Original Article

Association of carcinoma of the gallbladder with typhoid carriage in a typhoid endemic area using nested PCR

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Abstract

Background: Although well studied the association between chronic typhoid carrier state and carcinoma of the gallbladder (CaGB) remains unproven.

Methodology: The study was performed at a tertiary care medical center in North India and involved 52 patients with CaGB, 223 patients with benign gallbladder diseases, 508 healthy individuals and, 424 corpses. For the detection of *Salmonella enterica* serovar Typhi, hepatobiliary specimens were subjected to DNA extraction for specific nested- PCR amplification of the *S.* Typhi flagellin gene. Anti-Vi *S.* Typhi antibodies were detected in serum samples from patients by indirect haemagglutination.

Results: Thirty five of the 52 (67.3%) CaGB patients were PCR-positive for the *S.* Typhi flagellin gene; significantly higher than for patients with benign gallbladder diseases (95/223, 42.6%; $p<0.01$) and corpses (35/424, 8.2%; $p<0.001$). The numbers of individuals that had significant anti-Vi antibody titres (≥160) in their serum were 20/52 (38.5%) for CaGB patients, 31/223 (13.9%) for patients with benign gallbladder diseases, and 47/508 (9.2%) for healthy individuals.

Conclusions: Specific nested-PCR amplification of the *S.* Typhi flagellin gene in hepato-biliary specimens was more sensitive for detection of *S.* Typhi carriage than anti-Vi antibody titres in serum. The results demonstrate an association between typhoid carriage and gallbladder diseases, both CaGB and benign. *S.* Typhi specific immunosuppression is also suggested in patients with gallbladder diseases.

Key Words: *S.* Typhi, chronic typhoid carriers, Ca GB, nested PCR

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Introduction

Gallbladder carcinoma affects women two to six times more commonly than men and its incidence steadily increases with age [1]. There is a marked regional and ethnic variation in the incidence of gallbladder cancer. While it accounts for only 0.5% of all gastrointestinal malignancies in the United States and Europe, it is very common in Chile, Mexico, Peru and North India. The highest gallbladder cancer incidence rates worldwide were reported for women in North India (21.5/100,000), South Pakistan (13.8/ 100,000) and Quito, Ecuador (12.9/100,000) [2]. Carcinoma of the gallbladder (Ca GB) is the most common malignancy of the biliary tract. It is the third most common gastrointestinal malignancy in the Eastern part of North India [3].

There are several risk factors for gallbladder cancer. These are cholelithiasis, obesity, reproductive factors, environmental exposure to certain chemicals, congenital developmental abnormalities of the pancreaticobiliary junction and chronic infections of the gallbladder. There is also less studied genetic factor/s [1, 4].

A link has specifically been proposed between chronic infection of the gallbladder and *Salmonella enterica* subspecies *enterica* serotype Typhi (*S.* Typhi hence forth) [5-10]. A chronic biliary carrier state of *S.* Typhi with a focus usually in the gallbladder is considered far more common than a focus in any other site. Chronic microbial infection of an organ has often been observed with carcinogenesis of the same or related part of the body [11-13]. Typhoid fever remains one of the leading causes of morbidity and mortality in most

of the developing countries with an incidence rate of >100/100,000 population in most South East Asian countries including India [14]. It has been suggested that only 20% of all those infected by *S.* Typhi would develop classical disease whereas 80% remain asymptomatic [15, 16]. In India, approximately 15 million people acquire clinical/sub clinical infection each year and about 3 %(450,000) of them might be added to the pool of chronic carriers. We have recently reported a definite association between chronic typhoid carriers and Ca GB based either on culture isolation or serological methods [9, 10]. While serological testing has its limits and drawbacks, the culture isolation rate of *S.* Typhi from the gall bladder specimens has also been found to be very low [9]. Therefore, underreporting of association between typhoid carrier states with Ca GB can not be ruled out. This shortcoming can easily be overcome by using a highly sensitive and specific nested PCR method for the clinical specimen that would be capable of detecting the L forms, other non-culturable forms, and also low numbers of bacteria [17]. Therefore, the present study was designed to explore the association of the chronic typhoid carrier state and Ca GB by detecting *S.* Typhi in specimens of hepatobiliary origin from patients with malignant and benign gallbladder diseases using a flagellin gene specific nested protocol.

Materials and Methods

A total of 52 patients with carcinoma of the gallbladder, 223 with other gallbladder diseases, and 508 healthy adults were included in the present study conducted from July 2004 through June 2007. The study subjects were patients of the University Hospital of Banaras Hindu University, Varanasi, a tertiary care medical center located in the Eastern part of North India. The diagnosis of gallbladder pathology was made by ultrasonography, aspiration cytology, and/or by histopathology. Patients as well as healthy individuals with a history suggestive of enteric fever during the previous one year were excluded from the study. Further, a total of 424 corpses not having any visible gall bladder pathology were also included. The study design was approved by the Ethical Committee of the university.

Collection of specimen

a) Bile, stone and gallbladder tissue: Bile was aspirated by needle puncture either during laparotomy or via percutaneous route under ultrasound guidance. Gallbladder stones and gallbladder tissues were collected peroperatively. One or more of these specimens were collected from 275 patients suffering from gallbladder ailments based on the feasibility.

b) Liver tissue, bile, gallbladder tissue and bone marrow specimens were collected from a total of 424 dead bodies brought within 12 hours of their deaths to the Postgraduate Department of Forensic Medicine of the University Hospital.

c) Blood: Five ml blood was collected in a sterile tube by venipuncture and allowed to clot at room temperature for 30 minutes. Blood was collected from 275 patients (52 with Ca GB and 223 with benign gallbladder diseases) with gallbladder diseases and 508 healthy individuals (usually voluntary donors visiting the University Blood Bank) with no history of typhoid fever in the preceding one year. Serum was separated within 2 hours of collection.

The specimens were preserved at -20°C until used.

Processing of the specimen

a) Culture studies: Bile was inoculated directly onto blood agar, McConkey agar (MA), Desoxycholate citrate agar (DCA) and Selenite F broth (for enrichment). Inoculated media was incubated overnight at 37°C aerobically. Subcultures were made from Selenite-F broth on solid plates (DCA and MA). The bacterial growth was identified by using standard recommended techniques [18]. The gallstone and gallbladder tissue specimens were first homogenized by a sterile plastic microfuge adopted pestle, then processed in the same way as bile.

b) Serological studies: For detection of typhoid carriers, antibody titer against somatic (TO) and flagellar (TH) were determined by the Widal test using a colored antigen kit (Span Diagnostics, Surat, India). Antibodies against Vi antigen (ViAb) were measured following the method of Barrett using the Indirect Hemagglutination assay [19]. A titer $\geq$1:160 was considered as significant to diagnose typhoid carriers.

iii) Detection and sequencing of flagellin gene specific nucleotide sequences of *S.* Typhi.

DNA extraction from all 4 types of samples was done by using the phenol-chloroform method. Nested PCR was performed as described by Song *et al*. [20] and was modified according to Frankel [21].

Following were the primers:

For first round PCR to amplify a 495 bp fragment

ST1 5'-TAT GCC GCT ACA TAT GAT GAG-3' and
ST2 5'-TTA ACG CAG TAA AGA GAG-3'

For nested PCR to amplify a 364 bp fragment

ST3 5'-ACT CGT AAA ACC ACT ACT-3' and
ST4 5'-TGG AGA CTT CGG TCG CGT AG-3'

Randomly selected amplicons from 10 different specimens were sent for sequencing to Bangalore Genei, India. Sequences were analyzed using BLAST N (http://www.ncbi.nlm.nih.gov/BLAST/) to verify identity of the sequences as *S.* Typhi.

Statistical methods

Statistical analysis was conducted to examine the association between study variables using the chi square test. SPSS for Windows version 10.0 was used to calculate the chi square test. Odds-ratio, 95% confidence interval of odds ratio and its statistical significance were also determined.

Results

a) Culture studies

Overall isolation rate of *S.* Typhi from specimen derived from gallbladder was 1.4% (4/275). It could be isolated in 3.8% (2/52) and 0.9% (2/223) of the patients with malignant cancer and other diseases of the gallbladder respectively. The difference in the above isolation rates was statistically not significant ($p > 0.05$). *S.* Typhi could not be isolated from any of the 424 gallbladder tissues and liver tissue and 120 bile specimens from corpses.

b) Serological studies

Overall 38.4% (20/52) of the patients of Ca GB were positive for ViAb at a significant titer, i.e. ≥1:160 by IHA and was significantly higher (< 0.001) as compared to patients with benign GB diseases (13.9%, 31/223) and healthy controls (9.2%, 47/508) (Table 1). When the odds ratio (OR) was calculated taking the healthy group as reference, it was 6.13 for patients with Ca GB and 1.58 for patients with benign GB diseases.

Table 1. Detection of Vi antibody (ViAb) and flagellin gene specific sequences of *Salmonella enterica* serotype Typhi in different study groups.

	Age ranges in years and sex			
	1-30			
	Male		Female	
	ViAb	**PCR**	ViAb	**PCR**
Carcinoma of gallbladder	0/0 (0)	**0/0 (0)**	0/3 (0)	**2/3 (66.7)**
Benign gallbladder diseases	0/8 (0)	**1/8 (12.5)**	8/38 (21.1)	**22/38 (57.9)**
Healthy population	0/157 (0)	**ND**	15/147 (10.2)	**ND**
Corpses without gall bladder diseases	ND	**3/103 (3.9)**	ND	**2/51 (10.2)**
	31-60			
	Male		Female	
	ViAb	**PCR**	ViAb	**PCR**
Carcinoma of gallbladder	5/11 [a] (45.4)	**10/11† (90.0)**	12/30[a] (40)	**20/30 § (66.7)**
Benign gallbladder diseases	5/34[b] (14.7)	**15/34†† (44.1)**	18/120[b] (15)	**46/120‖ (38.3)**
Healthy population	11/107[c] (10.3)	**ND**	21/87 (24.1)	**ND**
Corpses without gall bladder diseases	ND	**16/158‡ (10.1)**	ND	**13/62‡‡ (20.9)**
	61-80			
	Male		Female	
	ViAb	**PCR**	ViAb	**PCR**
Carcinoma of gallbladder	0/2 (0)	**0/2 (0)**	3/6 (50)	**3/6 (50)**
Benign gallbladder diseases	0/9 (0)	**3/9 (33.3)**	0/14 (0)	**8/14 (57.1)**
Healthy population	0/5 (0)	**ND**	0/5 (0)	**ND**
Corpses without gall bladder diseases	ND	**1/41 (2.4)**	ND	**0/9 (0)**
	Total			
	ViAb		PCR	
Carcinoma of gallbladder	20/52 (38.5)		**35/52*** **(67.3)**	
Benign gallbladder diseases	31/223 (13.9)		**95/223**** **(42.8)**	
Healthy population	47/508 (9.2)		**ND**	
Corpses without gall bladder diseases	ND		**35/424***** **(8.2)**	

Figures in rows show: number positive/ total number and positive percentages in parenthesis. For ViAb detection: a Versus b: $p<0.001$; a Versus c: $p<0.001$. For PCR based detection: * vs ** $p<0.001$ (OR = 2.37), * vs *** $p<0.001$ (OR = 22.9), ** vs *** $p<0.001$ (OR = 2.3), § vs ‖ $p<0.01$ (OR = 3.2), § vs ‡‡ $p<0.001$ (OR = 7.5), ‖ vs‡‡ $p<0.05$ (OR = 2.3).

Males of age group 31-60 y were found positive for significant ViAb titer in 45.4% (5/11), 14.7% (5/34) and 10.2% (11/107) in patients with Ca GB (OR: 7.8), patients with benign GB

diseases (OR=1.47) and healthy controls respectively. In females of the above age group, prevalence of ViAb at titer ≥1:160 was found to be 40% (12/30) in patients with Ca GB, 15 % (18/120) in patients with benign GB diseases and 24.1% (21/87) in the healthy control group with OR 2.1 and 0.55 respectively. However, 50% (3/6) females of Ca GB group belonging to age group 61-80 y were found positive for the significant titer of ViAb.

Twelve of the 52 Ca GB (23.1%) patients were observed having titer ≥1:160 against TO which was significantly higher ($p < 0.05$) than those of patients belonging to benign GB diseases (11.7%, 26/223) and healthy controls (13.4%, 68/508). Eleven of the 52 Ca GB (21.1%) cases, 8.1% (8/223) of benign gallbladder diseases, and 10.2% (60/508) of the healthy adults were found to have ≥1:160 antibody titer against TH.

c) PCR studies

Table 1 shows that 35 of the 52 Ca GB (67.3%) were found to be positive by nested PCR for the flagellin gene specific sequences of *S.* Typhi, which was significantly higher in comparison to the rates in patients with benign GB diseases (42.6%, 95/223) and corpses without GB diseases (8.2%, 35/224) with p values of <0.01 and <0.001 respectively. Patients with benign GB diseases also had significantly higher ($p<0.001$) detection rate when compared with corpses. When the corpses group was taken as reference, the odds ratio for Ca GB and patients with benign GB diseases groups were 22.8 and 1.58 respectively. Female corpses of the age group 31-60 years had significantly higher (<0.001) positivity rate (20.9%, 13/62) as compared to male corpses (16/158, 10.1%) of the same age range.

Since most of the cases with gallbladder diseases were observed to be in the age group of 31-60 years, the gender based comparative analysis showed that the positivity rates by nested PCR in males of Ca GB, benign GB diseases, and corpses without GB diseases were 90.0% (10/11), 44.1% (15/34) and 10.1% (16/158) respectively, while in females the rates were 66.7% (20/30), 38.3% (46/120) and 20.9% (13/62) for the corresponding study groups as with the males. In this age group, the PCR positivity in males with Ca GB was observed to be significantly higher than that observed in males with benign GB diseases ($p<0.01$) and corpses ($p<0.001$, OR: 88.75). The difference in detection rates in males with benign GB diseases and corpses was also significant ($p<0.001$, OR: 7.01). The females aged 31- 60 years of the Ca GB group also had a significantly higher ($p<0.01$) detection rate as compared to corpses of the corresponding age group (odds ratio: 7.54). Females with benign GB diseases had a significantly higher detection rate for *S.* Typhi by PCR when compared with female corpses of this age range ($P<0.05$; OR: 2.34). Significantly ($p<0.001$) higher rate of PCR detection was seen in Ca GB patients not having stones in their gallbladder (85.15%, 23/27) as compared to those who had stones (48%, 12/25). A similar pattern was seen in patients with benign GB diseases where the significantly higher ($p<0.001$) detection was seen in patients without stones (83.3%, 53/60) than with stones (26.7%, 42/157).

Discussion

This is the first report of its kind where nested PCR has been used to detect flagellin gene of *S.* Typhi in hepatobiliary specimens along with detection of ViAb in the serum samples of diseases as well as a healthy population. Previous studies which were based either on culture isolation or serology have provided some clues for association between the chronic typhoid carrier state and Ca GB. In the present study, we observed that about 67.3% of the Ca GB patients were positive for the gene sequence in contrast to 8.2% in a healthy population not matched for age and sex. Thus the association of the bacterium was 22 times higher in Ca GB patients. Our previous report [9, 10] and many other retrospective and prospective studies [5-8] have shown a positive association between the chronic typhoid carrier state and Ca GB, but at a much lower magnitude. Age- and sex-matched analysis was conducted in the age group most affected by CA GB (31-60y) to check the reliability of the above findings. Since 90% of the males and 70% of the females of this age group were positive for *S.* Typhi carrier state, it may be suggested that this chronic infection may be the major etiological factor in carcinogenesis of the gall bladder in males. In females, however, 1/3rd of the Ca GB cases might be attributed to some other factor/s in this region. It was seen that males were 89 times

more associated with typhoid carriers as compared to their healthy counterparts. Females were found to be only 7 times more associated with typhoid carriers as compared to healthy women of the same age group. Apparently the lower proportion of chronic carrier state in females of Ca GB group may be explained on the basis of quite high (20.9%) *S.* Typhi specific PCR positivity in healthy females of this particular age group. It is interesting to note that only 10.1% of healthy males in the corresponding age group were found to be PCR positive. To determine why a healthy female of age group 31-60 years is more prone to a chronic carrier state requires further study.

The patients with benign gallbladder diseases also showed significantly higher ($p<0.001$) positivity for the flagellin gene of *S.* Typhi as compared to healthy controls. The males and females age 31-60 years belonging to the group of patients with benign gall bladder diseases also showed 7.5 and 2.3 times higher positivity respectively for the flagellin gene sequences. Seven times higher positivity in patients with cholecystitis and cholelithiasis indicates that the chronic typhoid carrier state might be one of the etiological factors for benign diseases of the gallbladder also. Since the PCR positivity rate for the *S.* Typhi gene was significantly higher in patients without stones irrespective of their malignant or nonmalignant status, gallstones may not be the predisposing factor for chronic typhoid carrier state.

Significant titers of ViAb ($\geq$1:160) were 24.1% in healthy females and 10.2% in healthy males. This shows host response against the pathogen. Interestingly, the PCR positivity (indicating direct presence of bacteria) rates in the specimens of hepatobiliary origin collected from the corpses of the same age group were statistically similar to those of live healthy subjects, i.e. 20.9% in females and 10.1% in males. But lower rates of detection of ViAb at significant titer in Ca GB (38.4%) and in the benign gallbladder disease group (13.9%) as compared to PCR positivity in the respective groups (Ca GB, 67.3% and in the benign gall bladder disease group, 42.6%) suggest *S.* Typhi specific immunosuppression in these subjects which warrants further study.

Extremely low isolation rate of *S.* Typhi from gallbladder (1.4%, 4/275) specimens does not support the hypothesis that chronic typhoid carrier state has a role in biliary tract carcinogenesis. Low isolation can be attributed to the bacteria's nonculturable state including L form or its facultatative intracellular location in some remote organ (biliary canaliculi of liver, bone marrow, spleen etc.). Low isolation can also be due to intermittent excretion through the gall bladder or insufficiency of the techniques employed to isolate it. Detection of *S.* Typhi by molecular method in liver, bone marrow, spleen etc. will overcome the non-culturability of bacteria and difficulty with culture techniques. Molecular method will help ascertain the probable nidus of *S.* Typhi in gallbladder.

Immunohistochemical localization would provide evidence of the location where *S.* Typhi resides.

Various carcinogens produced by *S.* Typhi and other bacteria have been suggested viz.: bacterial glucoronidase yielding some high energy intermediates after acting on bile which are potential mutagens [22]; bacterial products acting upon primary bile acids and producing high concentration carcinogenic secondary bile acids [23]; production of nitroso compounds from nitrates by the action of bacterial enzymes [24]; and chronic bacterial infection leading to obstruction and producing persistent chemical and mechanical injuries [25]. Therefore, on the basis of such high magnitude of typhoid carrier state in patients with gall bladder diseases, it may be proposed that this chronic infection may be one of the very important etiological factors in the genesis of gall bladder malignancy. It may also cause cholelithiasis, cholecystitis essentially in typhoid endemic areas.

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