World Journal of Epidemiology and Cancer Prevention

Original Article

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Helicobacter hepaticus does not Increases the Risk of Gallbladder Cancer: Results of A Case Control Study and Literature Review

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Abstract

Introduction: Gallbladder cancer is one of the few cancers that are associated with bacterial infections and inflammation. Of many bacteria, *Helicobacter* has been found to be associated with gastric MALToma, gastric adenocarcinoma and hepatobiliary neoplasms. We studied the presence of the *Helicobacter hepaticus* in carcinoma of the gallbladder using cholelithiasis as control.

Patients and methods: Fifty four gallbladder cancer and 55 controls with cholelithiasis were studied with *Helicobacter* culture and PCR for the *Helicobacter hepaticus* using flagellin-A gene primers. Relative risk and odds ratio with 95% CI were estimated. An extensive review of literature was carried out and data was analyzed using cumulative odds ratio.

Results: *Helicobacter hepaticus* was identified in 22/54 patients and 18/55 controls by culture, Flagellin A PCR products were seen in 14/54 cancer and 8/55 controls. The difference was statistically not significant (p=0.107). The relative risk of gallbladder cancer in *H. hepaticus* culture positive cases was 0.606 (95% CI 0.418 to 0.717), while risk of gallbladder cancer increased on detection by flagellin gene (OR 1.78 (95% CI 0.81-3.09) though this was statistically not significant. Overall after inclusion of all cases from literature 55/294 cases and 128/377 controls had H hepaticus infection the cumulative odds ratio was 0.4477 (95 % CI: 0.3116 to 0.6432) which was statistically significant.

Conclusions: The present study demonstrates no increased risk of gallbladder cancer in presence of *Helicobacter hepaticus*. The variability in methods of detection and use of variable cases and controls leads to heterogeneity in the sample that makes it difficult to interpret these results.

Key words: Cancer; neoplasm; hepatobiliary; pancreas; gallstones; cholangiocarcinoma

Introduction

Helicobacters are known as the enterohepatic bacteria that are shown to be associated with various intestinal and hepatobiliary disases [1]. *Helicobacter hepaticus* is one of the helicobacters which is slender curved to spiral-rods which form one to three spiral turns. They are gram negative bacteria and are motile having single bipolar sheathed flagellum [2]. *H. hepaticus* colonizes the lower gastrointestinal tract of mice in addition to its cecum, colon and hepatobiliary system [2, 3]. Flagellum plays an important role in the colonization and persistence within the host [4].

Various studies have concluded that *H. hepaticus* may play a role in the development of gastrointestinal diseases in humans [5]. In a study by Kobayashi, a higher level of antibodies against *H. hepaticus* has been found in patients

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Submitted: Sunday, August 6, 2017; Accepted Monday, August 21, 2017Published: Thursday, August 31, 2017

Table1: Sequence of primers, their specificity with genes and cycles program

S. No.	*Fla* –gene primer	Nucleotide Sequence	PCR Program	Size
1	HHE F 1	5◌ TGCGCGCTATGGATATGAGATTAG 3◌	30 cycles, Ta= 61^{0}C	680 bp
	HHE R 1	5◌ TTTCACCCTTGCCTTCATCGG3◌	30 cycles, Ta= 61^{0}C	680bp
2	HHE F 2	5◌ATGCGCCAAGATAAGGCATTG 3◌	40 cycles Ta= 59^{0}C	518 bp
	HHE R 2	5◌ TTGCCACAAGGACTATTTCC 3◌	40 cycles Ta= 59^{0}C	518 bp

Ta = Annealing temperature

having chronic liver diseases [5]. Another study suggested that *H. hepaticus* infection may contribute to the development of gallstones and the *H. hepaticus* is able to infect the liver, gallbladder epithelium, or intestine of human [6]. The aim of the present work was to identify the *Helicobacter hepaticus* in gallbladder cancer samples in comparison to cholelithiasis as control.

Material and methods

Subjects

Fifty-four histologically proven gallbladder cancer and 55 ultrasonographically proven cholelithiasis were included in the study. The tissue samples were collected from the surgical unit of Department of Surgical Oncology, Institute of Medical Sciences, Banaras Hindu University during 2012, the study protocol was approved by the Ethics committee. The samples were not matched for age and gender.

Identification of H. hepaticus

H. hepaticus was identified by two methods i.e. microbiological and molecular:

Microbiological methods

Culture: Primary bacterial culture was performed by using Brain Heart Infusion (BHI) agar medium. Autoclaved glass beads were taken in a conical flask and 10ml of defibrinated sheep blood was added and the mixture was kept at 4˚C. BHI was dissolved in distilled water and 2% Difco agar was added. All solutions were autoclaved and when temperature reached to 40-50˚C, they were supplemented with 7% defibrinated sheep blood, 0.4% IsoVitaleX and Skirrow selective supplement (vancomycin, 10µg/ml; polymixin B sulfate 2.5 IU/ml; trimethoprim lactate 5µg/ml) (Difco, USA). The biopsy specimens were ground together in an all glass homogenizer. An aliquot of this tissue homogenate was plated on media containing BHI agar (Difco, USA). Plates were incubated at 37^{0}C in an atmosphere of 5% O_2, 10% CO_2, and 85% N_2 for 3 to 7 days. *H. hepaticus* colonies were identified on the basis of their typical colony morphology, Gram negative spiral rods and Oxidase and Urease production.

Biochemical tests (Rapid Urease, Oxidase and catalase tests were carried out to identify the *Helicobacter hepaticus*:

Rapid Urease Test (RUT): KH_2PO_4, MH agar and glucose were dissolved in 100 ml distilled water. Then, phenol red was added as an indicator. All the constituents were autoclaved and when the temperature reaches 50°C, urea was added, and 3ml of above prepared media was poured in test tube and kept at 4°C. A colony was taken from the culture and inserted in the test-tube. Yellow color was observed in the RUT positive colonies.

Oxidase test: A piece of paper was soaked in the reagent solution. Some fresh growth was scraped from the culture plate with the help of disposable loop and rub onto the filter paper.

Table 2: Demonstration of Helicobacter by various techniques

Helicobacter	Culture		OR	RUT		OR	Fla A		OR
	Ca	Con		Ca	Con		Ca	Con	
Positive	22	18	0.6 (95% CI 0.418 to 0.877)	24	14	0.59 (95% CI 0.426-0.834)	14	8	2.0563 95 % CI:0.7829 to 5.4004
Negative	32	37		30	41		40	47	

Ca: cancer Con: control

Blue color was observed within 10 seconds in the oxidase positive colonies.

Catalase test: 0.2 ml hydrogen peroxide was placed in a test-tube. Colonies were picked with the help of inoculated loop and rub it inside the wall of test-tube containing hydrogen peroxide solution. Vigorous bubbling occurred within 10 seconds.

Molecular identification

DNA Isolation: The tissue sample was homogenized using mortar and pestle. The homogenized tissue sample was incubated with 1 mg lysozyme at 37^{0}C for 60 minutes. Then 1 ml of 0.1% Triton-X and 5 µl Proteinase-K was added and 30µl of SDS added and incubated again at 65^{0}C for 120 minutes. To this, equal volume of Chloroform: Iso-Amyl Alcohol (IAA) (24:1) was added and mixed by vortexing for 15 minutes, centrifuged at 10,000 rpm for 10 minutes and aqueous phase was collected. Then, 140 µl of Phenol: Chloroform: IAA (25:24:1) was added and mixed by vortexing for 15 seconds and again centrifuged at 10,000 rpm for 10 minutes. Aqueous phase was collected. Equal volume of Isopropanol was added to this aqueous phase. The solution was kept at room temperature for 5 minutes. The above was centrifuged at 10,000 rpm for 10 minutes and supernatant decanted. The pellet was washed by 200 µl 70% Ethanol. The above solution was centrifuged at 10,000 rpm for 10 minutes. The pellets were dried over at 37^{0}C for 30 minutes. Then the pellets were re-dissolved in 50 µl in TE buffer.

Nested-PCR: Gradient temperature PCR was performed to identify annealing temperature and then nested PCR protocol was performed for DNA amplification. Nested-Polymerase Chain Reaction (Nested-PCR) specific for the Flg-A gene was performed to identify the H. hepaticus strain. For this, two sets of primers were designed (Table 1). These were for conserved stretch of nucleotide in flagellar activity associated gene ORF Fla-A. This region of H. hepaticus ATCC 51449 is conserved and primers were analyzed on Clustal W2 version with three close bacteria viz. H. acinonychis, H. pylori and C. jejuni, to check their E value and specificity. It was found that the Fla A gene shows 100% specificity with H. hepaticus. The synthesis was completed by Metabion International Deutschland of 100.0 µM concentration and 0.02µmol synthetic scale. The outer sets of primers amplification located between 230 to 910 (680 bp) and the nested internal primer synthesized were between 298-816 (518bp) (http://www.ncbi.nlm.gov/BLAST). The sequence and their amplification program are given below in table.

PCR amplification was performed in a 25 µl volume using a thermal cycler (Biometra). The reaction mixture contained 1X PCR buffer, 0.2 mM of each oligonucleotide, 250 nM of each primer, 1.5 mM $MgCl_2$, 1 U of Taq polymerase and 50 ng (1 µl) of DNA. Agarose gel electrophoresis (1%) was carried out to check the amplification and visualized under UV light.

Result

Mean age of the cancer patients was 53.6 years while that of controls was 48.6 years. The difference was statistically not significant. Liver enzymes were significantly deranged in the gallbladder cancer patients compared to controls.

Table 3: Previous studies on Helicobacter hepaticus in biliary tract tumors

Reference and year	Type of study	Method of detection	Specimen	Disease	*Helicobacter* hepaticus positivity	OR
Kobayashi T et al	Case control	PCR culture	Bile	Mix. Incl cancer	16/30 benign 5/6 Ca	4.3750 95 % CI:0.4548 to 42.0822
Hamada T et al	Observational	cultures, nested PCR, or in situ hybridization	Bile	Benign gallbladder and non biliary	*40/126*	Higher in cholelithiasis and gastric cancer
Shimoyama T	Case control	western blot ELISA	serum	Gallstone -55 Bile duct or gallbladder Ca-18 Ca Pancreas 19	*14/55* *13/37*	1.5863 95 % CI:0.6401 to 3.9313
Pradhan SB, Dali S	observational	Histology	Gallbladder tissue	Mix	6/7 malignant 76/93 benign	1.0263 95 % CI:0.1140 to 9.2371
Segura-López FK et al	Case control	PCR	Tumor tissue	Benign diseases- 91 extrahepatic cholangiocarcinoma -103	*13/91* *17/103*	1.1860 95 % CI:0.5413 to 2.5989
Boonyanugomol W et al	Case control	PCR	Tissue	Benign hepatobiliary-53 Cholangiocarcinoma-87	*0/53* *0/87*	-
Present series	Case control	PCR	Tissue	Cholelithiasis Gallbladder Ca	*8/55* *14/54*	2.0563 95 % CI:0.7829 to 5.4004
Cumulative				Cases control	*55/294* *128/377*	0.4477 95 % CI:0.3116 to 0.6432

Microbiological results

The microbiological results are shown in Table 2. 22/54 cancer and 37/55 control patients were positive for *Helicobacter hepaticus* by culture method. Rapid urease test was positive in 24/54 cancer and 41/55 of the carcinoma patients (Table 2).

Molecular results

Fourteen samples out of fifty-four gallbladder cancer cases and only eight out of fifty-five control had shown to be positive for the PCR.

Review of world literature

Table 3 shows the results of literature review on H. hepaticus and hepato-biliary-pancreatic diseases. A detailed search of Pubmed, google Scholar and Cross ref along with hand search of back citations resulted in identification of only 6 studies, of which two were observational and 4 case control. Different studies used different methods of detection that varied from histological or serological demonstration to PCR identification. The cases also were varied ranging from cholngiocarcinoma to gallbladder carcinoma and pancreatic carcinoma, none of the studies however showed positive association of H. hepaticus with hepatic or extrahepatic pancreaticobiliary cancers. Cumulative odds were actually reduced by almost half; however this was due to one negative study that failed to identify even single H. hepaticus in all the benign and malignant samples.

Statistical Analysis

The relative risk of gallbladder cancer in *H. hepaticus* culture positive cases was 0.6 (95% CI 0.418 to 0.877), while no significance was obtained by flagellin gene [OR 1.78 (95% CI

0.81-3.09)]**.** The odds ratio by rapid urease test for all Helicobacter was 0.59 (95% CI 0.426-0.834). (Table 3)

Discussion

Since the discovery of various *Helicobacter* species, their role in variety of diseases of gastrointestinal tract is slowly becoming clearer. The involvement of bacterial infection in carcinogenesis, especially *Helicobacter* species which is known to colonize the gastric region of various murine organism as well as humans, is now almost certain. In an inbred strain of mice A/JCR persistent infection with *H. hepaticius* is linked to development of hepatic adenoma and hepatocarcinoma [7].

Although culture isolation has been standard method for detection of infectious agent, yet it may not be the most appropriate method for bacteria like *H. hepaticus*, which is both difficult and time consuming to grow. Rapid urease test (RUT) and histological staining technique may not prove to be specific [8]. In our work also using culture, biochemical tests (RUT, catalase, oxidase) and gram staining for screening purpose we have found colonies are appearing in 30 plates under microaerophilic condition followed by negative pressure. Of these plates 23 were positive with RUT, 19 with catalase and 20 for oxidase. All colonies (pin-point) were flagellated unipolar or bipolar.

H. hepaticus is a flagellated chronic pathogen which colonizes the hepatobiliary tract and it is a highly studied species among all Helicobacter species that are associated with intestinal tract of humans [1]. In this study, we have targeted flagellin gene (*fla A*) for the identification of the *H. hepaticus* in our samples. The flagellin gene is conserved gene and responsible for bacterial motility to host damage [9]. Because of this, it may be one of the best gene based diagnosis of *H. hepaticus* from clinical sample.

PCR is highly sensitive and specific test and results can be obtained in a short period of time and frequently used in various site specific detection[9-12]. Previous studies suggested that flagellin is an adhesion for epithelial cells and that motility is a virulence factor of some bacterium such as *C. jejuni* [13] but the wild type flagellar filament is a heteropolymer of *fla A* and *fla B* gene products [14]. Flagellin gene is transcribed for sigma 28 promoters of the bacterium. This gene product is capable of assembling independently into a functional filament and is conserved for *H. hepaticus*. In our study, at the time of primer designing we have matched in *Clustal W* format for other close relative bacterium. In this matching we have found 100% E value of the sequence BLAST and 100% specificity for *H. hepaticus*, so we have targeted *fla A* gene amplification in our nested protocol. Our study is unique because there is no other study applied this on human system in contrary to this site, in A/JCr mice [10] and commercial mouse [15].

PCR based method is highly specific and sensitive. The difference in results of our study by culture and PCR suggests over diagnosis of *H Hepaticus* as some of the other species could have been labeled as *H hepaticus* by culture. In contrast to our results where PCR based diagnosis showed increased risk of gallbladder carcinogenesis in *presence of H hepaticus*, Shimoyama et al., [16] also found an increased risk of bile duct cancer in presence of H. hepaticus. Pradhan and Dali [17, 18] too found the presence of *H hepaticus* in 82% of gallbladder cancer, however, in absence of molecular diagnosis presence of other Helicobacter in their study can not be ruled out. Results of a recent study by Segura-López et al., 2015 failed to show any role of *H hepaticus* in cholangiocarcinoma [19], though they observed a significant relation with *H. bilis*. Another study from Thailand failed to find any PCR product in cancer or controls [20]. Pandey and Shukla [21] in a meta analysis reviewed 15 studies on Helicobacter Species in hepatobiliary cancers and found a cumulative odds ratio of 8.72 for the development of cancer (95% CI 4.78-15.91). Another study by Pandey M (2007) [22]

reviewed 12 studies of Helicobacter Studies in benign disease and showed an increased risk (OR 1.77). Pandey et al., also reported results of case control study on Helicobacter bilis and meta analysis of previously published studies [23] . Though in their study the risk was not significant the results of meta analysis showed an odds ratio of 4.13 for hepatobiliary tract cancer and 1.24 for gallbladder cancer [23] . Hence, it is clear that the number of studies evaluating *H. hepaticus* are limited and are diverse, while those on *H. bilis* and other species showed increase risk of hepatobiliary tract cancer, although the risk of gallbladder cancer in particular is not clear. One of the latest review on Helicobacter and hepatobiliary studies has concluded that due to variability in the methods used and the results, it is not possible to come to any conclusions as of now and hence, more studies are needed to answer this question [24]. This is the first study reporting on detection of H. hepaticus in gallbladder cancer using Flagellin gene, and more studies on *H. hepaticus* are required to clearly elucidate its role in gallbladder cancer.

Conclusions

fla A gene specific nested PCR protocol is most appropriate for detection of *H. hepaticus* in clinical sample. This is particular valuable as it can be used as a non-invasive method for detecting *H. hepaticus* infection and results could be obtained quickly with high specificity. Results of the present study suggest that there is increased risk of gallbladder carcinogenesis in *H hepaticus* infection.

Conflict of Interests

The authors declare that there are no conflicts of interests

Authors' contributions

RD: Data collection, analysis and interpretation preparation of draft manuscript

VS: Data collection, analysis and preparation of manuscript

GN: Concept and design, interpretation of data and preparation of manuscript

MS: Editing of the manuscript, concept and design

MP: Concept, design, interpretation of data and editing of the final manuscript

Ethical considerations

The study was approved by the Institute Ethics committee; Informed consent was obtained from all participants.

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