRONIC CALCULAR CHOLECYSTITIS

By
Ahmed H, El-Defrawi I, Moussa M**, Helmy AH****
Biochemistry, Microbiology*, Pathology**, General Surgery*** Departments, Theodore Bilharz Research Institute, Imbaba Giza

Recent data support a role of bile-resistant Helicobacter sp. in the development of gallbladder disease. Our aim was to identify the presence of Helicobacter organism in association with calcular cholecystitis among Egyptian patients. Twenty eight patients were included in the study and subjected to laparoscopic cholecystectomy. The presence of Helicobacter sp. in bile and resected gallbladder tissue were investigated by culture, histopathological examination and polymerase chain reaction (PCR) using Helicobacter genus-specific primers. Aerobic and anaerobic cultures of gallbladder tissue, bile and gall stones were also done to exclude other bacteria which might be encountered in biliary infections. Culture for Helicobacter organisms were negative while histopathological examination of gallbladder tissue revealed curved bacteria suggestive of the organism in 2 specimens. helicobacter DNA was detected by PCR analysis in 57% and 21% of bile samples and resected gallbladder tissue respectively. All positive gallbladder specimens simultaneously revealed Helicobacter DNA in their bile samples. The current data support the concept of association of Helicobacter organisms with chronic calcular cholecystitis and a possible implication in the disease pathology. However, further epidemiological molecular studies are recommended for sequence analysis and identification of species distribution among Egyptian patients.

***Key words*: Helicobacter species, gall bladder, chronic caculaer cholecystitis, bile, Poly chain reaction**

INTRODUCTION

Since its culture by Marshall et al in 1982, Helicobacter pylori (H. pylori) has been found in gastric biopsy specimens and linked to many diseases in the gastrointestinal tract [1]. Several studies have established a causal role for H. pylori in the pathogenesis of duodenal ulcers, gastric ulcers and chronic gastritis [2]. H. pylori has also been linked to the development of gastric adenocarcinoma and gastric mucosa-associated lymphoma [3],[4]. Since the discovery of H. pylori, more than 25 additional helicobacter sp. have been isolated from the stomach, intestinal tract and liver of various mammalian and bird species [5]. Like Salmonella typhi, these Helicobacter organisms are tolerant to bile, colonize bile canaliculi and the gallbladder and cause hepatitis and cholecystitis in animals [6],[7],[8]. More recent studies have focused on investigating the possibility of pathogenesis of biliary tract diseases in humans by these organisms [9,10,11]. The aim of the present study is to ascertain whether Helicobacter sp. could be identified in resected gallbladder tissue and bile collected from Egyptian patients with chronic cholecystitis.

PATIENTS AND METHODS

Laparoscopic Cholecystectomy :

The study was conducted on 28 patients suffering from chronic calcular cholecystitis who were further subjected to laparoscopic cholecystectomy [12]. They comprised 7 males (age range: 23 - 63 years) and 21 females (age range: 18 –55 years). Patients were evaluated by routine ultrasonography and admitted to hospital 24 hours before procedures. Thorough preoperative assessment and investigations were done and informed consent obtained. No antibiotics were prescribed before surgery, while third generation cephalosporins were given

intra-operatively. Patients had four ports access and pneumoperitoneum using CO2 at 12 mmHg. Local anaesthetic was injected subcutaneously at port site immediately before insertion. Cholecystectomy was done meticulously through a dilated epigastric port to avoid contamination of the intact surgically removed gallbladder. Bleeding from the gallbladder bed was controlled by diathermy [13]. All patients were discharged on the same or next day and were prescribed antibiotics and pain killers. In this limited number study no patients developed jaundice and there were no recorded deaths. Port haematoma at the epigastric site occurred post-operatively and were treated conservatively.

Microbiological assessment:

Bile specimens were centrifuged at 1800 g for 10 minutes and deposits were Gram stained. Small portions of gall bladder tissue were homogenized using sterile motorized bacteriological loop. Fragmented gall bladder tissue and bile deposits were cultured specifically for Helicobacter sp., as well as aerobically and anaerobically for other bacteria that may be encountered. Culture for Helicobacter sp. was done by inoculation of modified Skirrow selective medium (Brucella agar base, 10% sheep blood and Skirrow supplement, Oxoid SR069E). Incubation was done at 37 C for 5 - 7 days in a microaerophilic atmosphere [14] using GasPak envelope (bioMerieux). All samples were cultured aerobically on blood agar, MacConkey agar and selenite broth at 37 C for 24 h. Subculture was done from the latter onto Hektoen enteric agar to exclude salmonella carriers. Anaerobic cultures were performed by inoculation of specimens onto fastidious anaerobe agar (Biotec, UK) enriched with 7 - 10% horse blood and in freshly prepared thioglycollate broth which were further incubated in anaerobic jar where Gas Generating Kit (Oxoid, Inc. , USA) was incorporated. No growth on the primary solid medium after anaerobic incubation for 48h necessitated reincubation for further 5 days. Subculture from the thioglycollate was also done onto blood agar (incubated aerobically for 48h) and enriched fastidious anaerobe agar (incubated anaerobically for 48h). Aerobic and anaerobic cultures were similarly done for gall stone wash and crush [15].

PCR analysis:

Bile aliquots and small amounts of bladder tissue in liquid nitrogen were immediately stored at -70 C until analysed for Helicobacter species by PCR.

D N A extraction:

DNA was extracted from gallbladder tissue [16]. Briefly 200 mg of the specimen were grinded into fine powder using a pre-chilled mortar. The powder was suspended in 1.2 ml digestion buffer per 100 mg tissue in tightly capped tubes which were further incubated with shaking at 50 C for 18h. The samples were then thoroughly extracted with an equal volume of phenol/chloroform/isoamyl alcohol and centrifuged at 1700 g in a swinging rotor for 10 minutes. This was followed by extraction with chloroform and the aqueous phase was transferred to a new tube to which 1/2 volume of 7,5%ammonium acetate and 2 volumes of 100% ethanol were added. After centrifugation at 10,000 rpm for 20 minutes, the pellet was washed with 70% ethanol and air-dried. DNA was re-suspended in 100 ul distilled water.

DNA was extracted from bile according to the technique of Fox et al (1998). Bile (100ul) was centrifuged with 900 ul phosphate buffered saline (PBS) at 12,000 rpm for 10 minutes and washed once with 1 ml PBS. The pellets were re-suspended in 10 ml distilled water and 20 ul GeneReleaser (BioVentures Inc.) were added to each tube. The samples were micro-waved at 700W for 7 minutes. After being centrifuged at 12,000 rpm for 5 minutes, the supernatants were transferred to another tube and diluted with 70 ul distilled water.

PCR Amplification:

For the gall bladder and bile samples that amplified the 400 base pair, helicobacter sp. were analyzed using helicobacter genus-specific primer pairs C97 and C98 or C97 and C05 (Research Genetics – USA). The primers have the following sequences: C97, 5'-GCT ATG ACG GGT ATC C-3' (276 - 291 forward); C98, 5'-GAT TTT ACC CCT ACA CCA-3' (681- 698 reverse) and C05, 5'-ACT TCA CCC CAG TCG CTG-3' (1478 - 1495 reverse). Ten microliters of the DNA preparation was added to a 100-ul reaction mixture containing 1X Taq DNA polymerase buffer (supplemented with 25mmol/L MgCl2 to a final concentration of 2.25 mmol/L), 0.5 □m each of the two primers (C98 and C97), 200 □m of each deoxynucleotide. Samples were heated at 94 for 4 minutes, briefly centrifuged and cooled to 55 C. At this time, 2 ul of Taq DNA polymerase and 1 ul polymerase enhancer (Perfect Match sigma, USA) were added followed by an overlay of 10 ul mineral oil. The following conditions were used for amplification: denaturation at 94 C for I minute, annealing at 55 C for 2 minutes and elongation at 72 C for 3 minutes. A 15 ul aliquot of the sample was then electrophoresed through 2% agarose gel.

Histopathological Examination:

After processing samples for microbiological and PCR testing, the gall bladder specimens were fixed in 10% formalin. They were opened along the longest diameter in the peritoneal side. Transverse multiple sections of the wall including mucosa and serosa were taken, embedded in paraffin, sectioned and stained with haematoxylin and eosin for routine histology, Masson's trichome stain for

studying fibrosis and Giemsa for detection of Helicobacter like organisms.

RESULTS

Molecular analysis of resected gallbladder tissue and bile samples by PCR with helicobacter genus specific primers produced 400 base pair amplicon in 21% (6/28) and 57% (16/28) respectively; (Figs 1 & 2.) Bile samples associated with gallbladder tissues successfully amplified with Helicobacter primers (in 6 patients) were simultaneously positive for Helicobacter DNA.

Results of detection of Helicobacter species in bile and resected gall bladder tissue by different techniques.

	Resected gall bladder tissue	*Bile*	*Both*
PCR	2/28 (7%)	16/28(57%)	6/28(21%)
Culture	Negative	Negative	Negative
Histopathology	2/28 (7%)	NA	NA

NA : Non applicable .

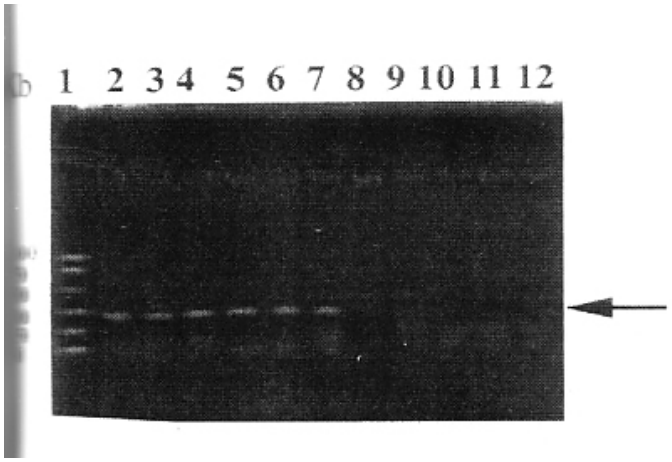

Fig. (1): PCR product of genus – specific heliobacter species. Positive bile sample (lane 2-7). Negative bile sample (lane 8-12) , lane (1)low molecular weight standard .

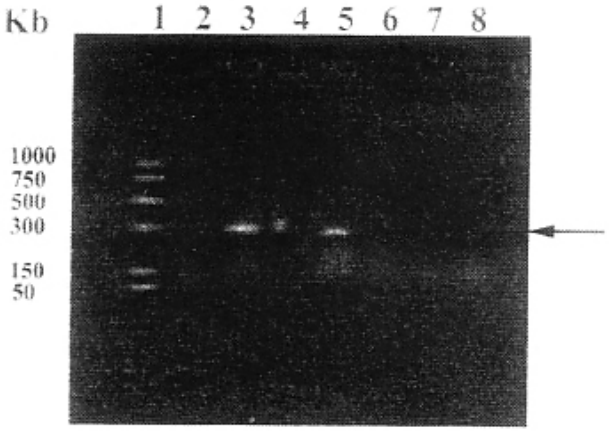

Fig. (2): PCR product of genus – specific Helicobacter species, positive gall bladder Sample (lane 3,5) Negative gall bladder sample (lane 2,4,6,7,8) , lane (1) low molecular weight standard.

Helicobacter organisms were not isolated from the tested bile or gallbladder tissue. Likewise aerobic and anaerobic cultures on various fluid and solid media revealed no growth in both specimens and stone wash.

Histopathological examination of resected gallbladder, all of which contained stones, revealed chronic cholecystitis with mononuclear cell infiltrate. Erosions and ulcers were found in 11% and 8% of examined gallbladder specimens respectively. Giemsa stain detected curved bacteria suggestive of helicobacter sp. in 2 specimens. The latter were among gallbladder specimens which amplified the 400 base pair helicobacter sp.

DISCUSSION

In recent years members of the genus Helicobacter have become the subject of intense research as colonizers of

the hepatobiliary tract of animals and pathogenic causes of hepatobiliary diseases [10]. The best model for helicobacter-induced liver disease to date is H. hepaticus. The organism persistently colonizes the colon and cecum and is associated with liver tumors in A/JCr mice as well as hepatitis in other susceptible inbred mouse strains [6],[8],[17]. The mechanism by which H. hepaticus infection leads to liver injury is unclear at present. H. hepaticus like other Helicobacter sp. expresses urease, which generates ammonia. This toxic product may damage hepatocytes adjacent to the bacteria. In addition, a soluble cytotoxin has been identified in H. hepatitis that produces significant in-vitro cytopathic effects in a murine hepatic cell line [18]. It is also possible that antibodies to Helicobacter sp. could also react with bile canaliculi and/or hepatocytes and be responsible for autoimmune-mediated damage of bile duct epithelium [19].

Other Helicobacter organisms capable of eliciting hepatitis in animals, e.g., H. pullorum, Flexispira rappini and H.canis, have also been isolated from the feces of diarrheic humans. Such data suggest a zoontic potential and raise the possibility that these or other Helicobacter organisms may cause liver disease in humans [7],[8].

The association of helicobacter sp. with chronic calcular chole-cystitis was studied in the current work. In the course of microbiological assessment, we could not isolate helicobacter organisms from neither gallbladder tissue nor bile samples. Lack of recovery of Helicobacter isolates from clinical specimens was reported [5]. They attributed failure of culture to the prolonged freezer storage of tissue and bile. Although direct culture of clinical specimens, as conducted in the present work, was expected to greatly increase the probability of in-vitro Helicobacter isolation and identification; still culture was not successful. The yield of histopathology was also poor revealing curved bacteria in only 2 gallbladder tissue samples. Such results were concordant with data reported [9],[20].Molecular identification was more successful where helicobacter DNA was detected in 57% and 21% of bile samples and resected gallbladder respectively. The detection rate of DNA in bile specimens was comparable to findings reported in previous studies [5],[9], while higher positivity (39%) in gallbladder tissue was recorded by the same authors. On the other hand, studies investigating the presence of H. pylori using different primers revealed low positive rates of 7.6 - 11.6 % [21],[11]. In the present work genus specific primers were used for PCR analysis since many of the named and unnamed helicobacter sp. are genetically very similar to one another [5]. Also, inspite of the high incidence of gastric H.pylori among Egyptians [22], no attempt was made to specify H. pylori DNA among gallbladder tissue and bile specimens since in-vitro studies indicated that H. pylori is unable to grow in the presence of bile products [23]. The sensitivity of H. pylori to bile acids is markedly contrasted by the ability of F. rappini, H. hepaticus, H. bilis, H. canis, H. cholecystus and H. pullorum to grow in the presence of bile [24]. It was reported that H.pylori DNA may be present in the bile when there are certain environmental changes, such as lowered pH [11].

For better interpretation of the significance of helicobacter prevalence among tested clinical specimens, our samples together with stone wash were cultured onto a variety of bacteriological media under both aerobic and anaerobic conditions. We aimed at exclusion of the possible association of other organisms that may be encountered in biliary infections. Typically gastrointestinal tract organisms may be isolated. The bacteria most commonly encountered are: E.coli, Proteus sp., other Enterobacteriaceae, B.fragilis, C.perfringens, anaerobic cocci and streptococci, usually enterococci. Salmonella sp., including S.typhi may be isolated from biliary carriers [25].

CONCLUSION

In conclusion, the sole identification of helicobacter sp. in bile and resected gallbladder tissue among patients in the current work provides strong evidence of the association of the organism with chronic calcular cholecystitis and its possible implication in the disease pathology. Bearing in mind the difficulty in obtaining normal gallbladder tissue from selected populations to ascertain the absence of helicobacter sp. in normal biliary tissue, there is a pressing need to develop noninvasive serological assays that allow determination of prevalence of hepatic helicobacter organisms in humans. Also, further molecular studies are mandatory for identification and typing of hepatic Helicobacter sp. for epidemiological purposes.

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