Section: Miscellaneous

Original Research Article

ROLE OF SALMONELLA TYPHI IN ETIOLOGY OF CARCINOMA GALLBLADDER

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ABSTRACT

Background: Several studies in literature point to a possible etiological role of Salmonella typhi in pathogenesis of carcinoma gallbladder (Ca GB). By reviewing the literature, it is evident that by eradicating the microbe we can reduce the incidence of microbe related cancers in endemic areas.

Aims and objectives: To study the role of Salmonella typhi in etiology of Ca GB by molecular, serology and culture methods and to detect the presence of DNA of Salmonella typhi using Flagellin gene in cells of gallbladder cancer and chronic cholecystitis (CC) by using DNA-PCR.

Material and Methods: 74 samples of biopsy tissues of Ca GB and CC were taken from an army hospital. These samples were processed for DNA extraction and real time PCR based on SYBR green chemistry done. Widal's test and stool culture for S. typhi was also performed for all patients. SPSS statistical software was used for analysis.

Results: In Ca GB tissues by PCR, only four cases were positive out of thirty-two patients (12.5 %) compared to two cases out of forty-two (4.7 %) patients of CC (chi square = 0.393). As for Widal's titre they were positive in only eight out of twenty-six (30.7%) cases in Ca GB patients compared to four out of twenty-eight (14.2%) patients of CC (chi square = 0.303). In Stool culture no case was positive for Salmonella typhi.

Conclusion: We found that there is low prevalence of S. typhi gene and antibodies in our patients who are all from defence background and vaccinated against S. typhi. A policy of eradication of microbes by antibiotic therapy and vaccination, may lead to reduction in the incidence of bacteria-induced cancers in areas where S. typhi is endemic.

Key Words: Carcinoma gallbladder, chronic cholecystitis, flagellin gene, Salmonella typhi, vaccination.

INTRODUCTION

Carcinoma gallbladder (Ca GB) is a common malignancy of the biliary tract. It has high incidence in certain world populations.[1] It is eight times more common in north India than in south India.[2] After geographic mapping of Ca GB patients at Tata Memorial Hospital, Mumbai (1990-95), Jagannath and colleagues,[3] found maximum incidence in Uttar Pradesh, Bihar, West Bengal and Assam. It is the third most common gastrointestinal malignancy in the Eastern part of North India.[4]

Several etiologies have been postulated for Ca GB which include cholelithiasis,[5] typhoid carrier state, [6-7] obesity,[8] genetic predisposition,[9] estrogens,[10] chemical carcinogens, [11-12] and diet.

Aims & Objective

- To study the role of Salmonella typhi in etiology of carcinoma gallbladder by molecular, serology and culture method.
- To detect the presence of DNA of Salmonella typhi using Flagellin gene in cells of gallbladder cancer and chronic cholecystitis with the help of DNA-PCR.

MATERIALS AND METHODS

The study was conducted in Command Hospital, Lucknow over a period of 3 years. Ethical clearance was taken for the study. Total 74 samples of biopsy tissues of Ca GB and CC were taken. These samples were processed for

1. DNA extraction and real time PCR
2. Widal's Test
3. Stool Culture

1. **DNA extraction and real time PCR based on SYBR green chemistry**

DNA (S. typhi) extraction from tissues

- Formalin embedded tissue of 25 mg was taken in 1.5 ml microcentrifuge tube containing 80 µl Phosphate buffer saline. It was vortexed briefly and homogenized using tissue rupture. Then 100-µl lysis buffer was added and the sample is grinded in presence of liquid nitrogen.
- With the help of Proteinase K, RNAase A, ethanol and QIA amp mini spin column, DNA were extracted
- The eluted sample was stored at -20ºC and used as template for PCR.

PCR set up

The primers for PCR amplification of samples are designed from the Flagellin gene of *Salmonella typhi*murium genome. The primers used are as following

Forward primer – a 30-bp forward primer

5'-CGGAACGTTATTTGCGCCATGCTGAGGTAG-3'

Reverse primer – a 27-bp reverse primer

5'-GCATGGATCCCCGCCGGCGAGATTGTG-3'

Master Mix preparation – PCR reaction mix was set up with designed primers, power SYBR green master mix (ABI), nuclease free water and template.

PCR components	Concentration
2X power SYBR Green Master Mix (ABI)	12.5 µL
Forward primer (10 picomol conc.)	1.0 µL
Reverse primer (10 picomol conc.)	1.0 µL
Template	8.0 µL
Nuclease free water	2.5 µL

PCR reaction was set up with the following PCR program

Step	Particulars	Temperature	Time	
Step 1	Holding	95°C	10 minutes	
Step 2	Denaturation	95°C	15 seconds	Repeat Step 2 to 4 for 40 cycles
Step 3	Annealing data capture	60°C	20 seconds	
Step 4	Extension	72°C	15 seconds	

Along with the samples, a positive control and a negative control are also analysed by PCR.

PCR was performed for 74 samples in batches. Each batch included five (labelled), one positive control and one negative control.

2. **Widal's test**

- Patient's serum was doubly diluted by mixing and transferring from 1:10 to 1:640 in three rows. Felix tube for somatic O antigen and Dreyer's tubes for H antigen were used.
- Reading the results – The control tubes were examined first, where they had no agglutination. Agglutination of `O` antigens appeared as "matt" or "carpet" at the bottom. Agglutination of `H` antigens appeared as loose, woolly or cottony. The highest dilution of serum that produces a positive agglutination was taken as titre. The titres for all the antigens were noted. The Widal's test is positive if TO antigen titre was equal to or more than 1:160 in an active infection, or if TH antigen titre was equal to or more than 1:160 in past infection or in immunized persons.

3. Stool culture:

Stool cultures were done using standard culture practice of lab by using MacConkey agar and Xylose Lysine Deoxycholate agar.

Statistical Analysis

SPSS statistical software was used for analysis. The Chi square test was applied for contingency tables and proportions. A p value of ≤0.05 was taken as significant.

RESULTS

1. PCR Results

In our study only six out of seventy-four samples were found to be positive for Salmonella typhi. All positive controls were picked up in real time PCR and negative controls were along with base line in different PCR profiles. In Ca GB tissues only four cases were positive out of thirty-two patients (12.5 %) compared to two cases out of forty-two (4.7 %) patients of CC (chi square = 0.393). The PCR results of Ca GB and CC is tabulated in Table 1. [Table 1]

2. Widal's and Stool culture results\

As for Widal's titre they were positive in only eight out of twenty-six (30.7%) cases in Ca GB patients compared to four out of twenty-eight (14.2%) patients of CC (chi square = 0.303). In Stool culture of both carcinoma gallbladder and chronic cholecystitis patients, none was positive for Salmonella typhi. Results of detection of Salmonella typhi by Widal's test and stool culture in gallbladder cancer and chronic cholecystitis patients are given in Table 2 and Table 3.

The positive PCR profiles generated from machine have been shown as Figure 1.

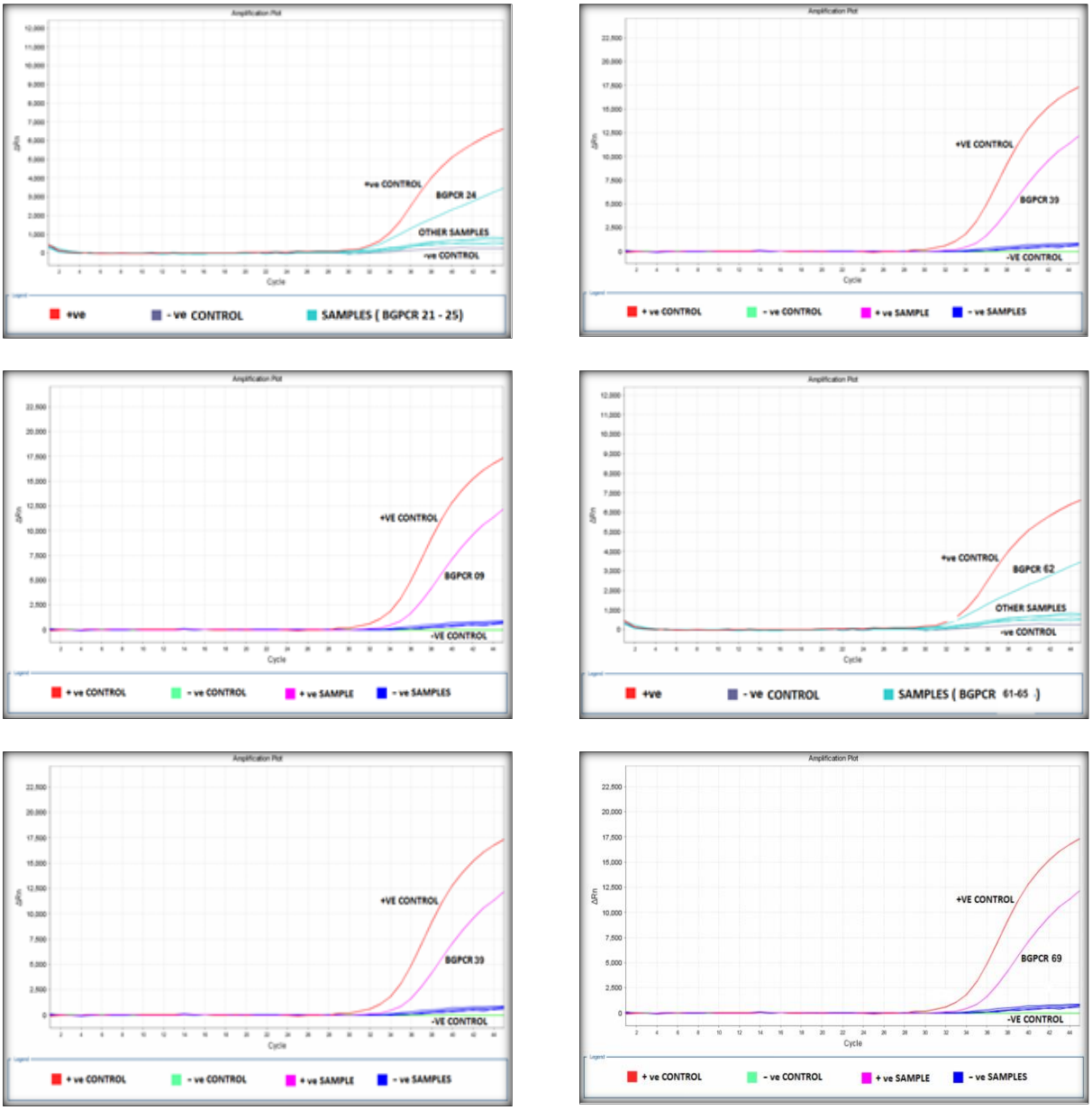

Figure 1: Positive PCR profiles

Table 1: PCR results for Salmonella typhi in carcinoma gallbladder (Ca GB) and chronic cholecystitis (CC)

No.	AGE	SEX	DIAGNOSIS	RESULT
PCR01	50	F	Ca GB	Negative
PCR02	65	F	Ca GB	Negative
PCR03	72	F	Ca GB	**Positive**
PCR04	61	F	Ca GB	Negative
PCR05	65	F	Ca GB	Negative
PCR06	56	M	Ca GB	Negative
PCR07	39	F	Ca GB	Negative
PCR08	29	F	Ca GB	Negative
PCR09	55	M	Ca GB	**Positive**
PCR10	70	M	Ca GB	Negative
PCR11	50	F	CC	Negative
PCR12	50	F	CC	Negative
PCR13	42	F	CC	Negative
PCR14	70	F	CC	Negative
PCR15	65	M	CC	Negative
PCR16	72	F	Ca GB	Negative
PCR17	68	M	Ca GB	Negative
PCR18	68	F	Ca GB	Negative
PCR19	55	F	Ca GB	Negative

PCR20	65	F	Ca GB	Negative
PCR21	47	F	Ca GB	Negative
PCR22	70	M	CC	Negative
PCR23	54	F	CC	Negative
PCR24	60	F	CC	**Positive**
PCR25	71	M	CC	Negative
PCR26	55	F	CC	Negative
PCR27	42	F	CC	Negative
PCR28	50	F	CC	Negative
PCR29	52	F	CC	Negative
PCR30	60	F	CC	Negative
PCR31	50	F	CC	Negative
PCR32	45	F	CC	Negative
PCR33	62	F	CC	Negative
PCR34	52	M	CC	Negative
PCR35	52	F	CC	Negative
PCR36	46	F	CC	Negative
PCR37	50	F	CC	Negative
PCR38	81	F	CC	Negative
PCR39	69	F	CC	**Positive**
PCR40	70	F	CC	Negative
PCR41	58	M	CC	Negative
PCR42	55	F	CC	Negative
PCR43	52	F	CC	Negative
PCR44	48	M	CC	Negative
PCR45	65	F	CC	Negative
PCR46	62	F	CC	Negative
PCR47	52	F	CC	Negative
PCR48	50	F	CC	Negative
PCR49	70	F	CC	Negative
PCR50	66	F	CC	Negative
PCR51	45	F	CC	Negative
PCR52	55	F	CC	Negative
PCR53	48	F	CC	Negative
PCR54	45	M	CC	Negative
PCR55	50	F	CC	Negative
PCR56	52	F	CC	Negative
PCR57	42	F	CC	Negative
PCR58	40	F	CC	Negative
PCR59	70	F	Ca GB	Negative
PCR60	65	F	Ca GB	Negative
PCR61	60	F	Ca GB	Negative
PCR62	50	F	Ca GB	**Positive**
PCR63	38	F	Ca GB	Negative
PCR64	75	F	Ca GB	Negative
PCR65	82	M	Ca GB	Negative
PCR66	55	F	Ca GB	Negative
PCR67	54	F	Ca GB	Negative
PCR68	82	M	Ca GB	Negative
PCR69	48	F	Ca GB	**Positive**
PCR70	65	F	Ca GB	Negative
PCR71	60	F	Ca GB	Negative
PCR72	58	F	Ca GB	Negative
PCR73	63	F	Ca GB	Negative
PCR74	72	F	Ca GB	Negative

Table 2: Widal's titre and Stool culture results in patients of Carcinoma Gallbladder (Ca GB)

No.	Age (Yrs.)	Sex	Diagnosis	Widal's titre TO	Widal's titre TH	Stool Culture
1.	23	F	Ca GB	N	N	Neg.
2.	65	F	Ca GB	1/80	1/320	Neg.
3.	53	F	Ca GB	1/160	1/80	Neg.
4.	55	M	Ca GB	N	N	Neg.
5.	50	F	Ca GB	1/160	1/160	Neg.
6.	53	F	Ca GB	N	N	Neg.
7.	65	F	Ca GB	N	N	Neg.
8.	58	F	Ca GB	N	N	Neg.
9.	52	M	Ca GB	1/80	1/160	Neg.
10.	65	M	Ca GB	N	N	Neg.
11.	45	F	Ca GB	N	N	Neg.
12.	46	F	Ca GB	N	N	Neg.
13.	65	M	Ca GB	N	N	Neg.

14.	62	F	Ca GB	1/320	1/160	Neg.
15.	45	F	Ca GB	N	N	Neg.
16.	55	F	Ca GB	N	N	Neg.
17.	62	F	Ca GB	1/160	1/80	Neg.
18.	80	M	Ca GB	N	N	Neg.
19.	72	F	Ca GB	N	N	Neg.
20.	70	F	Ca GB	N	N	Neg.
21.	62	F	Ca GB	N	N	Neg.
22.	56	F	Ca GB	1/160	1/160	Neg.
23.	50	F	Ca GB	N	N	Neg.
24.	48	F	Ca GB	1/80	1/160	Neg.
25.	76	F	Ca GB	N	N	Neg.
26.	70	M	Ca GB	N	N	Neg.

Table 3: Widal's titre and Stool culture results in patients of chronic cholecystitis

No.	Age	Sex	Chronic cholecystitis (CC)	Widal's titre (TO)	Widal's titre (TH)	Stool Culture
1.	27	F	CC	N	N	Neg.
2.	65	F	CC	N	N	Neg.
3.	32	F	CC	N	N	Neg.
4.	38	F	CC	N	N	Neg.
5.	60	F	CC	N	N	Neg.
6.	32	F	CC	N	N	Neg.
7.	50	F	CC	N	N	Neg.
8.	73	F	CC	N	N	Neg.
9.	50	F	CC	N	N	Neg.
10.	68	F	CC	N	N	Neg.
11.	65	F	CC	1/160	1/160	Neg.
12.	27	F	CC	N	N	Neg.
13.	65	F	CC	1/160	1/80	Neg.
14.	32	F	CC	N	N	Neg.
15.	40	M	CC	N	N	Neg.
16.	42	F	CC	N	N	Neg.
17.	50	F	CC	N	N	Neg.
18.	68	F	CC	N	N	Neg.
19.	65	F	CC	1/160	1/80	Neg.
20.	35	F	CC	N	N	Neg.
21.	52	F	CC	N	N	Neg.
22.	50	M	CC	N	N	Neg.
23.	65	F	CC	N	N	Neg.
24.	35	F	CC	1/320	1/160	Neg.
25.	40	M	CC	N	N	Neg.
26.	48	F	CC	N	N	Neg.
27.	38	F	CC	N	N	Neg.
28.	42	F	CC	N	N	Neg.

DISCUSSION

Ca GB is a common gastrointestinal malignancy seen in North India. It has a high prevalence mainly along the Indo Gangetic plains of UP and Bihar. It is estimated that over 18% of malignancies worldwide can be attributed to infections or about 1.2 million cases per year. According to Pisani et al,[14] infections involving viruses, bacteria and schistosomes have been linked to higher risks of malignancy.

The interplay of genetic susceptibility, life style factors and infections of the hepatobiliary system in carcinogenesis of the gallbladder is poorly understood; however, a link has been specifically proposed between chronic bacterial infections of the biliary tree with Salmonella typhi and Ca GB. An association of chronic typhoid carriage and Ca GB was first reported by Axelrod et al.[15] Welton et al,[7] observed increased incidence of cancer of the hepatobiliary system in patients of typhoid carriers. In a case control study typhoid carriers registered by the New York City Health Department between 1922 and 1975 were carried out to test for an association between the typhoid carrier state and death due to hepatobiliary cancer. 471 carriers were matched with 942 controls. The results showed that in chronic typhoid carriers, death due to hepatobiliary cancer was six times more often than the matched controls and that this difference is significant (P<0·001).

Caygill et al,[16] studied cancer mortality in people infected during the Aberdeen typhoid outbreak in 1964. Their results suggested a lifetime risk of developing gallbladder cancer in 6% of carriers.

In another study Shukla VK et al,[17] from Northern India, using Vi serology, showed a 7.9 times increased risk for Ca GB in chronic typhoid carriers. In this study authors aimed at finding out the association of typhoid carrier state in patients with cholelithiasis, carcinoma of the gallbladder and controls using indirect haemagglutination assay measuring antibodies against highly purified S. typhi Vi polysaccharide antigen. A significantly

high Vi positivity was observed in patients with gallbladder carcinoma (29.4%) compared to controls (5%) (chi2 = 6.325, P < 0.004, OR = 7.19) and patients with cholelithiasis (10.7%) (chi2 = 5.066, P < 0.01, OR = 3.86). There is 8.47 times more risk of developing carcinoma of the gallbladder in culture positive typhoid carriers than the noncarriers. This study suggests the typhoid carrier state to be one of the possible mechanisms of gallbladder carcinogenesis.

Earlier also from North India, Nath G et al,[18] investigated if typhoid infections and carcinoma of the gallbladder are linked. In their study, a total of 1001 bile specimens collected from cases of Ca GB, cholelithiasis and individuals without biliary pathology and subjected to aerobic cultures that had been enriched for salmonella. Salmonella typhi and S. Para typhi-A were detected in a significantly higher (P < 0.05) number in patients with Ca GB as compared with cholelithiasis and control groups.

In another study based on serology, Dutta U et al,[19] from North India, identified the risk factors for Ca GB and among patients with gallstones with special reference to role of chronic Salmonella typhi carrier state and concluded that chronic typhoid carrier state was the most important risk factor (OR=14) among patients with Ca GB and gallstones.

Gopal Nath and colleagues,[20] reported the prevalence of chronic typhoid carriers in Ca GB patients using a very sensitive and specific nested PCR technique in hepatobiliary specimens to exclude the limitations of serology-based detection and of culture isolation. They showed that 67.3% of the Ca GB patients were typhoid carriers, as compared to 8.3% of the healthy population in the typhoid endemic area of North India (Odds ratio 22.8).

In another study conducted at BHU, Varanasi, by Mallika Tewari and colleague,[21] a total of 101 specimens of gallbladder were collected and divided into three groups - group A (N=25) gallbladder cancers, group B (N=45) benign gallbladder diseases, group C (N=31) control (from cadavers, not having any gallbladder pathology). It was found on PCR that 20 out of 25 (80 %) Ca GB, 8 out of 45 (17.7 %) in benign gallbladder disease and 2 out of 31 (6.4%) in healthy control were positive for Flagellin gene of S typhi. In Vi serology 8 out of 25 (32%) Ca GB, 5 out of 45 (11.1%) in benign gallbladder disease and nil out of 31 healthy controls were positive. On culturing 13 out of 25 (52%) Ca GB, 7 out of 45 (15.5%) benign gallbladder disease and nil out of 31 healthy controls were positive for Salmonella.

Thus, authors concluded that PCR is the most sensitive and specific method for detection of bacteria followed by culture and serology. It is also positive in a significant (80%) number of patients of Ca GB and thus emphasizing an association between S. Typhi and Ca GB.

Ratnakar Shukla and colleague,[22] performed modified Widal's test for antibodies against Salmonella typhi (Vi and O) and Salmonella Para typhi (AO and BO) antigens in patients with Ca GB (n=100), xanthogranulomatous cholecystitis (XGC, n=24), chronic cholecystitis (CC, n=200) and healthy controls (n=200). They concluded that Salmonella carrier state was more common in Ca GB and XGC.

Enea Gino Di Domenico and colleagues,[23] in 2017 reviewed the epidemiological studies performed in regions where S. Typhi was endemic and revealed that the majority of chronically infected carriers also harbour gallstones which is the primary predisposing factor for the onset of Ca GB. S. Typhi is kwon to produce typhoid toxin which has carcinogenic potential by inducing DNA damage and cell cycle alterations in intoxicated cells. S. Typhi is also kwon to produce biofilm which promote persistent infection in the gallbladder, thus sustaining a chronic local inflammatory response and exposing the epithelium to repeated damage caused by carcinogenic toxins.

Jill Koshiol et al,[24] in Chile, evaluated the association between Salmonella enterica serovar Typhi (S. Typhi) antibodies and Ca GB. They tested 39 Ca GB cases, 40 gallstone controls, and 39 population-based controls for S. Typhi Vi antibodies and performed culture and quantitative polymerase chain reaction for the subset with bile, gallstone, tissue, and stool samples available. They also conducted a meta-analysis of >1000 Ca GB cases by combining their results with previous studies. Their results were consistent with the meta-analysis.

Kayafat Yusuf and colleagues,[25] in 2023 performed a comprehensive review on PubMed, Embase, and Web of Science databases and highlighted the complex relationship between bacterial infections and the development of several cancer types. They found a strong link between Salmonella typhi infection and gallbladder cancer.

On reviewing the literature, we found that the percentage of Ca GB patients positive for S. typhi gene on PCR varies from 33 to 67.3%. Compared to reported studies from literature we in our study found only four cases out of thirty-two Ca GB patients (12.5%) positive for S. typhi flagellin gene vs 2 out of 42 (4.7%) cases positive in patients of CC. This is statistically not significant.

The percentage of Ca GB patients positive for S. typhi, Widal`s serology, varies in literature from 38 to 44.4%. We also in our study found that Widal`s titre were positive in 8 out of 26 (30.7%) cases in Ca GB patients compared to 4 out of 28 (14.2%) of patients of CC. This is statistically not different significantly.

Stool cultures were negative in both cases of Ca GB and CC patients in our study.

The reason as to why low prevalence of S. typhi gene and antibodies is seen in our patients could be due to the fact that carcinoma gallbladder has a multifactorial etiology. It is possible that in our patients who are all from defence back ground are less likely to acquire S. typhi infection due to the

vaccination policy against S. typhi carried out by armed forces. It is therefore likely that other causative factors like chemical carcinogens or diet may have played a role in the etiopathogenesis of Ca GB in our patients.

CONCLUSION

Given the evidence from literature, that S. typhi is a possible etiological factor for Ca GB by residing in gallbladder for longer duration and causing tumorigenesis by repeated damage and cell cycle alterations. It is evident from our study, the sample patients being from defence background and immunized against S. Typhi, had low prevalence of S. typhi DNA incorporated within the Ca GB and CC cells.

A policy of eradication of microbes by antibiotic therapy and immunological potentiation by active immunization, may lead to reduction in the incidence of bacteria-induced cancers in areas where S. typhi is endemic.

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