Role of *Salmonella typhi* as a risk factor for Gallbladder cancer

By

PRACHI RAVINDRA PHADKE

[HLTH09201304004]

Centre for Cancer Epidemiology

Tata Memorial Centre

Mumbai

A thesis submitted to the Board of Studies in Health Sciences

In partial fulfillment of requirements for the Degree of

DOCTOR OF PHILOSOPHY

of

Homi Bhabha National Institute

April 2019

Homi Bhabha National Institute

Recommendations of the Viva Voce Committee

As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by **Ms. Prachi Phadke** entitled "**Role of *Salmonella typhi* as a risk factor for gallbladder cancer**" and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

Chairman – **Dr. Rajendra Badwe** **Date:** 16/04/19

Guide – **Dr. Rajesh Dikshit** **Date:** 16/04/19

Examiner – **Dr. J.S. Thakur** **Date:** 16/04/19

Member 1- **Dr. Mahesh Goel** **Date:** 16/04/19

Member 2- **Dr. Vivek Bhat** **Date:** 16/04/19

Member 3- **Dr. Pankaj Chaturvedi** **Date:** 16/04/19

Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to HBNI.

I hereby certify that I have read this thesis prepared under my direction and recommend that it may be accepted as fulfilling the thesis requirement.

Date: 16/04/19

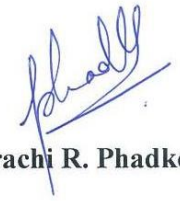
Place: Mumbai

Dr. Rajesh Dikshit

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at Homi Bhabha National Institute (HBNI) and is deposited in the Library to be made available to borrowers under rules of the HBNI.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the Competent Authority of HBNI when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.



Prachi R. Phadke

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

Prachi R. Phadke

List of Publications arising from the thesis

Journal

1. "Trends of Gallbladder Cancer Incidence in the High- and Low-Risk Regions of India", Prachi Ravindra Phadke, Sharayu Sitaram Mhatre, Atul Madhukar Budukh, Rajesh Prabhakar Dikshit, Indian J of Medical and pediatric oncology 2019; 40: 90-93.

Conferences:

1. Poster presentation on "Development of In-house ELISA for detection of Salmonella antibodies in human sera: Methodology and quality control"

   Conference: Scientific symposium frontiers in Epidemiology

   Date: 6th-7th March 2017

   Place: Tata Memorial Centre, Mumbai

2. Poster presentation on "An Ecological correlation between chronic infection of *Salmonella typhi* and Gallbladder Cancer in India"

   Conference: Indian Association of Cancer Research Convention

   Date: 23rd to 25th February 2018

   Place: Bose Institute, Kolkata

Prachi R. Phadke

CERTIFICATE

I certify that the thesis titled '**Role of *Salmonella typhi* as a risk factor for Gallbladder cancer**' submitted for the degree of Doctor of Philosophy by **Ms. Prachi R. Phadke** is a record of the research carried out by her during the period September 2013 to August 2018 under my supervision. This work has not formed the basis for the award of any degree, diploma, associateship or fellowship at this or any other institute or university.

Dr. Rajesh Dikshit

Acknowledgements

I would like to express gratitude to all of them who directly or indirectly contributed to successful completion of my Ph.D. work.

Firstly of all I pay sincere reverence to my mentor Professor Dr. Rajesh Dikshit. His immense knowledge, encouragement, constant support and patience were the key motivators during my Ph.D. journey. His valuable suggestions during tough times and the freedom he gave to conduct experiments helped me to grow intellectually and develop my research skills throughout this journey. I could not have imagined having a better mentor than him for this Ph.D. study.

I am greatly honored to have eminent surgeons-clinicians as my doctoral committee members. I am thankful to Dr. Rajendra Badwe, Dr. Mahesh Goel, Dr.Vivek Bhat and Dr. Pankaj Chaturvedi for their insightful comments and encouragement which helped to widen my research perspective. I also wish to thank Dr. Atul Budukh for his guidance and encouragement from time to time.

I am grateful to Dr. Sharayu Mhatre, my senior, who has always guided me throughout the journey. I convey my special thanks to you.

I wish to express my gratitude towards Dr. Shousun Szu and Dr. Rajesh Gupta for their invaluable support for procurement of antigen and reference standard for the assay and expertise regarding working of the assay.

I am thankful to the social investigators Janhavi, Swati, Sakshi, Shraddha and Sheetal for their important contribution to this study. I thank Ankita for providing me with the quality

data. I am also grateful to Sheetal, Kishor and Nilesh for helping me with the lab experiments.

I would like to express my gratitude to Dr. Rajini Nagrani for her guidance and boosting my morale whenever required. Huge thanks to my batch-mates Devyani, Abhinendra, Dr. Saurabh, Dr. Kavita, Jignasa and Aanchal for all the fun times, get-togethers and other good times besides wholehearted support during difficult times. This journey has been wonderful having them by my side.

I would like to thank Tata Memorial Hospital (TMH) and Department of Atomic energy (DAE) for providing me Research fellowship during my tenure.

I acknowledge and appreciate the cooperation of all the patients and healthy controls for participating in the study.

Eventually, I am thankful to my Mother, Father and Sister for their invaluable love and encouragement.

Last but not the least, I am grateful to the Almighty god for good health, wellbeing and spiritual insight that were necessary to complete this research work.

With Gratitude,

Prachi R. Phadke

Table of contents

Synopsis

Homi Bhabha National Institute

SYNOPSIS OF Ph. D. THESIS

1. **Name of the Student:** Prachi R. Phadke
2. **Name of the Constituent Institution:** Centre for Cancer Epidemiology, TMC
3. **Enrolment No. :** HLTH09201304004
4. **Title of the Thesis:** Role of *Salmonella typhi* as a risk factor for Gall Bladder Cancer
5. **Board of Studies:** Health Sciences

Introduction:

Gallbladder cancer shows highest incidence in some parts of India [1]. Number of factors such as gallstones, female gender, obesity and chronic infection contribute to risk of development of gallbladder cancer [2][3]. *Salmonella typhi* infection is assumed to be endemic in India and it is estimated that 3 -5 % of patients become carriers and may asymptomatically carry *Salmonella typhi* in gall bladder [4]. Asymptomatic chronic carriers play a major role in spreading of the disease. The gall bladder is the site of bile storage and has an environment that is habitable exclusively by organisms that are resistant to bile's detergent-like properties. Few studies previously have reported the positive association of S.typhi infection with GBC. A prospective case-control study, with 37 GBC cases and 80 controls, the risk was found to be 14 (95%CI: 2-92) [5]. One study showed that by activating AKT and MAPK pathways during infection, Salmonella irreversibly transforms mice, gallbladder organoids, and MEFs with mutated TP53 and amplified c-MYC [6]. In a case control study from Chile, GBC cases found to be having 4 times more risk of developing gallbladder cancer than controls [7].

Gaps in Literature:

There is no nation-wide prevalence data available for *S.typhi* infection. Few studies which address the association of *S.typhi* infection and gallbladder cancer are underpowered and methodologically poor. Selection of a control population in most studies is not appropriate according to study design norms. Many methods which are used to detect *S. typhi* meant to interpret acute infection and not the chronic one. To address the association with cancer, it is required to look for the chronic infection.

Hypothesis:

Exposure to chronic infection of *Salmonella typhi* increase the risk of gall bladder cancer

Aim:

To study association of chronic infection of *S.typhi* with gall bladder cancer

Objectives:

1. To study association of *Salmonella typhi* infection with gall bladder cancer.
2. To standardize ELISA for detection of chronic infection of *Salmonella typhi*

Objective 2: To standardize ELISA for detection of chronic infection of *Salmonella typhi*

Vi Polysaccharide antigen, purified from Citrobacter freundii WR2011 was used in the assay. It is structurally identical to the Vi purified from *Salmonella typhi*. Anti Vi IgG (Human), which pooled and purified sera containing 33 µg/ml of anti Vi-IgG was used as reference standard. Vi Polysaccharide Antigen (Lyophilized, purified) and Reference standard Anti Vi IgG (Lyophilized, purified) were obtained from Centre for Biologics Evaluation and Research (CBER), US Food and Drug Administration (FDA) and in collaboration with National Institutes of Health (NIH-US). The assay was performed according to the protocol and instructions provided by National Institutes of Health [8]. Polystyrene, flat bottom medium binding 96 well plates (Nunc) were coated with 2 µg/ml of Vi PS antigen in coating buffer (100µl/well). After Incubation at 20-25^{o}C for 16-20 hours coated wells were washed 6 times followed by addition of blocking buffer. Plates were then incubated for 2 hrs at 20-25^{o}C. The reference standards and serum samples diluted in dilution buffer and added in the plates according to the plate layout.

This is then kept at 20-25^{o}C for 16-20 hours. Subsequently, the plates were washed and secondary antibody (Alkaline phosphatase labeled Anti-human IgG produced in Goat) was added at 1:2000 dilution. After incubation for 4 hrs at 37^{o}C, the plates were washed and substrate (1mg/ml of p-nitrophenyl phosphate disodium salt) was added. This is then incubated for 30 minutes at 20-25^{o}C. OD was measured at 405- 630 nm using sunrise reader. Data were processed by Magllen^{TM} Software using Four Parameter Logistic model.

This Vi PS ELISA was standardized based on following parameters

A. Determination of Optimal dilution of secondary antibody: Range of dilutions of the secondary antibody (1:1000 to 1:12800) was checked against the Reference standard.

B. Determination of Optimal Development time for end point signal: Reference standard was run 8 dilutions and the absorbance was read at various time intervals 10 minutes, 20 minutes, 25 minutes and 30 minutes.

C. Generation of reference Standard curve: In this assay, availability of reference standard and the exact concentration value of anti Vi IgG ensured the estimation of anti Vi IgG concentration in the serum sample based on the absorbance reading. Standard curve was based on 4- parameter logistic model. Magellen^{TM} software was used for the same [9]. Anti Vi IgG (Ref std) was run at 8 dilutions to generate standard curve with each assay plate.

D. Parallelism: Approximately 15% of samples were run at 8 serial dilutions (1:50 to 1:6400) to check the phenomenon of parallelism. We calculated % CV using Anti-IgG Concentrations at multiple dilutions by nullifying the dilution factor. Samples having % CV of < 30% were considered to be showing presence of parallelism.

E. Quality control: Each serum sample was run in duplicate. CV of < 15% was considered acceptable for Intra-assay QC. Approximately 5% samples were rechecked for reproducibility. CV of < 30% was considered acceptable for Inter-assay QC (Reproducibility).

F. Determination of cut-off for chronic *Salmonella typhi* infection:

According to the study [8], protective level of anti-Vi IgG is approximately 4.3 μg/ml. We selected the cut-off value for the assay to be 5 μg/ml.

Results and Discussion:

For secondary antibody, 1:1000 resulted in highest absorbance, followed by 1:2000. Absorbance showed sharp decrease after the dilution of 1:8000. Hence, it was inferred that 1:1000, 1:2000 and 1:4000 were the optimum secondary antibody dilutions for the assay. For this assay, 1:2000 was considered as working dilution for the secondary antibody. Optimum absorbance range for the standard curve 3.2 to 0.09, resulted at the time points of 25 minutes and 30 minutes. Hence, the end point development time for this assay was decided to be 30 minutes. Average OD obtained at 1:100 was 3.25. Average concentration at the same dilution was 322.46 ng/ml. Average OD obtained at highest dilution of 1:12800 was 0.11, concentration for the same was 0.67 ng/ml. Recovery was calculated against the known concentration of reference standard which is 33μg/ml. Recovery was in the range of 85%-113%. Exception was at 1:12800, which showed 69.04% of recovery. R^2 values of all the standard curves were in the range of 0.98 to 0.99, suggesting a very good fit. Total 169 samples were run at 8 serial dilutions to check the parallelism. 72 samples showed the % CV of < 30, which were said to be showing presence of parallelism as per the definition. In this assay approximately 42.6 % of the samples followed parallelism. There can be multiple factors in serum samples like auto-antibodies, anti-coagulants,

enzyme inhibitors etc. due to which some samples failed to generate linear signal. For reproducibility check, 55 samples out of 60 showed inter-assay CV of < 30%, showing approximately 90% reproducibility. 1:100, 1:200 and 1:400 serum dilutions gave comparable results, hence can be considered as optimum dilutions for this assay. Dilutions lower than 1:100 and higher than 1:400 caused inconsistent results with higher variability.

Objective 1: To study association of *Salmonella typhi* infection with gall bladder cancer.

This study is part of a hospital based case-control study evaluating risk factors of Gallbladder cancer, conducted in Centre for Cancer Epidemiology, Tata Memorial Centre-Mumbai, India. The study has been approved by TMH institutional review board. Written Informed Consent was obtained from all study participants before enrolling them in study.

Eligibility criteria for cases and Controls: Cases were males or females with histopathologically/cyopathologically confirmed primary Gall bladder cancer patients coming to TMH for diagnosis/treatment with date of diagnosis less than one year from the date of enrollment. Patients were eligible to be enrolled in the study only if their age is between 20 to 69 years and if they are resident of India for atleast one year. Controls were recruited from visitors with no history of cancer, accompanying patients of any cancer site to the TMH. Visitor controls aged 20-70 years were recruited from all Disease Management Groups (DMGs) of the hospital (excluding relatives of GBC cases).The proportion of controls from visitors from each DMG did not exceed 20%.

A pre-tested structured questionnaire along with blood samples were collected for all study participants. Each case and control was interviewed by trained interviewers. Questionnaire covered demographic and socioeconomic variables, gallstone history, reproductive history,

occupational history, tobacco and alcohol habits and diet. A 10 ml blood sample was collected from each study participant and fractionated into serum and red blood cells (RBC's). The Serum sample was stored immediately at -80^{0}C and then transferred to -196°C in liquid nitrogen cylinder for long-term storage. Samples were randomly selected from the pool of 1170 GBC cases and 2525 Controls. Cases and controls were then frequency matched on the basis of age, gender and area of residence.

Serum samples from 1188 study participants, including 612 GBC cases and 576 controls were analysed in the present study. Serum samples were analysed by standardized In-direct, In-house ELISA to detect anti-Vi IgG antibodies to ascertain chronic *Salmonella typhi* infection. Final analysis of association of chronic *Salmonella typhi* infection and gallbladder cancer was done on 1085 study participants, including 554 cases and 530 controls, due to exclusions on the basis of quality control parameters and assay failures.

Statistical Analysis: Odds ratio with 95% CI for gallbladder cancer with respect to chronic *S.typhi* infection was estimated using unconditional logistic regression. Unconditional logistic regression models were adjusted for potential confounders like age, gender, region of residence, education and gallstones. Quartile analysis with test for linear trend was done on the continuous variable of anti-Vi IgG in μg/ml. Joint effect of chronic *S.typhi* infection and gallstones was estimated by fitting interaction term in the unconditional logistic regression model. All analyses were performed using STATA version 15.0 [10].

Results and discussion: Risk was observed for gallbladder cancer for those having chronic infection of Salmonella typhi. Odds ratio with 95% CI for all participants was found out to be 1.91 (1.48-2.46) when adjusted for age, gender, residence and education. Odds ratio went down

to 1.73 (1.30-2.31) when adjusted for age, gender, residence, education and gallstone. Quartile analysis revealed a significant increase in risk with increase in anti-Vi IgG concentration. Highest quartile showed the OR of 2.65 (1.72- 4.08) after adjusting for age, gender, residence, education and gallstones.

Separate analysis for gangetic belt states (Uttarakhand, Uttar Pradesh, Bihar & West Bengal) was done. There was no difference observed in the risk. Analysis was done by stratifying the education categories (Illiterate, Literate, Less than 5 years of schooling, 5-8 years of schooling, high school and college graduate- above). Risk was found to be higher 2.92 (1.61-5.31) in highest education category 'college graduate-above'.

Joint effect of chronic *S.typhi* infection and gallstones was found to be more than additive but was not statistically significant.

Summary and Conclusion:

1. ELISA was optimized for detection of anti-Vi IgG antibodies in serum samples indicative of chronic infection with *Salmonella typhi*. Strict adherence to several factors like quality control parameters, freeze-thaw cycles and automation is essential for accurate results.
2. Ecological correlation of Incidence of GB cancer and chronic *S.typhi* infection was observed in north-east and north regions of India.
3. Chronic infection of *S.typhi* significantly increases the risk of developing gallbladder cancer. Significant increase in trend in quartile analysis strengthens this association.

References:

1. Globocan 2012: Estimated Cancer Incidence, mortality and prevalence worldwide by IARC (International agency for research on cancer)
   Available from: http://globocan.iarc.fr/
2. Misra S, Chaturvedi A, Misra NC, Sharma ID. Carcinoma of the gallbladder. Lancet Oncol.2003 Mar;4(3):167–176
3. Randi G, Franceschi S, La Vecchia C. Gallbladder cancer worldwide: Geographical distribution and risk factors. Int J Cancer.2006; 118(7):1591–1602.
4. Gunn J. et al. Salmonella chronic carriage: epidemiology, diagnosis and gallbladder persistence. Trends Microbiol. 2014 November ; 22(11): 648–655.
5. Nath et al. Association of carcinoma of the gallbladder with typhoid carriage in a typhoid endemic area using nested PCR. J Infect Developing Countries 2008; 2(4): 302-307.
6. Tiziana Scanu et al. Salmonella manipulation of host signaling pathways provokes cellular transformation associated with Gallbladder carcinoma. Cell Host & Microbe 17, 763–774.
7. Koshiol Jill et al. Salmonella enterica serovar typhi and gallbladder cancer: a case–control study and meta-analysis. Cancer Medicine 2016; 5(11):3310–3325.
8. Shousun Szu et al. A human IgG anti-Vi reference for Salmonella typhi with weight-based antibody units assigned. Vaccine 31 (2013) 1970– 1974.
9. Magllen Data Analysis Software. Available on: https://lifesciences.tecan.com/products/software/magellan_data_analysis_software
10. STATA statistical software version 15.0 Available on: https://www.stata.com/new-in-stata/

List of abbreviations

CIS	Carcinoma in situ
GBC	Gallbladder Cancer
CI5	Cancer Incidence in Five Continents
AAR	Age Adjusted Incidence Rate
NCRP	National Cancer Registry Program
PBCR	Population based cancer registry
APC	Annual Percent Change
OR	Odds Ratio
HR	Hazard Ratio
CI	Confidence Interval
NTS	Non typhoidal Salmonella
ELISA	Enzyme linked immune-sorbent assay
PBS	Phosphate buffer saline
BSA	Bovine serum albumin
Vi-PS	Vi- Polysaccharide
CV	Coefficient of variation
OD	Optical Density

List of Tables

List of Figures

Chapter 1
Introduction

1.1 Gallbladder Cancer

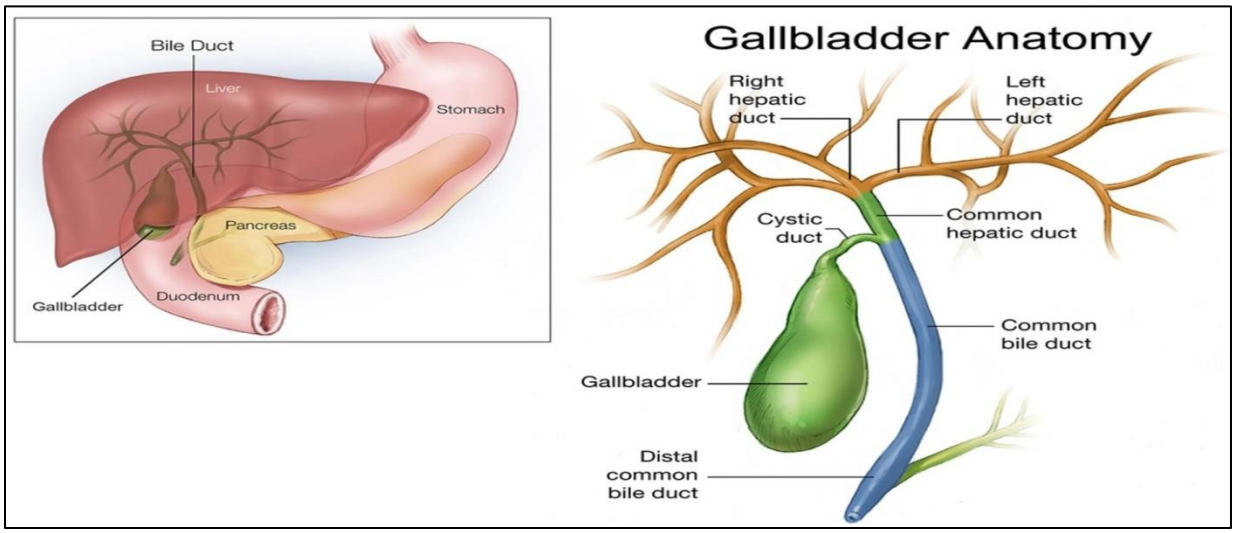
1.1.1 Anatomy and Physiological considerations of Gallbladder

The Gallbladder is reservoir of bile, situated in a fossa on the inferior surface of right hepatic lobe. The Gallbladder is divided into a fundus, a body and a neck or infundibulum. The neck tapers and connects to the biliary tree via the cystic duct, which then joins the common hepatic duct to become common bile duct [1]. The larger end of the gallbladder extends inferiorly and to the right while the tapered end points superiorly and medially. The tapered end of the gallbladder narrows into a small bile duct known as the cystic duct. The cystic duct connects to the common hepatic duct that carries bile from the liver. These ducts merge to form the common bile duct that extends to the wall of the duodenum [2]**[Figure 1.1].** In adults, the *gallbladder* measures approximately 8 cm in length and 4 cm in diameter when fully distended [3].

The histology of the gallbladder consists of mucosa with a single layer of epithelial cells, a lamina propria, a single layer of muscle and a serosal layer [4]. The mucosa, which forms the innermost layer of the gallbladder, lines the gallbladder with simple columnar epithelial tissue. The columnar epithelial tissue contains microvilli on its surface, increasing the surface area and allowing the lining to absorb water and concentrate the dilute bile. Beneath the columnar tissue is a thin lamina propria layer made of connective tissue and capillaries that support and anchor the epithelial layer. Deep to the lamina propria is the muscularis layer that contains smooth muscle tissue. Contraction of the muscularis pushes bile out of the gallbladder and into the cystic duct. Surrounding the muscularis is a thin layer of fibrous connective tissue that helps to reinforce and strengthen the wall of the gallbladder. Finally, the serosa forms the outermost layer of the gallbladder. The serosa gives the gallbladder a smooth, slick surface to prevent friction between

moving organs [4]. Approximately 60% of the gallbladder carcinoma originates in the fundus of gallbladder, with 30% occurring in the body and 10% in the neck [5][6].

Figure 1.1 Gallbladder Anatomy



The adult human gallbladder stores about 50 millilitres of bile, which is released into duodenum when food containing fat enters the digestive tract. The bile, produced by liver, emulsifies fats in partly digested food. After being stored in the gallbladder, the bile becomes more concentrated than when it left the liver, increasing its potency and intensifying its effect on fats.

1.1.2 Types of Gallbladder tumors

There are several types of gallbladder cancers [6].

Adenocarcinoma: This is the most common type of gallbladder cancer. More than 85 out of every 100 gallbladder cancers (85%) are adenocarcinomas. The cancer starts in gland cells in the gallbladder lining. These gland cells normally produce mucus.

There are three types of adenocarcinomas of the gallbladder; Non papillary adenocarcinoma, papillary adenocarcinoma and mucinous adenocarcinoma. Only about 6 out of every 100 gallbladder cancers (6%) are papillary adenocarcinomas. They develop in the connective tissue. This type of gallbladder cancer is less likely to spread to the liver and nearby lymph nodes. It tends to have a better outlook than most other types of gallbladder cancer. With mucinous adenocarcinomas, the cancer cells are often in pools of mucus. Only about 1 or 2 out of every 100 gallbladder cancers (1 or 2%) are mucinous adenocarcinoma.

Squamous cell: Squamous cell cancers develop from the skin like cells that form the lining of the gallbladder, along with the gland cells. **Adenosquamous cancer**: Adenosquamous carcinomas are cancers that have both squamous cancer cells and glandular cancer cells. Other rare types include, small cell cancer, neuroendocrine tumour and lymphoma.

Carcinoma of the gallbladder progresses from dysplasia, to carcinoma in situ (CIS), to invasive carcinoma over about 15 years. Dysplasia and CIS can be found in more than 90% of patients with gallbladder carcinoma although adenomas are thought not to be precancerous because molecular analysis of these lesions does not show the genetic changes associated with gallbladder carcinoma or its precursor lesions. Papillary cancer, however, may represent malignant degeneration of papillary adenoma. Gallbladder carcinoma usually produces asymmetric thickening of the gallbladder wall with infiltration to surrounding structures. Most cancers originate in the gallbladder fundus. As the tumour progresses, the gallbladder may fill with tumour or may contain pus, mucus, or stones. Early carcinomas can appear as a mucosal plaque, a polypoid or papillary outgrowth, or discrete thickening of the wall. Macroscopically, gallbladder carcinomas can be divided into papillary, tubular, and nodular forms. Papillary

tumours are less likely to invade the liver directly and have lower incidence of lymph-node metastasis.

1.1.3 Clinical Presentation of Gallbladder cancer

Early gallbladder carcinoma does not have any specific symptoms, but the general warning signs are pain in the right hypochondrium, weight loss, Anorexia, Nausea and vomiting, lump in right hypochondrium, Jaundice, Abdominal distention etc. [7].

These nonspecific symptoms have been grouped into five clinical syndromes. The first is acute cholecystitis—about 1% of patients operated on for acute cholecystitis have gallbladder carcinoma. Patients with this syndrome generally have an earlier stage of carcinoma than those who don't, and such patients have improved survival. Patients with chronic cholecystitis fall into the second category. The third syndrome is biliary-tract disease which includes symptoms of jaundice, weight loss, general weakness, and pain in the right upper quadrant. Patients with this clinical syndrome have extensive disease. In the fourth category are the clinical features of malignant tumours outside the biliary tract, which include: anorexia, weight loss, general weakness, and local complications of the tumour such as a fistula or invasion of adjacent organs. These patients usually have extensive disease. The final syndrome includes benign manifestations outside the biliary tract; the small group of patients with this syndrome present with gastrointestinal bleeding and upper gastrointestinal obstruction. Jaundice is common and is an indicator of poor prognosis. In 15–20% of patients gallbladder carcinoma is discovered incidentally at the time of routine cholecystectomy or postoperatively by the pathologist. Only 20% of patients have disease confined to the gallbladder at the time of diagnosis. The majority

(80%) have loco-regionally advanced disease with invasion of adjacent organs or distant metastases.

1.2 Descriptive Epidemiology

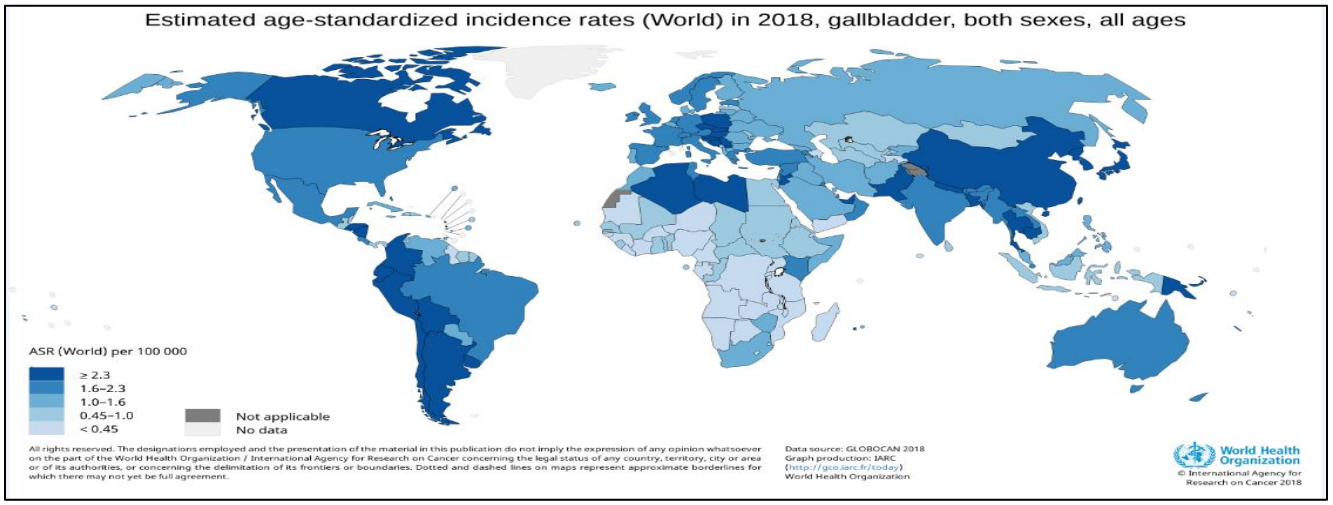
1.2.1 Incidence and Mortality (Worldwide)

GBC, though generally considered rare, is the fifth most common malignancy of the biliary tract, accounting for 80% to 90% of biliary tract cancers. GBC shows marked ethnic as well as geographical variation in its incidence [8].Worldwide, total number of GBC cases were 233,820 (Females: 123,366, Males:110,454) , as estimated by Globocan 2018 [9][10].

Worldwide Age standardized Incidence rate (ASR) for males in 2.2/100,000 and for females it is 2.4/100,000. [9]

GBC incidence is characterized by worldwide variation, being low in many European countries and U.S.A, relatively high in central European countries, and very high in Eastern Asia and South America. [**Figure 1.2**]

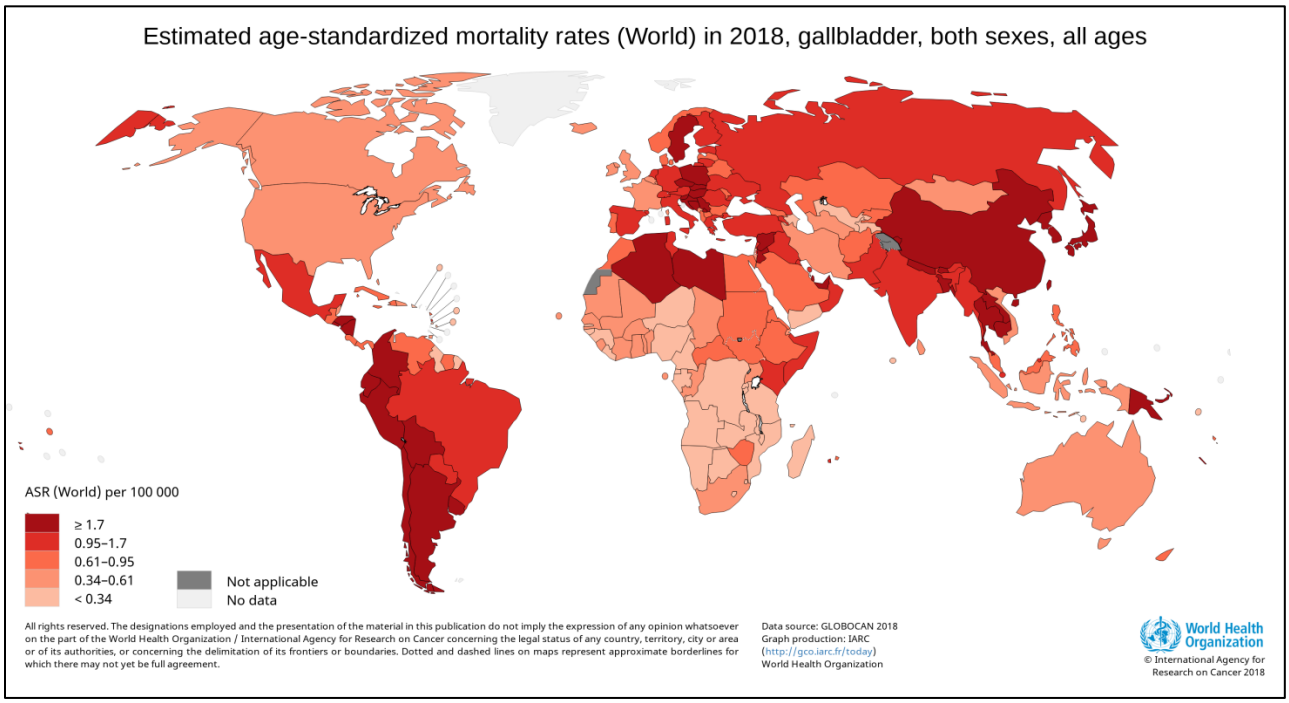
Figure 1.2 Age Standardised incidence rates (World) of Gallbladder cancer (Both genders, all ages)



According to Cancer Incidence in five continents report (CI5-Volume XI) [11], among females Chile was found to have highest Incidence (22.4) and among males Korea showed the highest incidence (10.1).

Worldwide Age standardized Mortality rate for males is 1.6/100,000 and for females it is 1.8/100,000. Due to poor prognosis and poor survival GBC mortality and incidence rates do not show remarkable difference, however mortality rates vary worldwide [10][**Figure 1.3**]

Figure 1.3 Age Standardised Mortality rates (World) of Gallbladder cancer (Both genders, all ages)



1.2.2 Disease Burden (India)

Gallbladder Incidence shows geographical variation in India. North-East region has the highest Incidence while South region has the lowest. [10]

Though the overall age-adjusted incidence rates of GBC in India are low, high incidence is reported in Kamrup, Cachar district, Delhi, Dibrugarh district, Sikkim state, Kolkata, Tripura, Manipur and Mizoram States. In Delhi, GBC was the fourth most common cancer (following cervix, breast and ovary) and the most common gastrointestinal cancer in women. Similarly in Bhopal, Kolkata, and Assam, GBC is among the five leading sites of cancer in women and commonest gastrointestinal cancer in women.

The rates for GBC are higher among women than men at virtually all ages, with gender difference decreasing slightly with increasing age. GBC is the leading cancer among digestive cancers in women in the northern Indian cities like Delhi.

1.2.3 Trend in Incidence of Gallbladder cancer in High and low risk regions in India:

Trends in Age adjusted incidence rates (AAR) for both the genders for the 14 year period (2001-2014) were estimated using National cancer registry programme (NCRP) database [12]. 18 registries having AAR for atleast three time periods, namely Aurangabad, Bangalore, Barshi, Bhopal, Cachar, Chennai, Delhi, Dibrugarh, Kamrup, Kolkata, Kollam, Manipur, Mizoram, Mumbai, Nagpur, Pune, Sikkim and Thiruvananthapuram were taken into consideration for the trend analysis. Indian states and territories were divided into high-.and low-risk regions using incidence rates extracted from PBCRs. States were considered to be in high-risk region if PBCR existing in the state observed average age-adjusted rates of >5.0 per100,000 persons [13]. Others were considered as low-risk regions. According to the definition Cachar, Delhi, Kamrup,

Dibrugarh, Kolkata and Sikkim were in high risk region and rest of the registries was considered to be in low risk region. For the comparison purpose, average AARs of all registries from high risk and that of from low risk regions were taken for four time periods (2001-2004 to 2012-2014). Trend was estimated using JoinPoint Trend Analysis Software by National Cancer institute [14][15]. APC was regarded as significant if the P value at 95% confidence interval was < 0.05.

Table 1.2 (A) and **1.2 (B)** show gender-wise age adjusted incidence rates for four time periods (2001-2004 to 2012-2014) in 18 registries. There are substantial differences in the rates for both the genders, with high rates in Delhi, Cachar, Dibrugarh, Kamrup and with lowest rates in Bangalore, Barshi, Chennai, and Aurangabad. For females, statistically significant increasing trends were observed in Bangalore, Cachar, Delhi, Kamrup and Pune; whereas rates were decreasing in Aurangabad, Barshi and Manipur. Among males increasing but statistically non-significant trends were observed in Cachar, Dibrugarh, Kamrup, Nagpur and Sikkim, whereas decreasing trends were observed in Bangalore, Barshi, Bhopal and Kolkata. Aurangabad showed significant decrease in trend (APC: - 14.5, P- value: <0.001) for males.

Table 1.1 Age Adjusted Incidence rates (AAR) of Gallbladder Cancer with Annual Percent Change (APC) in Indian registries (males)

Cancer Registry	AAR by Time period				APC	P value
	2001-2004	2004-2008	2009-2011	2012-2014		
Aurangabad	NA	0.7	0.3	0.2	- 14.5^	<0.001
Bangalore	0.8	2.6	0.3	1.2	- 4.6	0.75
Barshi Rural	1.0	1.7	1.0	1.0	- 1.7	0.67
Bhopal	2.8	3.4	3.3	2.6	- 0.6	0.76
Cachar District	2.1	4.7	5.1	5.2	7.4	0.19
Chennai	1.2	3.2	2.1	1.8	1.5	0.81
Delhi	3.7	6.5	4.2	5.3	1.2	0.76

Dibrugarh District	2.4	3.7	3.2	4.1	3.6	0.23
Kamrup	3.0	7.3	7.4	8.8	8.5	0.17
Kolkata	NA	3.7	2.5	3.3	- 2.0	0.72
Kollam	NA	0.9	1.1	1.4	5.5	0.15
Manipur	1.2	1.7	1.8	1.7	2.9	0.25
Mizoram	1.4	1.9	2.4	1.8	2.8	0.4
Mumbai	1.7	3.6	1.7	2.2	- 0.7	0.9
Nagpur	NA	0.8	1.3	1.3	6.7	0.24
Pune	NA	0.9	0.9	1.1	2.2	0.55
Sikkim	0.6	3.7	2.1	2.2	8.1	0.5
Thiruvananthapuram	NA	0.9	0.7	1.2	2.8	0.71

Source: National cancer registry programme (NCRP) database, **P-value**: For APC estimated at 95% confidence interval.

Abbreviations: AAR: Age Adjusted Incidence rate per 100,000, **NA:** AAR not available, **APC:** Annual Percent change

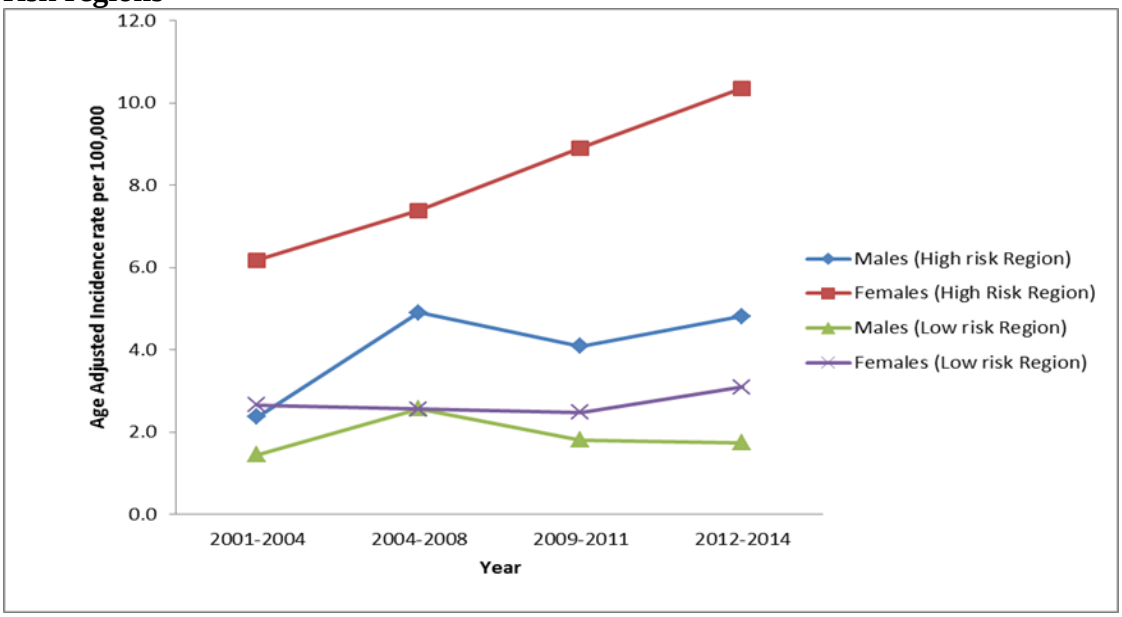
Table 1.2: Age Adjusted Incidence rates of Gallbladder Cancer with Annual Percent Change in Indian registries (Females)

Cancer Registry	Time period				APC	P value
	2001-2004	2004-2008	2009-2011	2012-2014		
Aurangabad	NA	0.3	0.1	0.1	- 13.1	0.09
Bangalore	1.0	1.4	1.5	2.0	5.7 ^	0.04
Barshi Rural	0.5	0.6	0.2	0.2	- 10.6	0.13
Bhopal	4.4	4.2	5.0	6.4	3.4	0.11
Cachar District	5.1	6.5	10.1	10.2	7.0 ^	0.02
Chennai	0.9	1.2	2.0	1.5	5.7	0.17
Delhi	7.4	8.1	9.2	11.8	4.0^	0.04
Dibrugarh District	5.8	7.5	7.7	8.6	3.1	0.09
Kamrup	10.2	12.6	14.0	17.1	4.3 ^	0.02
Kolkata	NA	4.5	5.6	7.7	6.7	0.14
Kollam	NA	0.6	0.8	1.0	6.4	0.09
Manipur	5.6	5.2	3.6	3.8	- 4.1	0.06
Mizoram	4.1	2.7	2.9	3.6	- 0.7	0.82
Mumbai	2.1	2.75	2.2	4.1	4.3	0.31
Nagpur	NA	0.8	1.0	1.4	6.9	0.21
Pune	NA	1.0	1.2	1.3	3.4^	0.04
Sikkim	2.4	5.2	6.8	6.7	9	0.12
Thiruvananthapuram	NA	0.8	1.3	1.1	4.7	0.47

Source: National cancer registry programme (NCRP) database, **P-value**: For APC estimated at 95% confidence interval.

Abbreviations: AAR: Age Adjusted Incidence rate per 100,000, **NA:** AAR not available, **APC:** Annual Percent change

Figure 1.4: Trends in Age adjusted incidence rates of gallbladder cancer in High and low risk regions



As shown in **Figure 1.4**, in high risk region, there was significant increase AAR for females (APC 5.4, P-value: <0.001); whereas for males trend was increasing but non-significant (APC: 6.3, P-value: 0.2). No significant in increase in AAR was observed in low risk region (Male: APC- 1.1, P-value-0.8, Females: APC-1, P-value- 0.5).

1.2.4 Survival

Many factors determine the survival and prognosis of the disease which include stage, type of tumour, grade, age of the patient etc. The 5-year survival rate for people with gallbladder cancer is 19%. The 5-year survival rate for people with stage 0 gallbladder cancer is 80%. The 5-year survival rate for stage I is 50%. If the cancer has spread outside the gallbladder to the lymph nodes, the 5-year survival rate is between 7% and 8%. Gallbladder cancer that has spread to

other parts of the body has a 5-year survival rate of 4% or less [17].In many patients the disease is discovered during operations to remove gallstones. At present, the therapeutic outcome of treatment for gallbladder carcinoma is extremely poor.

According to the recent analysis [16] using 1973 to 2009 data from the Surveillance Epidemiology and End Results database (SEER) among non-Hispanic whites, Hispanics, African American, and Asian/Pacific Islanders, Survival has improved considerably over time. It is better in females than males and in Asian/Pacific Islanders than other racial groups. The highest survival was in patients who received both surgery and radiation. Trend analysis revealed a recent increase of the incidence of late-stage gallbladder cancer. Highest survival was associated with receiving both surgery and radiation. Survival analysis of GBC in Indian population is not well documented.

1.3 Etiology

1.3.1 Gallstones: Gallstones, or choleliths, are solid masses formed from bile precipitates. These "stones" may occur in the gallbladder or the biliary tract. There are two types of gallstones: cholesterol and pigment stones. Cholesterol gallstones account for 80% of gallstones. Gallstones are considered to be very important risk factor for gallbladder carcinoma. However it is not clear if the association represents a causal link or the presence of common risk factors. Gallstones are found in 60 – 90% of the patients with gallstones carcinoma.

Reported relative risks (RRs) from case control studies vary greatly, and this variation probably results from difference in study design and method and definitions used to collect information on gallstone.

According to the case-control study conducted in Tata hospital, the risk of self-reported gallstone history was 28.94 [95% CI 21.55- 38.86], adjusted for age, gender, education, current residential region, waist to hip ratio and tobacco chewing and tobacco smoking.

A cohort study was performed for 396,720 South Korean men and women who underwent a health checkup from 2002 to 2012. Gallstones were found to be significantly associated with GBC, with multivariable-adjusted hazard ratios (95% confidence intervals) for GBC of 7.35 (2.60–20.8) [18].

Cholesterol and mixed gallstones account for 80% of the total and pigment stones (composed largely of calcium bilirubinate) account for the remaining fraction [19]. Cholesterol gallstones are caused by bile supersaturation with cholesterol or by abnormal motor function with consequent cholestasis. Cholesterol hypersecretion is related to increased cholesterol synthesis in the liver and associated with female gender, obesity and Western lifestyle. Constipation, which has been related to increased GBC risk, may facilitate the re-absorption into the bile of secondary bile acids formed in large quantities in the colon via enterohepatic circulation [20].

The worldwide distribution of gallstone prevalence shows a strong geographic as well as ethnic variation, and positive correlation with GBC [22]. Increased rates for both GBC incidence (per 100,000) and gallstone prevalence (%) occur in Pima Indian females (21/100,000 cancer incidence and 75.8% gallstone prevalence), North American Indian females (7.1/100,000 and 64.1%), Chilean Mapuche Indian females (27.3/100,000 and 49.4%) and East Indian females (22/100,000 and 21.6%) [8]. Low -risk areas for gallstones (i.e. where prevalence is <10% among women) included African countries, but also Thailand, China, Korea, and Japan which reported high GBC incidence rates [22].

1.3.2 Gallbladder anomalies and pathologies: Conditions like Anomalous Pancreaticobilliary duct junction and porcelain gallbladder are also suggested to be associated with gallbladder carcinoma. The anomalous ductal condition is seen in about 17% of the patients. Porcelain gallbladder refers to the pathological finding of a brittle gallbladder with bluish discoloration resulting from extensive calcification of the organ wall. It has been reported in 12.5 -62% of the patients with gallbladder cancer [7].

1.3.3 Reproductive and Hormonal factors: Higher incidence of gallbladder cancer in women, suggests a role of reproductive and /or hormonal factors in pathogenesis of GBC. Observational studies have suggested role of endogenous hormone in promoting gallstone, increasing cholesterol, and impairing gallbladder motility, and or through a direct carcinogenic effect on gallbladder. In this population based case- control study in China [23] indicated Parity [OR- 2.0, 95% CI 0.7-5.1], younger age at first birth [OR 1.2, 95% CI 0.99-1.6] and old age at menarche [OR 1.4, 95% CI 1.1-1.8] were the risk factors for gall bladder cancer. A population based prospective cohort study in Japan showed irregular and longer cycles[HR 2.12 , 95%CI 1.30-3.47 & HR 1.76, 95%CI 1.08-2.89 respectively] and older age at first pregnancy [HR 1.84, 95% CI 1.03- 3.29] were associated with GBC [24].

1.3.4 Obesity: There have been contradictory findings regarding the role of obesity with that of gallbladder cancer in case- control studies. However, in cohort studies overweight and obesity were strongly associated with incidence of GBC. In a meta-analysis [25] of excess body weight and GBC risk, the summary relative risk was found out to be 1.15

(95% CI 1.01-1.30. The association with obesity was stronger for women (1.88 , 95% CI 1.66 -2.13) than in men (1.35, 95% CI 1.09-1.68).

1.3.5 Tobacco and Alcohol: Tobacco smoking has been inconsistently associated with gallbladder cancer risk. In a systematic review and meta-analysis [26], summary relative risk was 1.19 (95% CI 1.12-1.28) for current smokers, 1.10 (95% CI 1.07-1.13) for former smokers and 1.15 (95%CI 1.13-1.18) for ever smokers. One study investigated the effect of alcohol on various cancer types in meta-analytic approach and found that heavy drinkers had the risk of 2.64 (95% CI 1.62 -4.30) for GBC [27].

1.3.6 Water supply: The etiologic relation of heavy metals and their compounds has not been adequately described for carcinoma of the gallbladder. In a review of environmental pollutants and GBC risk [28], a number of heavy metals like nickel, cadmium, lead, etc. have been implicated; however, the evidence is not robust. Presence of significant high concentration of heavy metals in GBC cancer tissue was also demonstrated in an Indo-Japan collaborative study [29]. There have been no studies regarding source of drinking water and GBC risk pertaining to *Salmonella* contamination.

1.3.7 Genetic and hereditary factors: There have been few studies regarding Familial risk for GBC.A nationwide epidemiological study from Sweden [30], reported Standardized Incidence ratios (SIR 5.21 95% CI 2.07-10.80), showing high risk for familial GBC. In a case-control genome-wide association [31] study found significant associations for several markers in the chromosomal region 7q21.12 harbouring both the ABCB1 and ABCB4 genes. GWAS heritability analysis suggested that common variants are

associated with substantial variation in risk of gallbladder cancer (sibling relative risk 3·15 [95% CI 1·80–5·49]).

1.3.8 Infection: The rationale behind idea of association of infections and GBC is inflammation of the organ due to chronic stage of infection by the bacteria and parasites. Few bacteriae like *Helicobacter spp.* and *Salmonella spp* and parasites like liverfluke have thought of been associated with GBC. A study [32] showed the presence of H. pylori in 75% of the patients with GBC. Another study [33] reported H. pylori colonies in 44% of GBC patients and 33% of Gallstone disease patients upon culture. Serology was positive in 32% of GBC patients and 28% of Gallstone disease patients. PCR with hsp60-nested primers showed presence of DNA in 33% of GBC patients and 28% of Gallstone disease patients. This result indicates endemic presence of *H.pylori* in the respective population, but no possible association with GBC. Other species of Helicobacter also have also been explored. In one case-control study and meta-analysis [34], *Helicobacter bilis* was identified in 32/54 patients and 32/55 controls, The relative risk of gallbladder cancer in *H. bilis* positive cases was 1.05 (95% CI 0.49 to 2.24). Pooled statistics of the meta-analysis on showed an odds ratio of 1.24 (95% CI 0.63-2.44).

Liver-fluke is well established risk factor cholangiocarcinoma, but there are no studies regarding association of the same with GBC, except one case report [35]. CT scan of gallbladder showed inflammation and *Clonorchis sinensis* (A liverfluke species) worms. Regarding, association with *Salmonella spp*, there have been few studies which are discussed in detail in coming section.

1.4 *Salmonella typhi* :

1.4.1 Structure and types of species:

Salmonella typhi is a gram negative bacillus belongs to the family enterobacteriaceae. Currently, 107 strains of this organism have been isolated. The nomenclature of Salmonella is controversial and still evolving. Currently, the Centers for Disease Control and Prevention (CDC) uses the nomenclatural system of Salmonella recommended by the World Health Organization (WHO) Collaborating Centre [37]. According to this system, the genus Salmonella is classified into two species, *Salmonella enterica* and *Salmonella bongori*, based on differences in their 16S rRNA sequence analysis. *S. enterica*, can be further classified into six subspecies based on their genomic relatedness and biochemical properties. The subspecies are denoted with roman numerals: I, *S. enterica subsp. enterica*; II, *S. enterica subsp. salamae*; IIIa, *S. enterica subsp. arizonae*; IIIb, *S. enterica subsp. diarizonae*; IV, *S. enterica subsp. houtenae*; and VI, *S. enterica subsp. Indica* **[Table 1.3]**. Among all the subspecies of Salmonella, *S. enterica subsp. enterica* (I) is found predominantly in mammals and contributes approximately 99% of Salmonella infections in humans and warm-blooded animals. In contrast, the other five Salmonella subspecies and *S. bongori* are found mainly in the environment and also in cold-blooded animals, and hence are rare in humans [36].

Table 1.3: Salmonella subspecies and serovars

Species	Subspecies number	Subspecies names	Number of serovars
S. enterica			2557
	I	*enterica*	1531
	II	*salamae*	505
	III	*arizonae*	99
	IV	*diarizonae*	336
	V	*houtenae*	73
	VI	*indica*	13
S. bongori			22
Total number of serovars			2579

1.4.2 Clinical manifestations:

Based on the clinical patterns in human salmonellosis, Salmonella strains can be grouped into typhoid Salmonella and non-typhoid Salmonella (NTS). In human infections, the four different clinical manifestations are enteric fever, gastroenteritis, bacteremia and other extra- intestinal complications, and chronic carrier state [36].

a. Enteric fever

Salmonella Typhi is the etiological agent of typhoid fever, while paratyphoid fever is caused by *S. Paratyphi* A, B and C. The clinical symptoms of paratyphoid fever are indistinguishable from typhoid fever. Humans are the sole reservoir for the two strains of typhoid Salmonella. The organisms are transmitted via the ingestion of food or water contaminated with the waste of

infected individuals. Enteric fever is characterized by an incubation period of one week or more, with prodromal symptoms such as headache, abdominal pain and diarrhea (or constipation), followed by the onset of fever. Besides fever, infected patients may also develop myalgia, bradycardia, hepatomegaly, splenomegaly, and rose spots on their chest and abdomen.

In endemic regions, approximately 15% of the infected patients develop gastrointestinal complications which include pancreatitis, hepatitis and cholecystitis.

b. Gastroenteritis:

Salmonella strains other than *S. typhi* and *S. paratyphi* are referred to as NTS, and are predominantly found in animal reservoirs. NTS infections are characterized by gastroenteritis, an inflammatory condition of the gastrointestinal tract which is accompanied by symptoms such as non-bloody diarrhea, vomiting, nausea, headache, abdominal cramps and myalgia. Gastrointestinal complications of NTS infections include cholecystitis, pancreatitis and appendicitis, while the perforation of the terminal ileum has no association with NTS infections.

c. Bacteremia and other extra-intestinal complications:

Salmonella bacteremia is a condition whereby the bacteria enter the bloodstream after invading the intestinal barrier. Almost all the serotypes of Salmonella can cause bacteremia. Similar to enteric fever, high fever is the characteristic symptom of bacteremia.

d. Chronic carrier state:

The status of chronic carrier is defined as the shedding of bacteria in stools for more than a year after the acute stage of *Salmonella* infection. Since humans are the only reservoir of typhoid Salmonella, carriers of *S. typhi* and *S. paratyphi* are responsible for the spreading of enteric fever

in endemic regions, as the common transmission route is the ingestion of water or food contaminated with the faeces of chronic carriers. About 4% of patients with enteric fever, predominantly infants, elderly people and women, may become chronic carriers. In contrast, the carrier state of NTS is less frequent, with an occurrence rate of 0.1% in patients with non-typhoidal salmonellosis. This is because the primary reservoir of NTS is animals, instead of humans [36].

1.4.3 Prevalence:

India is said to be endemic for *S.typhi* infection, but there is lack of data regarding the same. In 2000, it was estimated that over 2.16 million episodes of typhoid occurred worldwide, resulting in 216 000 deaths, and that more than 90% of this morbidity and mortality occurred in Asia.

A prospective, population based surveillance in five Asian countries including China, India, Indonesia, Pakistan and Vietnam was carried out by WHO to provide an update on burden of typhoid in Asia [38]. The incidence of typhoid among children aged 2–5 years was 340.1/100,000 in India and it was similar to that in school aged children and adults. However, this data is only from 2 government hospitals and 5 clinics in Kolkata, West-Bengal, India and hence cannot be generalized to the whole Indian population.

1.4.4 Modes of infection and pathogenesis:

The encounter of humans to *S. typhi* is made via fecal-oral route from infected individuals to healthy ones. Poor hygiene of patients shedding the organism can lead to secondary infection, as well as consumption of shellfish from polluted bodies of water. The most common source of infection, however, is drinking water tainted by urine and feces of infected individuals.

S. typhi has a combination of characteristics that make it an effective pathogen. This species contains an endotoxin typical of Gram negative organisms, as well as the Vi antigen which is thought to increase virulence. It also produces and excretes a protein known as "invasin" that allows non-phagocytic cells to take up the bacterium, where it is able to live intracellularly. It is also able to inhibit the oxidative burst of leukocytes, making innate immune response ineffective. The entry of this bacterial species into the human body is most commonly achieved by ingestion, with the importance of aerosol transmission unknown. Once ingested, the organisms multiply in the small intestine over the period of 1-3 weeks, breech the intestinal wall, and spread to other organ systems and tissues. The innate host defenses do little to prevent infection due to the inhibition of oxidative lysis and the ability to grow intracellularly after uptake.

Salmonellae must endure very challenging conditions to get to the intestine of the host, such as the low pH of the stomach, which in humans is as low as 1.5, high concentrations of bile salts, osmolytes, commensal bacteria metabolites, and low oxygen tensions [39]. Salmonella have developed survival strategies to cope with these challenging conditions including an acid tolerance response (ATR). The ATR is directed by several mechanisms that help the bacteria survive the drastic change to low pH in the stomach of the host. The intracellular pH of gram negative bacteria is pH 7.6-7.8. This pH is maintained by pumps that remove protons from the cytoplasm in a low pH environment. In addition, Salmonellae use inducible lysine decarboxylase and arginine decarboxylase systems to increase intracellular pH. Following external cues indicating low external pH Salmonellae activate transcription of the cadBA operon. In the presence of lysine, the CadA enzyme converts lysine to cadaverine with the consumption of a proton. The CadB antiporter exports cadaverine in exchange for extracellular lysine. Thus the intracellular pH is increased [39]. Furthermore, Salmonella produce acid shock proteins to

prevent/repair damage as a result of acid stress. The alternative sigma factor RpoS has been shown to control expression of at least 10 acid shock proteins. Most acid shock proteins are involved in; cell regulation, molecular chaperoning, energy metabolism, transcription, translation, fimbriae synthesis, regulation of cell envelopes, colonisation and virulence.

Other adaptations to acid conditions include modification of the bacterial membrane. Salmonella change the fatty acid composition of the membrane in response to low pH. Exposure to acid pH has been shown to result in a decrease of unsaturated fatty acids and increase in saturated fatty acids and cyclic fatty acids. The role of bile and bile salts in the digestive tract serves to digest lipids. Salmonella have developed mechanisms by which to resist digestion in bile. The shortening or loss of O antigen, results in a rough colony phenotype and increased bile sensitivity, indicating a role for LPS in resistance to bile. The lumen of gastrointestinal tract has a relatively high salt concentration, 0.3M NaCl. This increase in salt concentration might result in osmotic shock. Salmonella react to changes in osmolarity by stimulating uptake of potassium (and glutamate). This is followed by dramatic increase in the cytoplasmic concentration of so-called compatible solutes. Oxygen availability in the large intestine decreases and is largely anaerobic. Salmonella possesses cytoplasmic oxygen sensor, Fnr. Fnr binds promoter sequences and interacts with RpoA (RNA polymerase subunit), these interactions result in increased efficiency of transcription of genes important for anaerobic metabolism. The gastrointestinal tract is colonised by commensal microorganisms. Salmonella must compete with commensal bacteria to gain access to the epithelia. In addition, these commensal microorganisms produce metabolites such as bacteriocins and short chain fatty acids with anti-Salmonella activity. The specific mechanisms by which Salmonella escapes destruction by bacteriocins has not yet been elucidated [39].

Transmission of *S. typhi* has only been shown to occur by fecal-oral route, often from asymptomatic individuals. 3-5% of previously infected individuals become chronic carriers who show no signs of disease, but actively shed viable organisms capable of infecting others. A famous example is "Typhoid" Mary Mallon, who was a food handler responsible for infecting at least 78 people, killing 5. These highly infectious carriers pose a great risk to public health due to their lack of disease-related symptoms.

1.4.5 Prevention strategies:

Contaminated water or food is the major transmission route of enteric fever. At present, preventive measures for enteric fever concentrate on access to safe water and food, proper sanitation [36]. Besides water, *Salmonella spp*. can be found in a variety of foods, predominantly in poultry, eggs and dairy products. Proper handling and cooking of food are measures proposed to eradicate the bacterial contamination of food. In many countries, food irradiation has been greatly promoted owing to its effectiveness in reducing the risk of food contamination.

Asymptomatic chronic carriers play a major role in spreading of the disease. Screening of carriers and subsequently providing the proper treatment to those who screen positive is also a significant preventive strategy.

1.5 Rationale for Association of *Salmonella typhi* with Gallbladder Cancer

1.5.1 Salmonella Chronic carriage:

Approximately 3 -5% of infected individuals fail to fully clear the infection, progressing to the state of carriage. The basic requirements for establishment of long-term extraintestinal infection are likely to involve successful breach of the intestinal epithelial barrier, evasion of early innate immune-mediated response, and localization to a permissive niche. The permissive niche in humans is primarily the biliary tract and gallbladder [40]. In order to induce gallbladder chronic carriage, organisms must enter the biliary tract either via a descending route after systemic infection, or an ascending route directly from the small intestine. In the ascending route, the bacteria would enter the biliary system via the sphincter of Oddi, which if malfunctioning due to surgical intervention or pathology, may fail as a mechanical barrier. However, the more likely route is direct transfer into the gallbladder from the liver during the systemic phase of typhoid fever. Normally, Kupffer cells in the liver prevent toxic metabolites and bacteria from entering the hepatobiliary system and the continuous flushing action of bile and the bacteriostatic effect of bile salts keep the biliary tract relatively sterile. The failure of these, or other gallbladder functions, in addition to the organism's ability to bypass these systems, likely induces and helps to maintain long-term carriage.

Chronic carriers intermittently shed the bacteria for a prolonged, ill-defined period of time in the local environment and thus may spread the disease in the community and maintain a reservoir of infection. Although the precise role of chronic carriers in disease transmission remains unclear, these asymptomatic carriers presumably act as reservoirs for a diverse range of *S. typhi* strains [41][42].

During the course of infection with invasive Salmonella, carriage may be split into three different periods: convalescent, temporary, and chronic. Convalescent carriers shed the bacilli in feces for three weeks to three months post-infection. Temporary carriers shed the bacilli for between three and twelve months, and chronic carriers shed the bacilli for more than one year [43].

1.5.2 Survival strategies of Salmonella in Gallbladder:

The gall bladder is the site of bile storage and an environment that is habitable exclusively by organisms that are resistant to bile's detergent-like properties. Salmonellae, not only are highly resistant to bile, but also respond to the presence of bile by regulating the expression of many resistance-related genes. Salmonella exhibit rapid surface association and intracellular invasion of gallbladder epithelial cells, and can form biofilms on the epithelial surface. Salmonella can adapt to bile, as exposure to sublethal levels can result in increased survival when challenged with otherwise lethal levels. One study [44] suggests that this phenotype is both growth phase- and bile concentration-dependent. Pre-treatment of exponential-phase cells of *S. Typhimurium* with 15% bile resulted in increased survival when challenged with 24% bile. Exposing stationary-phase cells to the same concentration of bile did not increase their resistance when subsequently challenged with 24% bile. It was also noted that exposure of exponential-phase cells to low levels of bile (1–3%) did not affect resistance.

The epidemiological risk factors for becoming a persistent carrier have not been extensively investigated, primarily because this is a challenging population to prospectively identify. A study has shown that salmonellae form bile-mediated biofilms on human gallstones and cholesterol coated surfaces in vitro. Gallbladder carriage has been demonstrated to be mediated by biofilm

formation on gallstones and by intracellular persistence in the gallbladder epithelium of mice. [45].

In a study [46], a mouse model of acute typhoid fever was used to demonstrate that Salmonella could replicate extracellularly in the gallbladder lumen and could also infect and replicate intracellularly within gallbladder epithelial cells. *S. typhi* has been shown to colonize the human gallbladder and persist in an asymptomatic carrier state that is often associated with the presence of gallstones. Residing in this location may allow the bacterium to escape the host immune system and antibiotics, and to be released in bile back into the intestine to reinfect the same host or be shed in the faeces.

1.6 Association of Chronic *S. typhi* infection and GBC (Review of Literature)

Few studies previously have reported the positive association of *S.typhi* infection with GBC. A prospective case-control study conducted in tertiary care Centre in India, with 37 GBC cases and 80 controls, the risk was found to be 14 (CI: 2-92) on the basis of Vi serology [47]. Another study involved 52 patients with CaGB, 223 patients with benign gallbladder diseases, 508 healthy individuals and, 424 corpses [48]. 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. 67.3% CaGB patients were PCR-positive for the *S.* Typhi flagellin gene whereas proportion of PCR positives in benign gallbladder disease patients was 42.6%.

A study from Kathmandu, Nepal investigated microbiological and clinical characteristics of Salmonella spp. in Gallbladders from 1377 Cholecystectomy patients. 3.5% of individuals undergoing cholecystectomy in this setting showed a high concentration Salmonella in their bile suggesting a positive association [49].

A collaborative study [50] looked into the cellular transformation of Gall bladder cancer cells by manipulation of host signaling pathways by Salmonella. It showed that Salmonella enterica induces malignant transformation in predisposed mice, murine gallbladder organoids, and fibroblasts, with TP53 mutations and c-MYC amplification. Mechanistically, activation of MAPK and AKT pathways, mediated by Salmonella enterica effectors secreted during infection, is critical to both ignite and sustain transformation, consistent with observations in GBC patients from India. The findings indicated that Salmonella enterica can promote transformation of genetically predisposed cells and is a causative agent of GBC.

One study from North India assessed the prevalence of S.typhi in patients with GBC and Gallstone disease, by using a number of tests/methods which were Bile culture, Bile PCR, WIDAL, Tissue PCR and IHA. Study was carried out on 54 GBC and 54 controls with gallstone disease. They concluded that S. typhi was significantly associated with GBC [51].

A case control study from Chile with sample size of 39 GBC, 40 Controls with gallstones and 39 population based controls evaluated the association between S.typhi antibodies and GBC. They performed culture and PCR on a subset with bile, gallstone, tissue and stool samples available. GBC cases found to be having 4 times more risk of developing gallbladder cancer than controls [52].

A systematic review and meta-analysis [53] assessed 17 studies to evaluate the relationship between chronic Salmonella typhi carrier status and gallbladder cancer. The overall OR for

chronic S. typhi carrier state was 4.28 (95% CI: 1.84–9.96). Most of the studies were from South Asia especially India and China. When a subgroup analysis was performed according to region, a significant association was observed in South-East Asia (OR: 4.13, 95% CI: 2.87–5.94, P value <0.01). Chronic S. typhi carrier state was associated with carcinoma of the gall-bladder based on detection methods of S. typhi antibody levels (OR: 3.52, 95% CI: 2.48–5.00, P value <0.01) and even more so on culture (OR: 4.14, 95% CI: 2.41–7.12, P value <0.01).

1.7 Gaps in Literature

There are few studies in literature which address the question of role of *Salmonella typhi* Infection as a risk factor for Gallbladder Carcinoma. Although, many studies have reported the positive association of *S.typhi* and Gallbladder cancer, almost all studies are underpowered and methodologically poor. Therefore this question still remains unanswered.

Selection of a control population in most studies is not appropriate according to study design norms. Many methods which are used to detect *S. typhi* meant to interpret acute infection and not the chronic one. To address the association with cancer, it is needed to look for the chronic infection.

It is said that *Salmonella typhi* infection is endemic in India, however there is no quality data available to support this fact. True prevalence of *Salmonella typhi* in India is still unknown.

The present study is extension of a previous case- control study, designed to develop/standardize a reliable method to detect chronic *Salmonella typhi* infection, to estimate the prevalence of *S.typhi* in India and to find the role of Chronic *S. typhi* infection as risk factor for Gallbladder cancer.

1.8 Hypothesis:

Exposure to chronic infection of *Salmonella typhi* increase the risk of gall bladder cancer

Aim:

To study association of chronic infection of *S.typhi* with gall bladder cancer

Objectives:

1. To study association of *Salmonella typhi* infection with gall bladder cancer.
2. To standardize ELISA for detection of chronic infection of *Salmonella typhi*

Chapter 2

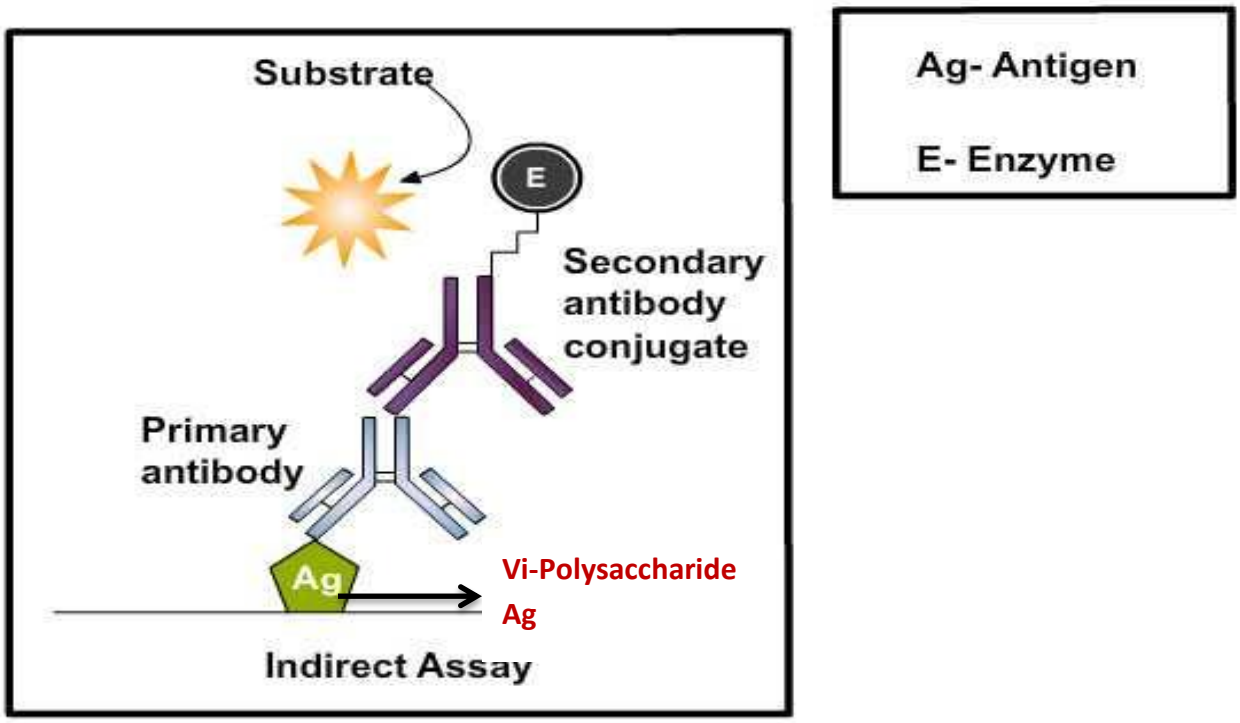
Optimization of ELISA for detection of Chronic *S.typhi* infection

Chapter 2: Optimization of ELISA for detection of Chronic S.typhi infection

2.1 Principle of the assay:

The method is based on standard In-direct ELISA technique [54] [**Figure 2.1**] which detects IgG antibody against *S.typhi* Vi capsular polysaccharide antigen. The plate is coated by Vi-polysaccharide antigen. Coating is achieved through passive adsorption of the antigen to the assay microplate. This process occurs though hydrophobic interactions between the micro titer plate and non-polar polysaccharide residues.

Figure 2.1 Schematic representation: principle of ELISA



After incubation any excess antigen is removed by washing steps by flooding and emptying the wells with neutral phosphate buffered saline containing surfactants like 30% Brij 35. Washing steps are necessary to remove nonbound reagents and decrease background, thereby increasing the signal: noise ratio. Insufficient washing will allow high background, while excessive washing might result in decreased sensitivity caused by elution of the antigen from the well. Blocking step with appropriate solution of protein like bovine serum albumin ensures unbound sites by on the microtitre plate are blocked. The ideal blocking buffer will bind to all potential sites of nonspecific interaction, eliminating background altogether, without altering or obscuring the epitope for antibody binding. Further appropriately diluted test serum samples and serially diluted reference standard are added. The target antibody present in the test serum which are specific to the antigen, binds the coated antigen on incubation. Each addition and incubation step is followed by washing to remove excess unbound antibodies or antigens.

The next step is the addition of secondary antibody, diluted antibody/serum dilution buffer directed against the primary antibody. Followed by incubation to achieve the binding of the enzyme-conjugated secondary antibody. The choice of antibody enzyme conjugate is determined by the goals of the assay. In this assay, alkaline phosphatase labeled goat anti human IgG is used. This secondary antibody binds to Anti-Vi IgG primary antibody (if present in test sample). After incubation and washing substrate is added. Substrates are critical for the detection and visualization steps of an ELISA. The step involves the addition of suitable substrate solution for the particular enzyme conjugated to the antibodies. The objective is to allow development of color reaction through enzyme catalysis. p-Nitro phenyl-phosphate is the substrate used against the secondary antibody alkaline phosphatase. Alkaline phosphatase catalyzes the hydrolysis of of p-nitro phenyl phosphate to p-nitrophenol, a chromogenic product with absorbance at 405 nm.

Hence, if a test sample contains Anti-Vi IgG antibodies (indicator of chronic S.typhi infection), the chain reaction of secondary antibody and substrate follows and consequently a yellow coloured product is formed, which is the end point of assay.

2.2 Materials

2.2.1 Biological:

I. Coating antigen: Vi polysaccharide (Purified from *Citrobacter freundii* WR 7011). It is structurally identical to the Vi purified from *S.typhi*. Each vial of the antigen contains 1 mg of lyophilized Vi PS.

II. Reference standard Anti-Vi IgG: This is the reference standard used in the assay. Each vial contains 33 μg of purified anti Vi IgG. This reference is prepared from pooled plasmas (of volunteers injected with Vi recombinant conjugate vaccine) with highest levels of anti Vi IgG [55].

III. Secondary antibody: Alkaline phosphatase labeled goat anti-human IgG (AffiniPure $F(ab')_2$ fragment, Fcy fragment specific)

2.2.2 Buffers and Solutions:

I. Coating buffer (1XPBS, pH 7.4)

II. Wash buffer (0.85% NaCl, 0.33% Brij 35, 0.02%NaN_3)

III. Blocking Buffer (1X PBS with 1% of BSA)

IV. Antibody or Serum dilution Buffer (1X PBS, 0.1% Brij 35, 1% BSA)

V. Substrate buffer: (1M Tris with 0.3% of 1M $MgCl_2$)

VI. Substrate: p-nitrophenyl phosphate disodium salt hexahydrate

(All the required buffers were prepared according to the protocol provided by NIH. Optimum Shelf life of all the buffers was 2 weeks.)

2.3 ELISA Procedure

The assay procedure was run and optimized according to the protocol provided by NIH.

2.3.1 Reconstitution of Vi PS antigen and anti-Vi IgG reference standard:

- Vi PS antigen and reference standard were provided in a lyophilized form. In order to use them in the assay, they were reconstituted with 1.0 ml/vial of sterile pyrogen-free water. After reconstitution Vi PS antigen had the concentration of 1 mg/ml per vial and Reference standard had 33 µg/ml of anti Vi IgG per vial.
- 10 aliquots each of both ViPS antigen and reference standard were prepared and stored at – 80^{o}C

2.3.2 Antigen Coating (Day 1 of the Assay)

- Concentration of antigen required for coating is 2 µg/ml**.** Hence, 1 mg/ml of Reconstituted Vi PS Antigen was diluted to 2 µg/ml using 1X PBS (Coating buffer).
- All wells of 96 well plate were coated with 100 µl/well of the 2 µg/ml Vi PS solution.
- Plates were incubated for 16- 20 hrs at 20- 25^{o}C

2.3.3 Blocking and Addition of Reference standard, serum samples (Day 2 of the Assay)

- Plates were washed 3 times with wash buffer. Blocking buffer was added 100 µl/well and incubated for 2 hrs at 20- 25^{o}C.
- Reference standard and serum samples were diluted in Antibody/serum dilution buffer according to the experiment plan and added to the washed plate (100 µl/well of diluted sample)
- Plates were incubated for 16- 20 hrs at 20- 25^{o}C

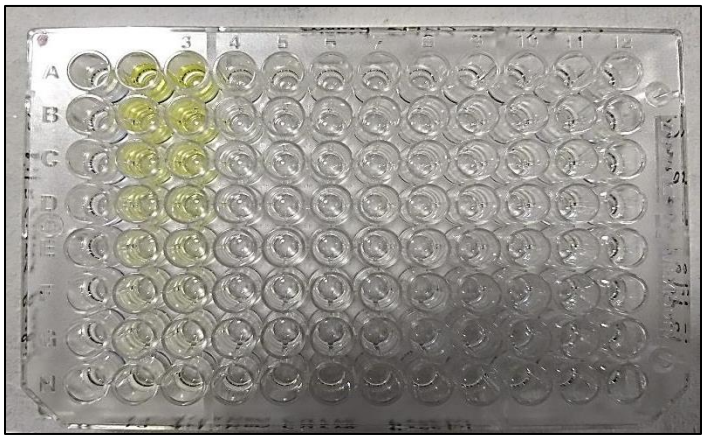
2.3.4 Addition of Secondary antibody and substrate (Day 3 of the Assay)

- After 2nd day incubation, plates were washed 6 times. Secondary antibody was diluted to the optimal dilution (Discussed in next sections) in Antibody/serum dilution buffer. Addition (100 µl/well) of Diluted secondary antibody was followed by incubation at 37^{o}C for 4 hrs.
- 1 mg/ml solution of p-nitro phenylphosphate was prepared in substrate buffer and added to the washed plates (100 µl/well).
- Incubate plates at 20- 25^{o}C for 10 to 30 minutes. The development time was optimized for the assay and discussed in next sections.
- Absorbance was read at 405 nm with 620 as reference filter on Tecan's Sunrise^{TM}.

2.3.5 Read-out and Analysis:

An automated reader, with appropriate software program (Magellan^{TM}) [56] was used for calculation of results. Absorbance data were converted to antibody concentration using the software program Magellan^{TM} for interpretation. Anti Vi IgG concentration (against OD at 1:400 dilution) for each sample was calculated based on Reference standard curve. 4-parameter logistic regression model was used for generation of the standard curve.

Figure 2.2 Assay plate after last step of ELISA for read-out
(Yellow color indicates a positive reaction)



2.3.6 Use of Automated Liquid handling system:

Addition of antigen, blocking buffer, secondary antibody, substrate and serum sample dilutions were carried out on TECAN's automated liquid handling platform [57] (**Figure 2.3**). Appropriate software program was generated and used for the same.

Figure 2.3 TECAN's Automated Liquid Handling system

2.4 Assay Optimization

This Vi PS ELISA was optimized based on following parameters

2.4.1 Determination of Optimal dilution of secondary antibody

2.4.2 Determination of Optimal Development time for end point signal

2.4.3 Generation of reference Standard curve

2.4.4 Parallelism

2.4.5 Optimum dilution factor for the assay

2.4.6 Determination of cut-off value for Chronic *S.typhi* sero-positivity

2.4.1 Determination of Optimal dilution of secondary antibody

Procedure: Range of dilutions of the secondary antibody (1:1000 to 1:12800) was checked against the Reference standard. Following was the plate layout for the same (**Figure 2.4**)

Figure 2.4: Plate layout for determination of Optimal dilution of secondary antibody

Plate 1	1	2	3	4	5	6
A	Blank	(1:1000)	(1:1000)	(1:32000)	(1:32000)	Blank
B	Blank	(1:2000)	(1:2000)	(1:64000)	(1:64000)	Blank
C	Blank	(1:4000)	(1:4000)	(1:12800)	(1:12800)	Blank
D	Blank	(1:5000)	(1:5000)			Blank
E	Blank	(1:6000)	(1:6000)			Blank
F	Blank	(1:7000)	(1:7000)	Dilution range of secondary antibody		Blank
G	Blank	(1:8000)	(1:8000)			Blank
H	Blank	(1:16000)	(1:16000)			Blank

2.4.2 Determination of Optimal Development time for end point signal

The range of color development time stated in the protocol is 10 to 30 minutes. This needed to be further optimized according to the secondary antibody dilution. According to the protocol, development time less than 10 minutes could lead to inconsistent results and more than 30 minutes could give high background noise.

Reference standard was run 8 dilutions and the absorbance was read at various time intervals 10 minutes, 20 minutes, 25 minutes and 30 minutes.

2.4.3 Generation of Reference standard curve:

In this assay, availability of reference standard and the exact concentration value of anti Vi IgG ensured the estimation of anti Vi IgG concentration in the serum sample based on the absorbance reading. Standard curve was based on 4- parameter logistic model.

The model is recommended for dose- response and receptor- ligand binding assays [58]. As the name suggests, this has 4 parameters in order to fit the curve. It forms a sort of S shaped curve.

Equation of the same is as follows;

$$y = d + \frac{a - d}{1 + \left(\frac{x}{c}\right)^{b}}$$

2.4.4 Parallelism:

The ELISA provides a quantitation of specific analyte concentration in samples, in this case anti-Vi IgG concentration. Concentration of the required analyte is calculated from the standard curve. Hence, it is important to ensure that characteristics of standard curve and sample dilution curve are similar. The assumption behind the idea of parallelism is that the standard and the sample are said to be having similar antibody- binding characteristics when these two curves are parallel [59]. However, in biological systems multiple factors like auto-antibodies, anti-coagulants, enzyme inhibitors which can contribute to failed parallelism response.

For determination of parallelism, % CV was calculated using Anti-IgG Concentrations at 8 serial dilutions by nullifying the dilution factor from linear part of the curve. Samples having % CV of < 30% were considered to be showing presence of parallelism. For parallelism estimation, 169 samples which are 15 % of the total sample size were run at 8 serial dilutions (1:50 to 1:6400). Graphs of samples vs. standard curve were also plotted in order to visualize the concept of parallelism.

2.4.5 Optimum dilution factor for the assay:

Assessing a set of samples at 8 serial dilutions i.e. 1:50 to 1:400 for parallelism, also helped to determine the optimum dilution factor for serum samples. Dilution factor is a crucial parameter in ELISA for determination of accurate concentration of the target analyte. Optimum dilution factor was selected on the basis of Absorbance reading (In linear part of the curve) and recovery calculated with reference standard concentration at that particular dilution.

2.4.6 Determination of cut-off value for Chronic *S.typhi* sero-positivity:

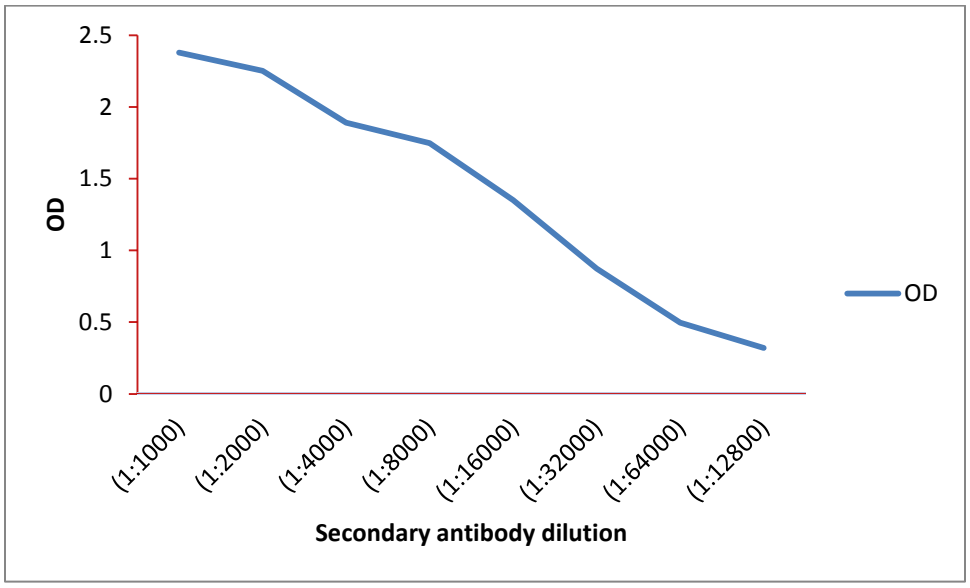
Cut-off value determination depends on the study purpose whether it is to check vaccine efficacy or to screen the population for *S.typhi* infection. The purpose of this study is to assess chronic infection of *S.typhi* in the population by determining concentration of anti Vi IgG in serum samples. The cut-off value for this assay was determined according to a vaccine efficacy trial in Vietnam, which is high endemic region like India. The estimated protective level of anti-Vi IgG is 4.34 μg/ml [55]. Hence, cut-off value for chronic *S.typhi* sero-positivity was decided to be 5 μg/ml.

2.5 Results (Assay optimization)

2.5.1 Determination of Optimal dilution of secondary antibody

The following plot of OD vs. Secondary antibody dilution, clearly shows that 1:1000 resulted in highest OD, followed by 1:2000. OD showed sharp decrease after the dilution of 1:8000. Hence, it was inferred that 1:1000, 1:2000 and 1:4000 were the optimum secondary antibody dilutions for the assay. For this assay, 1:2000 was considered as working dilution for the secondary antibody.**[Figure 2.5]**

Figure 2.5: Plot of OD at various dilutions of secondary antibody

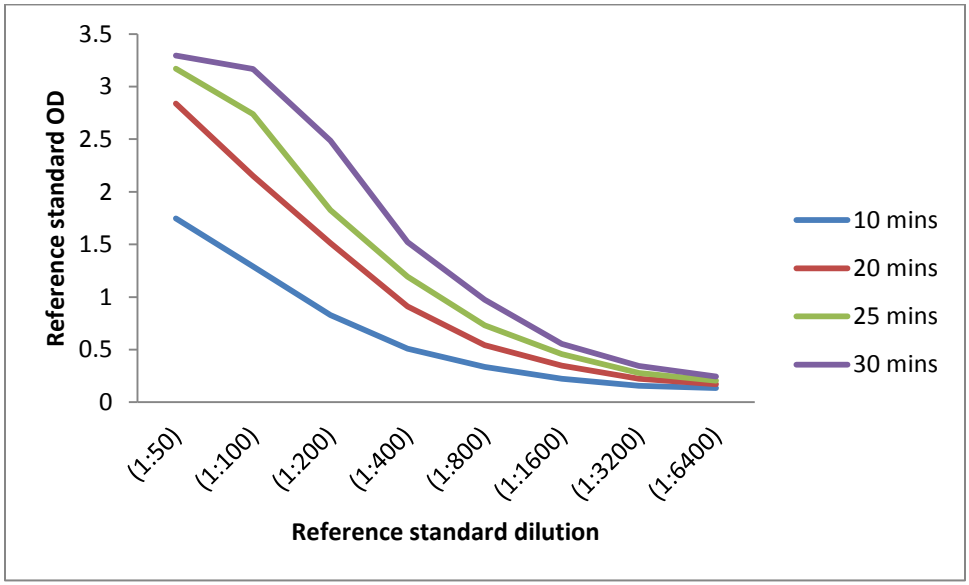


2.5.2 Determination of Optimal Development time for end point signal

The following plot shows Absorbance (OD) of Reference standard curve at various time points. Optimum absorbance range for the standard curve 3.2 to 0.09, resulted at the time points of 25

minutes and 30 minutes. Hence, the end point development time for this assay was decided to be 30 minutes.**[Figure 2.6]**

Figure 2.6: Absorbance (OD) of Reference standard curve at various time points



2.5.3 Generation of Reference standard curve

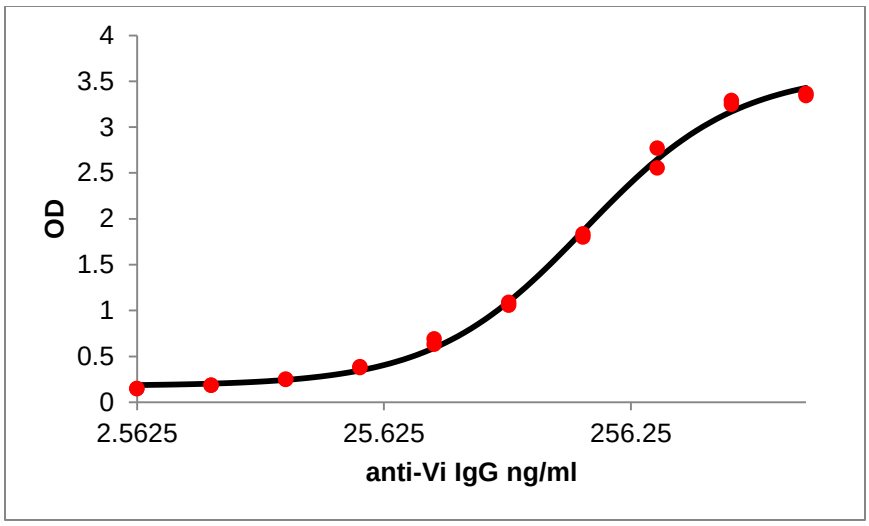
Table 2.1: Anti Vi-IgG reference standard OD values and IgG Concentration at 8 serial dilutions

Anti Vi-IgG Ref standard Dilutions	Absorbance	Avg conc in ng/ml	Avg conc in µg/ml	Recovery
(1:100)	3.25	322.46	32.246	97.72
(1:200)	2.67	161.52	32.304	97.89
(1:400)	1.82	79.62	31.848	96.51
(1:800)	1.04	42.45	33.96	102.91
(1:1600)	0.44	22.12	35.392	107.25

(1:3200)	0.32	11.67	37.344	113.16
(1:6400)	0.18	4.48	28.672	86.88
(1:12800)	0.11	0.67	22.784	69.04

Table 2.1 shows Anti Vi-IgG reference standard Average OD values and Concentration in ng/ml at 8 serial dilutions. Average OD obtained at 1:100 was 3.25. Average concentration at the same dilution was 322.46 ng/ml. Average OD obtained at highest dilution of 1:12800 was 0.11, concentration for the same was 0.67 ng/ml. Recovery was calculated against the known concentration of reference standard which is 33µg/ml. Recovery was in the range of 85%-113%. Exception was at 1:12800, which showed 69.04% of recovery. Typical Reference standard curve is shown in **Figure 2.7**.

Figure 2.7: Reference standard curve (Representative Image)



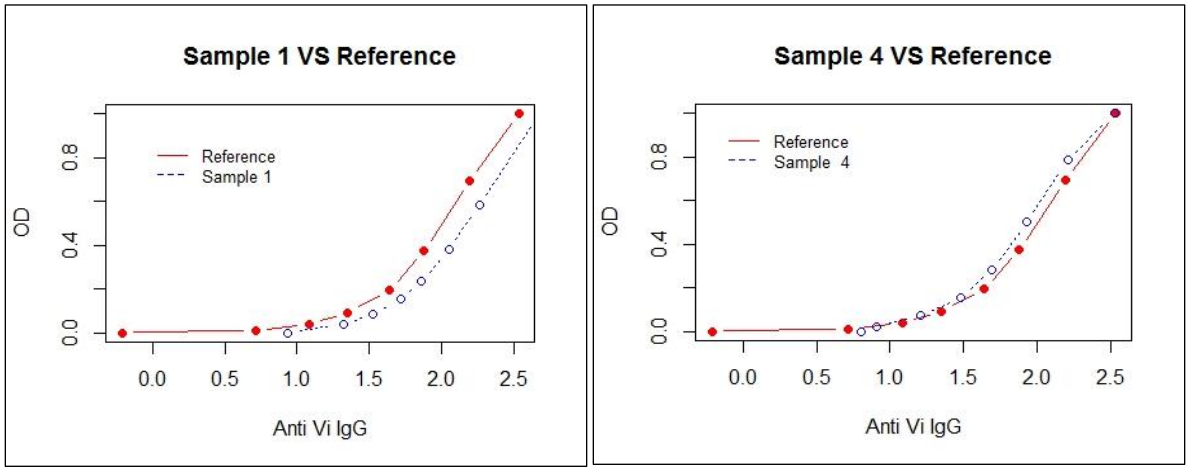
2.5.4 Parallelism:

Table 2.2 Proportion of serum samples showing parallelism:

Samples run at 8 dilutions	No. of samples with CV ≤ 30% (Parallel)	No. of samples with CV < 30% (Non-Parallel)
169	72 (42.60%)	97 (57.4%)

Total 169 samples were run at 8 serial dilutions to check the parallelism. 72 samples showed the % CV of ≤ 30, which were said to be showing presence of parallelism as per the definition. CV ranged from 4.5 % to 29.38 %, in samples showing presence of parallelism.

Figure 2.8 Representative graphs showing parallelism and non-parallelism



Graphs are prepared by R-Version 3.4.2 software.

In Figure 2.8: Sample 1 with parallelism CV= 4.5%, Reference standard and sample 1 dilution curves are parallel

Sample 2 with parallelism CV= 48%, Reference standard and sample 2 dilution curves are not parallel

2.5.5 Optimum dilution factor for the assay: A set of 169 samples were run at 8 serial dilutions (1:50 to 1:6400). Absorbance at 1:50 dilution was out of the range for linear part of reference standard curve. 1:100, 1:200 and 1:400 serum dilutions gave comparable results for absorbance and recovery. Dilutions lower than 1:100 and higher than 1:800 caused inconsistent results with higher variability. Optimum serum dilution factor for this assay could be anyone of the three: 1:100, 1:200 and 1:400. We selected the dilution factor to be 1:400. Anti Vi IgG concentration (unit: μg/ml) in serum sample at 1:400 dilution was considered for the final analysis.

2.5.6 Determination of cut-off value for Chronic *S.typhi* sero-positivity:

Table 2.3 Cut-off value for Chronic S.typhi sero-positivity:

Anti Vi IgG concentration in serum	**Interpretation**
≥ 5 μg/ml	Positive for *S.typhi* chronic infection
< 5 μg/ml	Negative for *S.typhi* chronic infection

2.6 Final assay plan and plate layout:

The assay was planned and completed on total 1084 samples. Samples were tested in duplicates at two dilutions 1:100 and 1:400. GBC cases and controls both were planned on each plate. Each assay plate consisted of 8 serial dilutions of reference standard (1:100 to 1:12800), unknown serum samples to be tested in duplicates in two dilutions (1:100 and 1:400), one positive control sample, one inter-assay QC and one intra assay QC samples. Plate layout was planned as showed in the **figure 2.9**

Figure 2.9 Plate layout of the assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1:100	Std 1:100	S1	S1	S9	S9	S1	S1	S9	S9	PC	PC
B	Std 1:200	Std 1:200	S2	S2	S10	S10	S2	S2	S10	S10	Inter QC	Inter QC
C	Std 1:400	Std 1:400	S3	S3	S11	S11	S3	S3	S11	S11	Intra QC	Intra QC
D	Std 1:800	Std 1:800	S4	S4	S12	S12	S4	S4	S12	S12	PC	PC
E	Std 1:1600	Std 1:1600	S5	S5	S13	S13	S5	S5	S13	S13	Inter QC	Inter QC
F	Std 1:3200	Std 1:3200	S6	S6	S14	S14	S6	S6	S14	S14	Intra QC	Intra QC
G	Std 1:6400	Std 1:6400	S7	S7	S15	S15	S7	S7	S15	S15	Blank	Blank
H	Std 1:12800	Std 1:12800	S8	S8	S16	S16	S8	S8	S16	S16	Blank	Blank

(1:100) Dilution

(1:400) Dilution

(1:100) Dilution

(1:400) Dilution

Chapter 3

Association of *Salmonella typhi* and Gallbladder cancer

Chapter 3: Association of *Salmonella typhi* and Gallbladder cancer

3.1 Background:

This study is part of a hospital based case-control study evaluating risk factors of Gallbladder cancer, conducted in Centre for Cancer Epidemiology, Tata Memorial Centre-Mumbai, India. The study has been approved by the Tata Memorial Hospital's Institutional review board. Written informed consent was obtained from all study participants before enrolment in the study.

ELISA for detection of chronic *Salmonella typhi* infection was optimized (Chapter 2). This standardized assay was then utilized for analyzing the association of *Salmonella typhi* and Gallbladder cancer. Blood samples analysed in this study were collected during the period of September 2010 to June 2015 along with a structured questionnaire.

3.2 Eligibility criteria for cases and Controls:

Cases: Cases were male or female patients with primary GBC (International Classification of Diseases for Oncology Version 3 [ICDO-3] site code C24) visiting TMH, Mumbai for diagnosis and/or treatment.

Case eligibility criteria were:

(i) Primary GBC confirmation based on histological/cytological diagnosis from gallbladder, or unequivocal clinical and imaging diagnostic such as abdominal ultrasound and/or abdominal computed tomography/magnetic resonance imaging from gallbladder and histological/cytological finding from metastatic site

(ii) Date of diagnosis less than one year from date of enrolment

(iii) Age between 20-70 years

(iv) Resident of India for at least one year

Patient with malignancies arising from the ampulla of Vater (C24.1), overlapping lesions of the biliary tract (C24.8), unspecified regions of the biliary tract (C24.9), and any other malignancies were excluded from the study. Patient's diagnostic details were acquired from hospital electronic medical record (EMR) system.

Controls: Controls were recruited from visitors with no history of cancer accompanying patients of any cancer site to the TMH. Visitor controls aged 20-70 years were recruited from all Disease Management Groups (DMGs) of the hospital (excluding relatives of GBC cases).The proportion of controls from visitors from each DMG did not exceed 20%. The majority of controls were friends, neighbors, colleagues, in-laws and spouses and other than first degree relatives.

3.3 Data from structured Questionnaire:

Every study participant was interviewed by trained staff on the basis of a structured questionnaire designed at Centre for Cancer Epidemiology, Tata Memorial Centre. Interviews had been conducted according to the working manual for Multi-site case-control studies [60].

Information on following variables from the questionnaire was taken into consideration for this study:

1. Age
2. Gender
3. Area of residence

4. Education: Study participants were categorized into six categories on the basis of education namely illiterate, literate, less than 5 years of schooling, 5-8 years of schooling, High school and college & above.
5. Gallstone history: Ascertainment of gallstone status was based on self-reports of gallstone history by study participant. Following the two different definitions were used for categorizing study participants on their gallstone history:

   Self-reported gallstone: as reported by study participants i.e. either present / not present

   Self-reported gallstone using stringent definition: Gallstone history was ascertained using definition of self-reported gallstone; however those gallstones diagnosed within a year prior to the date of diagnosis of gallbladder cancer for cases or within a year prior to the date of interview for controls were categorized as ―not present. Otherwise was categorized as ―present.

3.4 Blood sample collection:

A 10 ml blood sample was collected from each study participant and fractionated into serum and red blood cells (RBC‘s). The Serum sample was stored immediately at -80^{0}C and then transferred to -196°C in liquid nitrogen cylinder for long-term storage.

3.5 Frequency matching of cases and controls:

Cases and controls were then frequency matched on the basis of age (± 10 years) and area of residence. For area of residence, India was divided into five regions: North, north-east, west, central and south.

1. North (Uttar Pradesh, Bihar, Delhi, Haryana, Himachal Pradesh, Jammu and Kashmir, Punjab, Rajasthan, Chandigarh and Uttarakhand)
2. North-East (Arunachal Pradesh, Assam, Meghalaya, Nagaland, Manipur, Tripura, West Bengal, Jharkhand and Orissa)
3. West (Goa, Gujarat, Daman & Diu, Dadra & Nagar Haveli, Maharashtra)
4. South (Andhra Pradesh, Karnataka, Kerala, Lakshadweep, Andaman & Nicobar, Tamil Nadu, Telangana)
5. Central (Madhya Pradesh and Chhattisgarh)

3.6 Sample size:

Samples were randomly selected from the pool of previously collected 1170 GBC cases and 2525 Controls. For the assay, 1188 serum samples, including 612 GBC cases and 576 controls were analysed in the present study. Sample size was determined taking into consideration prevalence of *S.typhi* in Indian population (~ 10%), power of 80%, Case to control ratio of 1 and expected Odds ratio of 1.7.

3.7 Quality Assessments:

3.7.1 Quality control of Questionnaire data: Questionnaire data on variables such as age, gender, education, gallstone-history and residential information utilized in the present study had been previously subjected to data quality checks according to a comprehensive instruction manual [61]. Quality /logical checks had been introduced in the electronic database to minimize transcription and typing errors. Data cleaning was commenced once data was entered twice in the electronic database. Double data entry had been carried out by two different data entry typists to capture data entry errors or missingness. Regular examinations had been carried out on all questionnaires for human errors and missingness in data after conducting the interviews and after the data were entered in the electronic database. Completeness of data assessment was accomplished at three different levels: data were first examined by the interviewer, immediately after taking the interview, second by the project co-coordinator, on the day following the interview, and by the data entry typist before entering the data. Reproducibility of the questionnaire was ensured by an abbreviated reproducibility questionnaire.

3.7.2 Quality Control of the Assay: Stringent quality control measures were followed at various stages of the assay.

3.7.2 (A) Laboratory operations: All the assays were performed using Good Laboratory Practices (GLP). Laboratory operations involved, buffers preparations/handlings, equipment calibration and monitoring/ maintenance of laboratory temperature/humidity. Sterile filters, containers and sterile, de-pyrogenated reagent grade water were used for reagents and buffers preparation. All glassware used for preparing buffers/solutions were washed and rinsed with pyrogen free water, de-pyrogenated by heating in an oven at 180°C for minimum 2 hours to

remove endotoxin, or endotoxin free plastic was used. All containers for solutions were labeled with the reagent name, date prepared, name of preparer, and expiration date. All reagents/buffers and plastic/glassware were stored aseptically at required temperature. Before use, buffers/solutions were checked for signs of any contamination, which may include flocculence, unusual color or cloudiness. Cleaning, sterilization and de-pyrogenation of supplies were documented for investigations of atypical results or unacceptable rate of invalid assays.

All equipments, particularly automated liquid handling platform were periodically monitored for maintenance and calibration purpose for expected performance.

3.7.2 (B) Monitoring freeze- thaw cycles: Serum samples to be evaluated were aliquoted and stored at – 80^{o} C. Antigen, reference standard and secondary antibody were also aliquoted and stored at minus 80^{o} C. Usage of all the aliquots was documented. Aliquot was discarded after the 3rd freeze-thaw cycle of the usage. This precautionary measure was taken to avoid variations in assay results due to temperature storage fluctuations.

3.7.2 (C) Quality control for serum samples and assay validity criteria: Two sero-positive samples with acceptable results, obtained by at least 10 assays on each serum samples (The range represents Mean + 2SD determined in 10 assays) , were selected as in-house positive control samples. Positive Quality control sample values were checked for established range (which is ± 2 SD of mean), or the plate was rejected and samples re-analyzed. The absorbance reading of serial dilution of the reference standard was checked for their absorbance, which should be in the range of 3.0-3.4 (at 1:100 dilution) and 0.09-0.1 (at 1:12800 dilution), or else the assay plate was re-analysed. Serum samples were tested in duplicates. If the CV of the absorbance value of duplicates (termed as intra-assay CV) is > 15%, the corresponding anti-Vi IgG concentration of

the said sample was excluded from the final analysis. The absorbance reading of serial dilution of the reference standard was checked for their absorbance, which should be in the range of 3.0-3.4 (at 1:100 dilution) and 0.09-0.1 (at 1:12800 dilution), or else the assay plate was re-analysed.

Inter-assay Quality control or reproducibility was checked on approximately 5% of the total sample size. The criterion for reproducibility was, if the CV of inter-assay check is $\leq$ 30%.

3.8 Statistical Analysis:

Proportion of chronic *S.typhi* sero-positivity for present study population was analysed according to gender, age, education and current residence. Proportion was according to the cut-off definition of the present study; calculated by region-wise stratification of the study population irrespective of case/control status. Correlation of region-wise incidence of GBC and proportion of chronic *S.typhi* sero-positivity was analysed. Region-wise incidence rates were average of incidence rates of PBCRs of that particular region published in NCRP report (2012-2014). [12].

Crude and adjusted odds ratios with 95% confidence interval were calculated for chronic *S.typhi* infection by unconditional logistic regression models [62]. Potential confounders like age, area of residence (North, north-east, west, south and central), education, gender, gallstone history (present/not present) were taken into consideration in the analysis. Analyses were also done separately for each confounder including gender, education, gall-stone history by stratification. Study participants were classified into four groups according to anti-Vi IgG concentrations in serum using quartiles of the control population. Quartile analyses were done taking into account each confounding variable. P-value for linear trend was estimated for each quartile analysis. Analysis was done separately for Gangetic belt states (Uttarakhand, Uttar Pradesh, Bihar and

West- Bengal) and non- gangetic belt states (states other than gangetic belt). Joint effect of gallstone and chronic *S.typhi* infection was also analysed by fitting interaction term in unconditional logistic regression model. All analyses were done by STATA 15.0 [63].

3.9 Results:

1188 serum samples, including 612 GBC cases and 576 controls were analysed for detection of chronic *S.typhi* infection.

3.9.1 Quality Assessment: According to assay validity criteria based on intra-assay %CV, reference standard range and in-house positive control range; 58 GBC cases and 46 controls sample readings were excluded from the association analysis (**Figure 3.1**). Hence, final analysis was conducted on 554 GBC cases and 530 controls after quality assessment. Reproducibility or inter-assay quality control was assessed for approximately 5% of the total samples. 90.1% of the samples showed reproducible results.

3.9.2 Demographic characteristics of study participants: Table 3.1 shows characteristics of total 1084 study participants with respect to age, gender, current residential region, education and gall-stone history. Male to female ratio in GBC cases was found to be 1:2.4 in the present study population, which is in accordance with the literature. Mean age of cases and controls were 49.94 and 49.20 years respectively. P-values by chi-squared test were non-significant (for males and females). This indicates, there is no significant difference between cases and controls with respect to age and current residence; confirming the successful frequency matching. Greater percentage of GBC cases were from North and North-east regions of India.

3.9.3 Proportion of Chronic *S.typhi* sero-positivity: Chronic *S.typhi* sero-positivity was higher in GBC cases (Females: 64.12% & Males: 64.59%) than in controls (Females: 46.94% and Males: 48.36%)(**Table 3.2**). As mentioned in **Table 3.3**, North [(GBC cases= Females: 30.02% & Males: 24.22%) & (Controls=Females: 18.30%& Males: 22.22%)] and North-east [(GBC cases=Females: 21.88% & Males: 28.57% & (Controls= Females: 18.56% & Males: 20.26%)] regions showed higher proportion of chronic *S.typhi* sero-positivity as compared to other three regions. As showed in **Table 3.6**, there was region-wise correlation of GBC incidence and chronic *S.typhi* sero-positivity; showing higher proportion in the regions of high incidence (North= Incidence:8.5, proportion:23.98% & North-east= Incidence:8.1 & proportion:21.49%) and lower proportion in the regions of lower incidence (West= Incidence:2, proportion:7.19% & Central= Incidence:4.5, Proportion:3.22%).

Table 3.4 shows *S.typhi* sero-positivity proportion in various educational categories, which does not show any notable difference. *S.typhi* sero-positivity proportion is higher in older age-groups for both cases and controls (**Table 3.5**).

3.9.4 Association of Chronic *Salmonella typhi* infection and Gallbladder Cancer (Overall):

In an unconditional multivariate logistic regression model, adjusted for age (continuous variable), gender, current residence (North, north-east, west, central & south) and education (Illiterate, Literate, <5 years schooling, 5-8 years of schooling, high school, college and above) moderate and statistically significant risk was observed for all participants ($OR_{\text{all participants}}$: 1.91, 95% CI:1.48-2.46). Gender specific analysis found no remarkable difference in the magnitude of the risk ($OR_{females}$: 1.91, 95%CI: 1.41-2.59 & OR_{males}: 2.01, 95%CI: 1.25-3.22). In addition to age, gender, current residence and education; analysis was done taking into account gallstone-

history. A slight decrease in the risk was observed (OR $_{\text{all participants}}$: 1.73, 95% CI: 1.30-2.31) **[Table 3.7].**

Table 3.8 shows quartile (based on control distribution) analysis of anti-Vi-IgG Concentration. A strong, statistically significant, dose- response effect was observed, with highest quartile showing OR $_{\text{all participants}}$=3.18 (95% CI: 2.17-4.66). Gender specific analysis also showed a strong, statistically significant dose- response effect.

As mentioned in **Table 3.11**, the risk was estimated in three strata of education (Illiterate, literate-5-8 years of schooling, high school & above). There was no notable difference in the risk across the strata.

3.9.5 Association of Chronic *Salmonella typhi* infection and Gallbladder Cancer (for Gangetic belt states):

A separate analysis was done to find out the risk in Gangetic belt states (Uttarakhand, UP, Bihar, West Bengal). **Table 3.9** shows; the risk was higher in states excluding gangetic belt (OR$_{\text{all participants}}$= 2.25, 95% CI: 1.49-3.41) than in gangetic belt states (OR $_{\text{all participants}}$= 1.77, 95% CI: 1.28-2.45). Quartile analysis of the same shows a strong, statistically significant, dose-response effect with higher OR in highest quartile (Q4) (**Table 3.10**).

3.9.6 Joint effect of Chronic *S.typhi* sero-positivity and Gallstones:

Table 3.12 shows joint effect of chronic *S.typhi* infection and gallstones. More than additive but less than multiplicative, statistically non-significant interaction was observed (OR $_{\text{interaction}}$=28.71, 95%CI: 15.70-52.52). The same analysis was done using the gallstones (by stringent definition), no statistically significant interaction was observed (**Table 3.13**).

Figure 3.1: Flow chart for Quality assessment

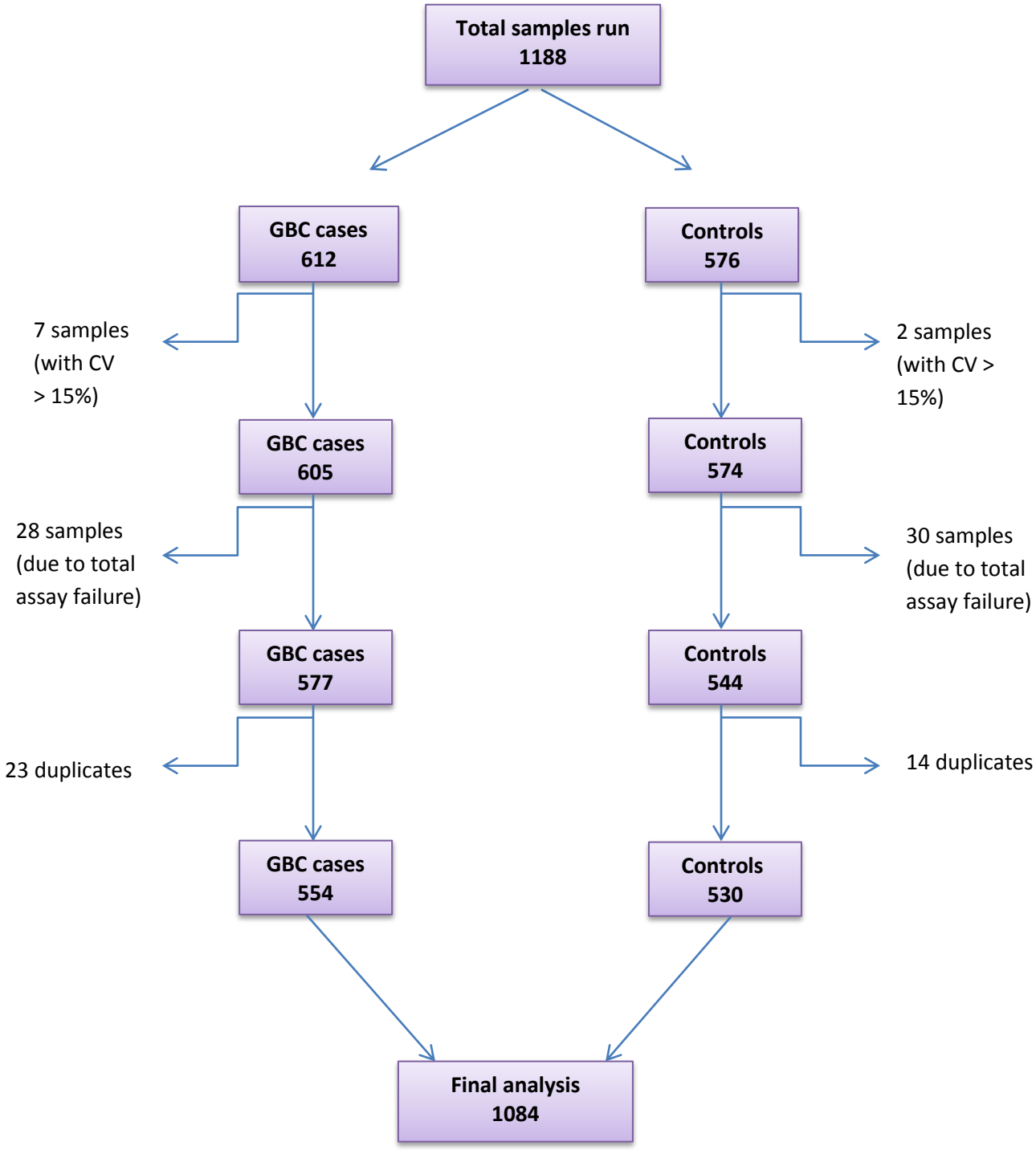


Table 3.1: Demographic characteristics of study participants

Demographic Information	Category	GBC cases (N= 554)		Controls (N= 530)	
		Males (N=161)	Females (N=393)	Males (N=153)	Females (N=377)
Age	18 -29	8 (4.97%)	5 (1.27 %)	9 (5.88%)	5 (1.33%)
	30 -39	22 (13.66%)	40 (10.18%)	19 (12.42%)	47 (12.47%)
	40 -49	38 (23.60%)	131 (33.33%)	33 (21.57%)	137 (36.34%)
	50 -59	51 (31.68%)	149 (37.91%)	49 (32.03%)	131 (34.75%)
	60+	42 (26.09%)	68 (17.30%)	43 (28.10%)	57 (15.12%)
	Mean	49.94		49.2	
	P-value	Male= 0.514 & Female= 0.505			
Current Residence	North	66 (40.99%)	185 (47.07%)	64 (41.83%)	163 (43.24%)
	South	0	0	1 (0.65%)	0
	West	22 (13.66%)	53(13.49%)	19 (12.42%)	57 (15.12%)
	North-East	66 (40.99%)	127 (32.32%)	60 (39.22%)	121 (32.10%)
	Central	7 (4.35%)	28 (7.12%)	9 (5.88%)	36 (9.55%)
	P-value	Male = 0.812		Female= 0.503	
Education	Illterate	12 (7.45%)	152 (38.68%)	6 (3.92%)	61(16.18%)
	Literate	3 (1.86%)	20 (5.09%)	5 (3.27%)	16 (4.24%)
	Less than 5 years of schooling	15 (9.32%)	30 (7.63%)	7 (4.58%)	16 (4.24%)
	5-8 years of schooling	37 (22.98%)	85 (21.63%)	27 (17.65%)	84 (22.28%)
	High School	47 (29.19%)	69 (17.56%)	46 (30.07%)	109 (28.91%)
	College graduation and more	47 (29.19%)	34 (8.65%)	62 (40.52%)	91 (24.14%)
	P-value	Male=0.115		Female= <0.001	
Gallstone History	Yes	58 (36.02%)	182 (46.31%)	3 (1.96%)	20 (5.31%)
	No	101 (62.73%)	201 (51.15%)	150 (98.04%)	355 (94.16%)
	P-value	Male = <0.001		Female = <0.001	

Table 3.2 Chronic *S.typhi* reactivity in study participants

Chronic S.typhi reactivity	GBC (N=554)		Controls (N= 530)	
	Females (N=393)	Males (N=161)	Females (N=377)	Males (N=153)
***S.typhi* sero-negative**	141 (35.87%)	57 (35.4%)	200 (53.05%)	79 (51.63%)
***S.typhi* sero-positive**	252 (64.12%)	104 (64.59%)	177 (46.94%)	74 (48.36%)

Table 3.3 Proportion of Chronic *S.typhi* positivity, stratified by residential status

Residential region	GBC (N=554)		Controls (N= 530)	
	Females (N=393)	Males (N=161)	Females (N=377)	Males (N=153)
North	118 (30.02%)	39 (24.22%)	69 (18.30%)	34 (22.22%)
South	0 (0%)	0 (0%)	0 (0%)	1 (0.65%)
West	34 (8.6%)	14 (8.69%)	27 (7.16%)	3 (1.96%)
North-east	86 (21.88%)	46 (28.57%)	70 (18.56%)	31 (20.26%)
Central	14 (3.56%)	5 (3.10%)	11 (2.91%)	5 (3.26%)

Table 3.4 Proportion of Chronic *S.typhi* positivity, stratified by Education

Education	GBC (N=554)		Controls (N= 530)	
	Females (N=393)	Males (N=161)	Females (N=377)	Males (N=153)
Illeterate	100 (25.44%)	8 (4.96%)	32 (8.48%)	4 (2.61%)
Literate	13 (3.30%)	1 (0.62%)	8 (2.12%)	3 (1.96%)
Less than 5 years of schooling	23 (5.85%)	10 (6.21%)	9 (2.38%)	4 (2.61%)
5-8 years of schooling	50 (12.72%)	24 (14.9%)	34 (9.01%)	12 (7.84%)
High school	39 (9.92%)	30 (18.63%)	52 (13.79%)	23 (15.03%)
College graduation and above	25 (6.36%)	31 (19.25%)	42 (11.14%)	28 (18.3%)

Table 3.5 Proportion of Chronic *S.typhi* positivity, stratified by Age-group

Age-group (Years)	GBC (N=554)		Controls (N= 530)	
	Females (N=393)	Males (N=161)	Females (N=377)	Males (N=153)
18-29	4 (1.01%)	3 (1.86%)	2 (0.53%)	2 (1.30%)
30-39	28 (7.12%)	11 (6.83%)	19 (5.03%)	8 (5.22%)
40-49	81 (20.61%)	24 (14.9%)	66 (17.50%)	10 (6.53%)
50-59	90 (22.9%)	35 (21.73%)	64 (16.97%)	28 (18.3%)
60 and above	49 (12.46%)	31 (19.25%)	26 (6.89%)	26 (16.99%)

Table 3.6 Region-wise correlation of Incidence of GB cancer and proportion of Chronic *S.typhi* Infection

Region	North	North-east	West	Central
Average Incidence rate (AIR)/100,000	8.5	8.1	2	4.5
Proportion of chronic *S.typhi* infection	23.98%	21.49%	7.19%	3.22%

Average Incidence rate/100,000: Average of incidence rates of PBCRs of that particular region published in NCRP report (2012-2014)

Proportion of chronic *S.typhi* sero-positivity: according to the cut-off definition of the present study; calculated by region-wise stratification of the study population irrespective of case/control status

Table 3.7: Association of Chronic *Salmonella typhi* infection and Gallbladder Cancer (For All participants and gender-wise stratification)

Chronic S.typhi infection	N(Cases|Controls)	OR[a]	95% CI[a]	P value[a]	OR[b]	95% CI[b]	P value[b]
All Participants (N=1084, Cases=554, Controls=530)							
Sero-negative	198|279	Reference			Reference		
Sero-positive	356|251	**1.91**	(1.48 -2.46)	<0.001	**1.73**	(1.30 -2.31)	<0.001
Females (N=770, Cases=393, Controls=377)							
Sero-negative	141|200	Reference			Reference		
Sero-positive	152|177	**1.91**	(1.41 -2.59)	< 0.001	**1.7**	(1.20 -2.40)	0.002
Males (N=314, Cases=161, Controls=153)							
Sero-negative	57|79	Reference			Reference		
Sero-positive	104|74	**2.01**	(1.25 -3.22)	0.004	**1.8**	(1.06 -3.04)	0.029

Abbreviations:CI – Confidence Interval, N – Number, OR – Odds Ratio

[a]Adjusted for Age (Continuous), gender, current residence (North,north-east,west,central & south), education (Illeterate,Literate, <5 years schooling, 5-8 years of schooling, high school, college and above)

[b]Adjusted for Age (Continuous), gender, current residence (North,north-east,west,central & south), education (Illeterate,Literate, <5 years schooling, 5-8 years of schooling, high school, college and above) and Gallstone history (Present/not present)

Table 3.8: Association of Chronic *Salmonella typhi* infection and Gallbladder Cancer (Quartile analysis) (For All participants and gender-wise stratification)

Anti-Vi IgG concentration quartiles (Mean, Median, IQR)	N(Cases|Controls)	OR[a]	95% CI[a]	P value[a]	OR[b]	95% CI[b]	P value[b]
All participants (N=1084, Cases=554, Controls=530)							
Q1 (1.62,1.86,1.7)	63|133	Reference			Reference		
Q2 (3.86, 3.86, 1.01)	123|132	**1.79**	(1.20 -2.68)	0.004	**1.53**	(0.97 -2.42)	0.062
Q3 (6.01,5.97,1.27)	149|133	**2.15**	(1.45 -3.18)	<0.001	**1.85**	(1.18-2.88)	0.006
Q4 (16.20,11.32,7.16)	219|132	**3.18**	(2.17 -4.66)	<0.001	**2.65**	(1.72- 4.08)	<0.001
P trend				<0.001			<0.001
Females (N=770, Cases=393, Controls=377)							
Q1 (1.62,1.86,1.7)	48|90	Reference			Reference		
Q2 (3.86, 3.86, 1.01)	87|99	**1.49**	(0.92 -2.40)	0.098	**1.1**	(0.64 -1.88)	0.724
Q3 (6.01,5.97,1.27)	106|100	**1.75**	(1.10 -2.79)	0.018	**1.34**	(0.79 -2.26)	0.269
Q4 (16.20,11.32,7.16)	152|88	**2.97**	(1.87 -4.69)	< 0.001	**2.15**	(1.29 -3.59)	0.003
P trend				<0.001			<0.001
Males (N=314, Cases=161, Controls=153)							
Q1 (1.62,1.86,1.7)	15|43	Reference			Reference		
Q2 (3.86, 3.86, 1.01)	36|33	**3.11**	(1.45 -6.66)	0.004	**4.04**	(1.61 -10.14)	0.003
Q3 (6.01,5.97,1.27)	43|33	**3.84**	(1.80 -8.16)	<0.001	**4.6**	(1.85 -11.43)	0.001
Q4 (16.20,11.32,7.16)	67|44	**4.59**	(2.20 -9.56)	<0.001	**5.44**	(2.23 -13.23)	<0.001
P trend				<0.001			0.001

Abbreviations:CI – Confidence Interval, N – Number, OR – Odds Ratio

[a]Adjusted for Age (Continuous), gender, current residence (North,north-east,west,central & south), education (Illeterate,Literate, <5 years schooling, 5-8 years of schooling, high school, college and above)

[b]Adjusted for Age (Continuous), gender, current residence (North,north-east,west,central & south), education (Illeterate,Literate, <5 years schooling, 5-8 years of schooling, high school, college and above) and Gallstone history (Present/not present)

Table 3.9: Association of Chronic *Salmonella typhi* infection and Gallbladder Cancer for all participants (Gangetic belt states and other states excluding gangetic belt states)

Chronic *S.typhi* infection	N(Cases|Controls)	OR[c]	95% CI[c]	P value[c]	OR[d]	95% CI[d]	P value[d]
(Gangetic Belt states) (N= 651, Cases= 349, Controls=302)							
Sero-negative	127|155	Reference			Reference		
Sero-positive	222|147	**1.77**	(1.28 - 2.45)	0.001	**1.64**	(1.13 -2.38)	0.009
Other states excluding gangetic belt (N=433, cases=205 , controls=228)							
Sero-negative	71|124	Reference			Reference		
Sero-positive	134|104	**2.25**	(1.49 -3.41)	<0.001	**1.94**	(1.22 -3.08)	0.005

Abbreviations:CI – Confidence Interval, N – Number, OR – Odds Ratio

[c]Adjusted for Age (Continuous), gender, education (Illeterate,Literate, <5 years schooling, 5-8 years of schooling, high school, college and above)

[d]Adjusted for Age (Continuous), gender, education (Illeterate,Literate, <5 years schooling, 5-8 years of schooling, high school, college and above) and Gallstone history (Present/not present)

Gangetic belt states include: Uttarakhand, Uttar Pradesh, Bihar, West Bengal, Non-gangetic belt states: All States excluding gangetic belt states

Table 3.10: Association of Chronic *Salmonella typhi* infection and Gallbladder Cancer for all participants (Quartile Analysis) (Gangetic belt states and other states excluding gangetic belt states)

Anti-Vi IgG concentration quartiles Chronic *S.typhi* infection (Mean, Median, IQR)	**N(Cases|Controls)**	**OR[c]**	**95% CI[c]**	**P value[c]**	**OR[d]**	**95% CI[d]**	**P value[d]**
(Gangetic Belt states) (N= 651, Cases= 349, Controls=302)							
Q1 (1.62,1.86,1.7)	47|76	Reference			Reference		
Q2 (3.86, 3.86, 1.01)	76|75	1.37	(0.83 -2.26)	0.215	1.15	(0.65 -2.05)	0.621
Q3 (6.01,5.97,1.27)	98|76	1.77	(1.09 -2.89)	0.021	1.49	(0.85 -2.61)	0.154
Q4 (16.20,11.32,7.16)	128|75	2.46	(1.53 -3.97)	<0.001	2.15	(1.25 -3.71)	0.005
P trend				<0.001			0.002
Non-gangetic belt states (N=433, cases=205 , controls=228)							
Q1 (1.62,1.86,1.7)	19|57	Reference			Reference		
Q2 (3.86, 3.86, 1.01)	38|57	2.37	(1.17 -4.80)	0.016	2.1	(0.97 -4.56)	0.059
Q3 (6.01,5.97,1.27)	64|57	4.14	(2.12 -8.08)	<0.001	3.68	(1.77 -7.65)	<0.001
Q4 (16.20,11.32,7.16)	84|57	4.9	(2.55 -9.41)	<0.001	3.62	(1.76 -7.42)	<0.001
P trend				<0.001			<0.001

Abbreviations:CI – Confidence Interval, N – Number, OR – Odds Ratio

[c]Adjusted for Age (Continuous), gender, education (Illeterate,Literate, <5 years schooling, 5-8 years of schooling, high school, college and above)

[d]Adjusted for Age (Continuous), gender, education (Illeterate,Literate, <5 years schooling, 5-8 years of schooling, high school, college and above) and Gallstone history (Present/not present)

Gangetic belt states include: Uttarakhand, Uttar Pradesh, Bihar, West Bengal, Non-gangetic belt states: All States excluding gangetic belt states

Table 3.11: Association of Chronic Salmonella typhi infection and Gallbladder Cancer for all participants, stratified by education

Chronic S.typhi infection	Illterate (N=231, cases=164, controls=67)			Literate (upto 8 years of schooling) (N=345, cases=190, controls=155)			High school and Above (N= 505, cases=197, controls=308)		
	(Ca|Co)	OR(95% CI)[e]	P- value[e]	(Ca|Co)	OR(95% CI)[e]	P- value[e]	(Ca|Co)	OR(95% CI)[e]	P- value[e]
Sero-negative	56|31	Reference		69|85	Reference		72|163	Reference	
Sero-positive	108|36	1.79 (0.93-3.42)	0.07	121|70	1.77 (1.10-2.86)	0.019	125|145	1.78 (1.14-2.76)	0.01
Anti-Vi IgG concentration quartiles Chronic *S.typhi* infection (Mean, Median, IQR)									
Q1 (1.62,1.86,1.7)	15|10	Reference		25|44	Reference		23|79	Reference	
Q2 (3.86, 3.86, 1.01)	36|20	1.41 (0.46 -4.27)	0.53	39|39	1.63 (0.79 -3.37)	0.18	47|73	1.87 (0.92 -3.77)	0.08
Q3 (6.01,5.97,1.27)	48|20	1.67 (0.56 -4.97)	0.35	51|36	2.27 (1.12 -4.61)	0.02	50|77	1.92 (0.96 -3.86)	0.06
Q4 (16.20,11.32,7.16)	65|17	3.38 (1.14 -10.01)	0.02	75|36	2.70 (1.35 -5.43)	0.005	77|79	2.94 (1.51 -5.72)	0.001
P trend			0.012			0.003			0.002

Abbreviations:CI – Confidence Interval, N – Number, OR – Odds Ratio

OR[e] is adjusted for Age (Continuous), gender, current residence (North,north-east,west,central & south), Gallstone history (Present/not present)

Table 3.12: Joint effect of Chronic *S.typhi* infection and Gallstones

Chronic S.typhi infection	Gallstone				P value (Interaction)
	No		Yes		
	N(Cases|Controls)	OR[f] (95% CI, P-value)[f]	N(Cases|Controls)	OR[f] (95% CI, P-value)[f]	
Sero-negative	114|269	Reference	81|9	23.45 (11.20 -49.09 ,<0.001)	0.417
Sero-positive	194|236	1.80 (1.33 -2.45, <0.001)	159|14	28.71 (15.70 -52.52)	

Abbreviations:CI – Confidence Interval, N – Number, OR – Odds Ratio

OR[f] is adjusted for Age (Continuous), gender, current residence (North,north-east,west,central & south), education (Illeterate,Literate, <5 years schooling, 5-8 years of schooling, high school, college and above)

Table 3.13: Joint effect of Chronic *S.typhi* infection and Gallstones (By stringent definition)

Chronic S.typhi infection	Gallstone				P value (Interaction)
	No		Yes		
	N(Cases|Controls)	OR[g] (95% CI, P-value)	N(Cases|Controls)	OR[g] (95% CI, P-value)	
Sero-negative	160|263	Reference	35|8	7.76 (3.45-17.46,<0.001)	0.109
Sero-positive	310|231	2.12 (1.62- 2.79, <0.001)	43|11	6.87 (3.37-13.98)	

Abbreviations:CI – Confidence Interval, N – Number, OR – Odds Ratio

OR[g] is adjusted for Age (Continuous), gender, current residence (North,north-east,west,central & south), education (Illeterate,Literate, <5 years schooling, 5-8 years of schooling, high school, college and above)

Chapter 4

Discussion

4. Discussion

The present study was carried out considering two objectives, 1. To develop a reliable tool for detection of chronic *Salmonella typhi* infection and 2. To find association of chronic *Salmonella typhi* infection and gall bladder cancer. This is the first study of its kind which has been carried out on a large population; on 1084 individuals.

4.1 Standardization of ELISA for detection of chronic infection of *S.typhi*:

The protocol used for this assay was initially developed for the purpose of studying immune response of Vi conjugate vaccines by National Institutes of Health . We optimized and validated this protocol for the detection of Anti-Vi-IgG antibodies in serum samples. Sample storage is very essential aspect which should be considered to get optimum results in serological assays [64]. We maintained aliquots of serum samples, antigens, reference standard and secondary antibody to nullify the effect of temperature fluctuations at each freeze and thaw cycle. All dilutions and pipetting work was done on automated liquid handling platform. Above two points improved precision of the overall assay.

Stringent quality control criteria were followed for entire assay procedure. All equipments, particularly automated liquid handling platform were periodically monitored for maintenance and calibration purpose for expected performance.

Another strength of this study is that the entire assay was done on two dilutions which further helped in validation. We observed the hook effect in few positive samples, where we got low signal at 1:50 dilution as compared to that of 1:100 dilution. 1:100, 1:200 and 1:400 serum dilutions gave comparable results, hence can be considered as optimum dilutions for this assay.

15 % of samples were also run at 8 serial dilutions 1:50 to 1:6400 to check the phenomenon of parallelism. This is the first study with large sample size which assessed the concept of parallelism. Approximately 42.6 % of the samples followed parallelism. There can be multiple factors in serum samples like auto-antibodies, anti-coagulants, enzyme inhibitors etc. due to which some samples failed to show the presence of parallelism. Reproducibility which is a crucial criterion in validation of any serological assay [65] was also found to be satisfactory, with 90.1% of samples showing Inter-assay CV of < 30%. R^2 of each reference standard curve was in the range of 0.97 to 0.99, which is considered ideal.

Cut-off value to determine the positivity depends on the study purpose whether it is to check vaccine efficacy or to screen the population for *S.typhi* infection. The commonly used strategy for cut-off value estimation in ELISAs is Mean value of negative control samples +3SD. However, we did not have known *Salmonella typhi* negative control population to consider this strategy. According to the assay-purpose which is to detect chronic infection, the cut-off value for this assay was determined according to a vaccine efficacy trial in Vietnam, which is high endemic region like India.

The only limitation regarding the standardization of this assay is that, we were unable to estimate the sensitivity and specificity. However, with the proper gold standard technique it can be done in future.

4.2 Association of Chronic infection of *Salmonella typhi* and Gallbladder cancer:

A detailed analysis was undertaken to study the association of chronic *S.typhi* infection and gallbladder cancer. Although it is a hospital based case-control study, Tata Memorial hospital being the referral cancer centre in India caters to patients from all over the country. This makes

the present study population a closest representative of Indian population. One of the strengths of this study is the population in which it was carried out. Indian population is the ideal study population for this hypothesis, because of its epidemic status for *Salmonella typhi* infection as well as for high incidence of GBC. All cases were histopathologically/cytopathogically confirmed. Controls were visitors in TMH from different DMGs, frequency matched on age, gender and area of residence to minimize the selection bias. Samples were randomly selected from the pool of previously collected samples, further minimized the selection bias. Detailed information of each study participant on various variables like age, gender, current residence, education and gallstone history allowed for assessment and control for confounders.

This study to find the association of chronic *Salmonella typhi* infection and GBC was based only on serology. We could not do PCR/sequencing for *S.typhi* in gall bladder tumor tissues, which would have further strengthened the association. Proportion of sample exclusions on the basis of assay validity criteria, was same in both case and control population, further minimized the bias.

Moderate and statistically significant positive association was estimated between chronic *S.typhi* infection and GBC. This finding is consistent with previous studies. However, there are differences in magnitude of the risk and measurement of the association **[Table 4.1]**. Although, these studies have reported the positive association of *S.typhi* and Gallbladder cancer, almost all studies are underpowered and methodologically poor. Our study is first of its kind with large sample size, appropriate assay methodology and statistical analysis.

Several lines of biological evidence suggest that *S. typhi* may contribute to gallbladder carcinogenesis. The capsular polysaccharide expressed by *S. typhi* has been shown to suppress the inflammatory response of intestinal mucosa. The gall bladder is the site of bile storage and an

environment that is habitable exclusively by organisms that are resistant to bile's detergent-like properties. Salmonellae, not only are highly resistant to bile, but also respond to the presence of bile by regulating the expression of many resistance-related genes [40]. This explains the biological plausibility of the association.

Table 4.1 Comparison of the results with previous studies

Study site	Sample size (Case|Control)	Assay method	Result	Study Reference	Remark
Chile (2016)	39 GBC cases| 40 GS controls+ 39 population controls	Vi-ELISA PCR Culture	Odds ratio: 4.0	[52]	Inadequate sample size
Varanasi, India (2008)	52 GBC cases| 223GC+ 508 HC	WIDAL, Bile culture, Indirect haemaglutination & Detection of flagelin gene	38.5% cases, 13.9% GB condition cases and 9.2% of healthy controls were positive by serology 67.3% cases, 42.8% Gb condition cases were positive by PCR	[48]	Low sample size Inappropriate statistical methodology Not conclusive
Varanasi, India (2010)	54 GBC cases |54 controls with GC	Culture PCR Widal test	44.4% cases and 24% controls were positive by widal test 33% cases and none of the controls were positive by PCR	[51]	Low sample size Inappropriate statistical methodology Not conclusive

Abbreviations: GBC- gallbladder cancer, GS- gall-stones, GC- gallbladder conditions/disorders, HC- healthy controls

There was region-wise correlation of GBC incidence and chronic *S.typhi* sero-positivity proportion observed; showing higher proportion in regions with high incidence (North and

North-east) and lower proportion in regions with lower incidence (West and Central). This further strengthens the association. Quartile analysis estimated the strong, statistically significant dose-response effect, where there was increase in magnitude of risk as the concentration of anti-Vi IgG increases. As a case-control study design this study only proves the association of *Salmonella typhi* and GBC, but not the causality. We also cannot rule out the possible reverse causality.

No difference in the magnitude of OR in gender specific analysis. Odds Ratio was also found to be similar in three strata of education. After adjusting with gallstone history, magnitude of the risk did not show any remarkable difference. This suggests that gender, education and gallstones are not potential confounders.

Joint effect analysis between *Salmonella typhi* and gallstones estimated a more than additive and less than multiplicative but statistically non-significant interaction. In spite of this being the inconclusive result, a possible synergistic effect of *Salmonella typhi* and gallstones could not be ruled out. This further needs to be confirmed on larger sample size. Salmonella, especially *S. typhi*, is well known to form biofilms upon contact with cholesterol gallstones and similar substrates [66]. This explains the possible mechanism of interaction between gallstones and *S.typhi* bacteria.

A separate analysis was done for gangetic belt states due to the fact of high incidence rates of GBC in regions in the gangetic belt. However, the analysis revealed no notable difference in the association in gangetic and non gangetic regions. Risk factors other than *Salmonella typhi* infection such as presence of heavy metals in the water could be the reason of high incidence of GBC in gangetic belt states.

Chapter 5

Summary and Conclusion

5.1 Standardization of ELISA for detection of chronic infection of S.typhi:

5.1.1 Summary

There was a need of reliable method for detection of chronic *Salmonella typhi* infection. An In-house, In-direct ELISA for detection of anti-Vi IgG antibodies in human serum was optimized for the same.

Vi Polysaccharide Antigen (Lyophilized, purified) and Reference standard Anti Vi IgG (Lyophilized, purified) for the assay were obtained from Centre for Biologics Evaluation and Research (CBER), US Food and Drug Administration (FDA) and in collaboration with National Institutes of Health (NIH-US). The assay optimization was based on the protocol and instructions provided by National Institutes of Health. This entire assay was carried out at two dilutions 1:100 and 1:400. In addition, to check the parallelism and linearity approximately 15% of the samples were run at 8 dilutions (1:50 to 1:6400). Automated Liquid handling system was utilized for all pipetting work including dilutions.

The parameters included for the assay optimization were a] determination of Optimal dilution of secondary antibody, b] determination of Optimal Development time for end point signal, c] generation of reference Standard curve, d] parallelism, e] optimum dilution factor for the assay and f] determination of cut-off value for Chronic S.typhi sero-positivity. A secondary antibody dilution 1:2000 was selected for the assay. The end point development time was determined to be 30 minutes. In reference standard curve, average OD obtained at 1:100 was 3.25. Average concentration at the same dilution was 322.46 ng/ml. Average OD obtained at highest dilution of 1:12800 was 0.11, concentration for the same was 0.67 ng/ml. Optimum serum dilution factor

was determined to be 1:400. Out of the total samples tested for parallelism, 42.6% of the samples showed presence of parallelism. Cut-off value for chronic *S.typhi* sero-positivity was decided to be 5 µg/ml.

5.1.2 Conclusion

The ELISA was successfully optimized and validated for detection of chronic *Salmonella typhi* infection. This methodology should be checked with other study designs and populations for external validity. Once, generalizability of the same is confirmed this assay methodology can be used for screening of chronic *S.typhi* infection in large scale public health settings. Cut-off from mean $\pm$ 3SD of known negative population of *Salmonella typhi* should be determined in the future studies and analysed.

5.2 Association of Chronic infection of Salmonella typhi and Gallbladder cancer:

5.2.1 Summary:

The present study utilized the optimized ELISA procedure described in chapter 2 to assess the association of chronic *Salmonella typhi* infection and risk of GBC.

1. After quality assessment as per the assay validity criteria, final analysis was performed on 554 GBC cases and 530 controls. On inter-assay quality check, 90.1% of the samples showed reproducible results.
2. Proportion of chronic *S.typhi* sero-positivity was higher in GBC cases than in controls. There was region-wise correlation of GBC incidence and chronic *S.typhi* sero-positivity proportion observed; showing higher proportion in regions with high incidence (North and North-east) and lower proportion in regions with lower incidence (West and Central).

3. Moderate and statistically significant risk for GBC was estimated by unconditional logistic regression model after adjusting with age, gender, current residence and education. There was no difference in magnitude of risk upon gender specific analysis. A strong, statistically significant, dose- response effect was observed after quartile (based on control distribution) analysis of anti-Vi-IgG concentration.
4. A separate analysis for gangetic belt states, revealed higher risk in states excluding gangetic belt than in gangetic belt states.
5. Joint effect analysis between Chronic *S.typhi* seropositivty and Gallstones showed more than additive but less than multiplicative, statistically non-significant interaction.

5.2.2 Conclusion:

Chronic infection with *Salmonella typhi* was found to be positively associated with gallbladder cancer having the moderate risk. Consistent results as compared with previous studies, strong dose- response effect, evidence of biological plausibility from few basic science studies as well as the region-wise correlation of GBC incidence rate and Chronic *S.typhi* proportion further strengthen the association. However, to prove the causality, trans-disciplinary studies with the help of basic science and prospective epidemiological designs are required.

Chapter 6
Future Prospective

6 Future prospective:

The present study observed moderate association of GBC with respect to chronic infection of *Salmonella typhi*. Nation-wide prevalence of *Salmonella typhi* is still not estimated in Indian population. Screening of the same should be investigated so that prevention strategies and policies could be planned and implemented.

Role of other Non-typhoidal Salmonella species are likely to be associated with GBC. This should be investigated further. Detection of *Salmonella typhi* DNA in gallbladder tissue or bile samples can be done to validate the association further. Whole-genome sequencing can also be planned to identify SNPs related to Salmonella infection.

A detailed microbiome study should be planned to identify role of other micro-organisms for development of gallbladder cancer.

Standardization of the assay for detection of chronic *S.typhi* infection from Dried Blood spots (DBS) should be planned. This will help in large scale screening of the *S.typhi* infection.

Interaction of gallstones with Salmonella infection should be further investigated with larger sample size, in the association of Salmonella and GBC.

Bibliography

1. Netter's Atlas of human anatomy, 5th Edition
2. Nagral S. Anatomy relevant to cholecystectomy. J Minimal Access Surg. 2005 Jun;1(2):53–8.
3. W. J. Dodds, W. J. Hogan, and J. E. Geenen, ―Motility of the biliary system,‖ in Handbook of Physiology: Gastrointestinal System. Volume Motility and Circulation, J. Wood and S. Schultz, Eds., chapter 28, pp. 1055–1101, Oxford University Press, New York, NY, USA, 1989.
4. R. D. Odze and J. R. Goldblum, Eds., Surgical Pathology of the GI Tract, Liver, Biliary Tract and Pancreas, chapter 29, part 2, Saunders, Philadelphia, Pa, USA, 2009.
5. Shaffer EA. Gallbladder Cancer. Gastroenterol Hepatol. 2008 Oct;4(10):737–41.
6. http://www.cancerresearchuk.org/about-cancer/gallbladder-cancer/types
7. Misra, S., Chaturvedi, A., Misra, N. C., & Sharma, I. D. (2003). Review Carcinoma of the gallbladder Aetiology and pathogenesis. *Lancet Onco.*, *4*(March), 167–176.
8. Hundal R, Shaffer EA. Gallbladder cancer: epidemiology and outcome. Clin Epidemiol. 2014;6:99–109.
9. Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R., Torre, L., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Journal for Clinicians, 00(00), 1–31. https://doi.org/10.3322/caac.21492.

10. Globocan 2018: Estimated Cancer Incidence, mortality and prevalence worldwide by IARC (International agency for research on cancer)
Available from: http://globocan.iarc.fr/

11. Cancer Incidence in five continents (Volume XI)
Available from: http://ci5.iarc.fr/CI5-XI/Default.aspx

12. National Cancer Registry Programme by Indian Council of Medical Research.
Available from: http://www.ncrpindia.org/

13. Mhatre, S., Nagrani, R., Budukh, A., Chiplunkar, S., Badwe, R., Patil, P., … Dikshit, R. (2016). Place of birth and risk of gallbladder cancer in India. *Indian Journal of Cancer*, *53*(2), 304. https://doi.org/10.4103/0019-509X.197723

14. Joinpoint trend analysis software (Version 4.6.0.0) by National Cancer Institute. Available from: https://surveillance.cancer.gov/joinpoint/

15. Kim HJ, Fay MP, Feuer EJ, Midthune DN. Permutation tests for join-point regression with applications to cancer rates. Stat Med. 2000; 19(3):335-51.

16. Rubayat Rahman1, Eduardo J. Simoes et al.Trend analysis and survival of primary gallbladder cancer in the United States: a 1973–2009 population-based study. Cancer Medicine. 2017, 6(4): 874–880

17. https://www.cancer.net/cancer-types/gallbladder-cancer/statistics

18. Ryu S et al. Gallstones and the Risk of Gallbladder Cancer Mortality: A Cohort Study. Am J Gastroenterol. 2016 Oct;111(10):1476-1487

19. Randi, G., Franceschi, S., & La Vecchia, C. (2006). Gallbladder cancer worldwide: Geographical distribution and risk factors. International Journal of Cancer, 118(7), 1591–1602. https://doi.org/10.1002/ijc.21683
20. Yagyu K, Lin Y, Obata Y, Kikuchi S, Ishibashi T, Kurosawa M, Inaba Y, Tamakoshi A; JACC Study Group. Bowel movement frequency,medical history and the risk of gallbladder cancer death: a cohort study in Japan. Cancer Sci 2004;95:674–8
21. Kim YT, Kim J, Jang YH, Lee WJ, Ryu JK, Park YK, et al. Genetic alterations in gallbladder adenoma, dysplasia and carcinoma. Cancer Lett. 2001 Aug 10;169(1):59–68
22. Hsing AW, Gao Y-T, Han T-Q, Rashid A, Sakoda LC, Wang B-S, et al. Gallstones and the risk of biliary tract cancer: a population-based study in China. Br J Cancer. 2007 Dec 3;97(11):1577–82
23. Andreotti, G., Hou, L., Gao, Y.-T., Brinton, L. A., Rashid, A., Chen, J., … Hsing, A. W. (2010). Reproductive factors and risks of biliary tract cancers and stones: a population-based study in Shanghai, China. British Journal of Cancer, 102(7), 1185–1189. https://doi.org/10.1038/sj.bjc.6605597
24. Makiuchi Takeshi et al (2017). Reproductive factors and gallbladder/bileduct cancer: a population based cohort study in Japan. European J of Cancer Prevention, 26, 292-300. https://doi.org/10.1097/CEJ.0000000000000260
25. Larsson, S. C., & Wolk, A. (2007). Obesity and the risk of gallbladder cancer: A meta-analysis. British Journal of Cancer, 96(9), 1457–1461. https://doi.org/10.1038/sj.bjc.6603703
26. Aune, D., Vatten, L. J., & Boffetta, P. (2016). Tobacco smoking and the risk of gallbladder disease. European Journal of Epidemiology, 31(7), 643–653.

https://doi.org/10.1007/s10654-016-0124-z

27. Bagnardi, V., Rota, M., Botteri, E., Tramacere, I., Islami, F., Fedirko, V., … La Vecchia, C. (2015). Alcohol consumption and site-specific cancer risk: A comprehensive dose-response meta-analysis. British Journal of Cancer, 112(3), 580–593. https://doi.org/10.1038/bjc.2014.579
28. Manoj Pandey (2006). Environmental pollutants in gallbladder carcinogenesis. J of Surgical Oncology, 93, 640-643
29. Deepak Chabra et al (2012). Chronic Heavy Metal Exposure and Gallbladder Cancer Risk in India, a Comparative Study with Japan. Asian pacfic J of cancer Prev,13,187-190.
30. K hemminki, X Li. (2003) Familial liver and gallbladder cancer.Gut,52,592-596
31. Mhatre Sharayu et al.Common genetic variation and risk of gallbladder cancer in India: a case-control genome-wide association study The Lancet Oncology , Volume 18 , Issue 4 , 535 - 544
32. Hassan et al. (2015) The role of H. pylori infection in gallbladder cancer: clinicopathological study. Tumour biology, 36, 7093-7098
33. Mishra, R. R., Tewari, M., & Shukla, H. S. (2011). Helicobacter pylori and pathogenesis of gallbladder cancer. Journal of Gastroenterology and Hepatology (Australia), 26(2), 260–266. https://doi.org/10.1111/j.1440-1746.2010.06435.
34. Manoj pandey et al (2010). Helicobacter bilis in Human Gallbladder Cancer: Results of a Case-control Study and a Meta-analysis. Asian Pacific Journal of Cancer Prevention,11, 343-347
35. Y.H. Kim (2003). Extrahepatic cholangiocarcinoma associated with clonorchis: CT

evaluation. Abdominal imaging,28,68-71

36. Kenneth J. Ryan & C George Ray: Sherri's Medical Microbiology, 4th Edition
37. F. W. BRENNER et al. Salmonella Nomenclature: JOURNAL OF CLINICAL MICROBIOLOGY,July 2000, p. 2465–2467
38. RL., O., CJ, A., & Danovaro MC, Baiqing D, Bhattacharya SK, A. M. et al. (2014). Bulletin of the World Health Organization A study of typhoid fever in five Asian countries : disease burden and implications for controls. WHO, 86, 8–14.
39. Álvarez-Ordóñez, A., Begley, M., Prieto, M., Messens, W., López, M., Bernardo, A., & Hill, C. (2011). Salmonella spp. survival strategies within the host gastrointestinal tract. Microbiology, 157(12), 3268–3281. https://doi.org/10.1099/mic.0.050351-0
40. Gonzalez-Escobedo G, et al. Chronic and acute infection of the gall bladder by Salmonella Typhi: understanding the carrier state. Nature reviews. Microbiology. 2011; 9:9–14
41. Roumagnac P, et al. Evolutionary history of Salmonella Typhi. Science. 2006; 314:1301–1304
42. Gunn J. et al. Salmonella chronic carriage: epidemiology, diagnosis and gallbladder persistence. Trends Microbiol. 2014 November ; 22(11): 648–655
43. Parry CM, et al. Typhoid fever. The New England journal of medicine. 2002; 347:1770–1782
44. Van Velkinburgh, J. C., & Gunn, J. S. (1999). PhoP-PhoQ-regulated loci are required for enhanced bile resistance in Salmonella spp. Infection and Immunity, 67(4), 1614–1622
45. Gonzalez-Escobedo G, et al. Histopathological analysis of Salmonella chronic carriage

in the mouse hepatopancreatobiliary system. PloS one. 2013; 8:e84058

46. Menendez et al. Salmonella infection of gallbladder epithelial cells drives local inflammation and injury in a model of acute typhoid fever. J Infect Dis. 2009 Dec 1;200(11):1703-13
47. Dutta U. et al. Typhoid carriers among patients with gallstones are at increased risk for carcinoma of the gallbladder. Am J Gastroenterol. 2000 Mar;95(3):7847.
48. Nath et al. Association of carcinoma of the gallbladder with typhoid carriage in a typhoid endemic area using nested PCR. J Infect Developing Countries 2008; 2(4): 302-307
49. Dongol et al. The Microbiological and Clinical Characteristics of Invasive Salmonella in Gallbladders from Cholecystectomy Patients in Kathmandu, Nepal. PLoS One. 2012; 7(10).
50. Tiziana Scanu et al. Salmonella Manipulation of Host Signaling Pathways Provokes Cellular Transformation Associated with Gallbladder Carcinoma. Cell Host & Microbe 17, 763–774, June 10, 2015
51. Mallika T. et al. Salmonella typhi and gallbladder cancer: report from an endemic region. Hepatobiliary Pancreat Dis Int, Vol 9, No 5 • October 15, 2010.
52. Koshiol Jill et al. Salmonella enterica serovar Typhi and gallbladder cancer: a case–control study and meta-analysis. Cancer Medicine 2016; 5(11):3310–3325.
53. V. Nagaraja & G. D. Eslick. Systematic review with meta-analysis: the relationship between chronic Salmonella typhi carrier status and gall-bladder cancer. Aliment Pharmacol Ther 2014; 39: 745–750.
54. John Crowther.The ELISA guidebook, 2nd Edition. Available from:

https://www.springer.com/la/book/9781603272537

55. Shousun Szu et al. A human IgG anti-Vi reference for Salmonella typhi with weight-based antibody units assigned. Vaccine 31 (2013) 1970– 1974

56. Magllen™ software. Available from: https://lifesciences.tecan.com/software-magellan

57. TECAN Automated liquid handling system. Availablefrom:https://lifesciences.tecan.com/products/liquid_handling_and_automation/freedom_evo_series.

58. https://elisaanalysis.com/knowledge-base/elisa-software-4-parameter-logistic-4pl-nonlinear-regression/

59. Brian d. Plikaytis et al. Determination of Parallelism and Nonparallelism in Bioassay Dilution Curves. Journal Of clinical microbiology (1994), p. 2441-2447

60. Dikshit RP, Nagrani R, Mhatre S. Guidelines and Working Manual for conducting interviews for Multi-site Case Control studies. Mumbai, India: Tata Memorial Centre; 2011

61. Dikshit RP, Nagrani R, Mhatre S. Guidelines and Working Manual of data entry for Multi-site Case Control studies. Mumbai, India: Tata Memorial Centre; 2012

62. Breslow NE, Day NE. Statistical methods in cancer research. Volume I - The analysis of case-control studies. IARC Sci Publ 1980:5–338

63. STATA software 15.0. Available from: https://www.stata.com/

64. Jill Tate, Greg Ward. (2004) Interferences in Immunoassay. Clin Biochem Rev Vol 25

65. Ulf Andreasson1, Armand Perret-Liaudet et al (2015) A practical guide to immunoassaymethod validation. Frontiers in Neurology August 2015 | Volume 6 | Article 179

66. Robert W.Crawford et al. Gallstones play a significant role in Salmonella spp. gallbladder colonization and carriage. Proc Natl Acad Sci U S A. 2010 Mar 2; 107(9): 4353–4358

Original Article

Trends in Gallbladder Cancer Incidence in the High- and Low-Risk Regions of India

Prachi Ravindra Phadke[1,2], Sharayu Sitaram Mhatre[1], Atul Madhukar Budukh[1], Rajesh Prabhakar Dikshit[1,2]

[1]Centre for Cancer Epidemiology, Tata Memorial Centre, Navi Mumbai, [2]Homi Bhabha National Institute, Mumbai, Maharashtra, India

Abstract

Background: Gallbladder cancer (GBC), a common cancer surrounding the Gangetic belt of India, accounts for 80%–90% of biliary tract cancers. GBC incidence shows striking geographical variation in India. **Materials and Methods:** We used the data from the National Cancer Registry Programme for the year 2001–2014 to study the time trends of GBC in the high- and low-risk geographical regions of India. Annual percentage change (APC) in age-adjusted incidence rates was computed by log-linear regression model. **Results:** Among females, a statistically significant increase in trend was observed in Cachar (APC: 7.0, $P = 0.02$), Delhi (APC: 4.0, $P = 0.04$), and Kamrup (APC: 4.3, $P = 0.02$) marked under high-risk region and in Bengaluru (APC: 5.7, $P = 0.04$) and Pune (APC: 3.4, $P = 0.04$) marked under low-risk region. Among males, increasing but statistically nonsignificant trends were observed in Cachar, Dibrugarh, Kamrup, Nagpur, and Sikkim, whereas decreasing trends were observed in Bengaluru, Barshi, Bhopal, and Kolkata. Aurangabad showed a statistically significant decrease in trend (APC: −14.5, $P < 0.001$) among males. **Conclusion:** The time trend and pattern of GBC have striking differences within the country as well as in state. Further large-scale region-wise studies are needed to find the risk factors of GBC.

Keywords: *Gallbladder cancer, incidence rate, India, trend*

Introduction

Gallbladder cancer (GBC) is less common in developed countries while its incidence is highest in some parts of India, Chile, and Mexico.[1] Within India, the states such as Assam (rate ratio [RR] [95% confidence interval [CI]] – female: 7.18 [4.89–10.55] and male: 3.61 [2.44–5.36]) and Delhi (RR – female: 4.70 [3.93–5.61] and male: 2.04 [1.61–2.60]) showed highest rate compared to South India (RR – female: 0.39 [0.24–0.66] and male: 0.53 [0.33–0.85]).[2] To investigate secular trends in GBC over the past 14 years, we conducted this study to estimate the annual percentage change (APC) in the incidence rate within different regions of India and performed a comprehensive analysis to understand the time trends in different geographical regions of India.

Materials and Methods

Incidence data

We retrieved age-adjusted incidence rate (AAR) per 100,000 of GBC from the National Cancer Registry Programme (NCRP) database,[3] for the period of 14 years (2001–2004 to 2012–2014). The NCRP database includes 30 population-based cancer registries (PBCRs), namely, Ahmedabad, Aurangabad, Bengaluru, Barshi, Bhopal, Cachar, Chennai, Delhi, Dibrugarh, Kamrup, Kolkata, Kollam, Manipur, Meghalaya, Mizoram, Mumbai, Nagaland, Nagpur, Naharlagun, Pasighat, Patiala, Pune, Sikkim, Thiruvananthapuram, Tripura, Wardha, Sangrur, Mansa, Chandigarh, and SAS Nagar. However, for trend analysis, we considered only 18 registries having AAR available for at least three time periods.

Definition of high- and low-risk regions

We divided Indian states and territories into high- and low-risk regions using incidence rates extracted from the PBCRs. States were considered to be in the high-risk region if PBCR existing in the state observed average AARs of >5.0 per 100,000 persons.[2] Others were considered as low-risk regions. According to the definition, Cachar, Delhi, Kamrup, Dibrugarh, Kolkata, and Sikkim were in the high-risk region, and the rest of the registries were considered to be in the low-risk region. For comparison, average

How to cite this article: Phadke PR, Mhatre SS, Budukh AM, Dikshit RP. Trends in gallbladder cancer incidence in the high- and low-risk regions of India. Indian J Med Paediatr Oncol 2019;40:90-3.

Address for correspondence:
Dr. Rajesh Prabhakar Dikshit, Centre for Cancer Epidemiology, Tata Memorial Centre, Kharghar, Navi Mumbai - 410 210, Maharashtra, India.
E-mail: dixr24@hotmail.com

Access this article online

Website: www.ijmpo.org

DOI: 10.4103/ijmpo.ijmpo_164_18

Quick Response Code:

AARs of all registries from high- and low-risk regions were taken for four time periods (2001–2004 to 2012–2014).

Estimation of annual percentage change

APC is a summary measure of the trend over a prespecified fixed interval. APC was estimated independently for both the genders for the selected 18 registries, namely, Aurangabad, Bengaluru, Barshi, Bhopal, Cachar, Chennai, Delhi, Dibrugarh, Kamrup, Kolkata, Kollam, Manipur, Mizoram, Mumbai, Nagpur, Pune, Sikkim, and Thiruvananthapuram for the time period of 2001–2004 to 2012–2014.

Trend was estimated using Joinpoint trend analysis software by the National Cancer Institute.[4,5] APC was considered statistically significant if P value at 95% CI was ≤0.05.

Results

Tables 1 and 2 show gender-wise AAR for four time periods (2001–2004 to 2012–2014) in the 18 registries. There are substantial differences in the rates for both the genders, with high rates in Delhi, Cachar, Dibrugarh, and Kamrup and with lowest rates in Bengaluru, Barshi, Chennai, and Aurangabad. Among females, statistically significant increasing trends were observed in Bengaluru, Cachar, Delhi, Kamrup, and Pune, whereas the rates were decreasing in Aurangabad, Barshi, and Manipur. Among males, increasing but statistically nonsignificant trends were observed in Cachar, Dibrugarh, Kamrup, Nagpur, and Sikkim, whereas decreasing trends were observed in Bengaluru, Barshi, Bhopal, and Kolkata. Aurangabad showed a statistically significant decrease in trend (APC: −14.5, $P < 0.001$) among males.

As shown in Figure 1 [Supplementary Table 1], in the high-risk region, there was a statistically significant increase in AAR among females (APC: 5.4, $P < 0.001$), whereas the trend was increasing but statistically nonsignificant among females (APC: 6.3, $P = 0.2$). No significant increase in AAR was observed in the low-risk region (male – APC: −1.1, $P = 0.8$ and females – APC: −1, $P = 0.5$).

Table 3 shows the within-state variation of AAR (2004–2014) in Maharashtra, northeast region, and south region. In Maharashtra, Mumbai showed slightly higher rates than other three regions, for both genders. There was no variation observed in rates within the four regions in South India. Variability in rates was observed in northeast region, with Cachar, Kamrup, and Dibrugarh registries in Assam showing high rates.

Discussion

This trend analysis has shown that the rates of GBC incidence in the high-risk region are significantly increasing among females, whereas increasing but statistically nonsignificant trend was observed among males. Several factors such as diagnosis and completeness of reporting can affect the reported incidence of cancer.

In states with low risk of GBC, the rates of GBC were higher only in metro cities (Mumbai, Pune, and Bengaluru), whereas the rates were lower in small towns. This indicates the role of migration from high-risk areas to the metro cities. The role of migration in GBC was previously investigated.[2] It would be interesting to explore if the rising trend is because of high rates of migration

Table 1: Age-adjusted incidence rates of gallbladder cancer with annual percentage change in Indian registries (males)

Cancer registry	AAR by time period				APC	*P*
	2001-2004	2004-2008	2009-2011	2012-2014		
Aurangabad	NA	0.7	0.3	0.2	−14.5^	<0.001
Bengaluru	0.8	2.6	0.3	1.2	−4.6	0.75
Barshi rural	1.0	1.7	1.0	1.0	−1.7	0.67
Bhopal	2.8	3.4	3.3	2.6	−0.6	0.76
Cachar	2.1	4.7	5.1	5.2	7.4	0.19
Chennai	1.2	3.2	2.1	1.8	1.5	0.81
Delhi	3.7	6.5	4.2	5.3	1.2	0.76
Dibrugarh	2.4	3.7	3.2	4.1	3.6	0.23
Kamrup	3.0	7.3	7.4	8.8	8.5	0.17
Kolkata	NA	3.7	2.5	3.3	−2.0	0.72
Kollam	NA	0.9	1.1	1.4	5.5	0.15
Manipur	1.2	1.7	1.8	1.7	2.9	0.25
Mizoram	1.4	1.9	2.4	1.8	2.8	0.4
Mumbai	1.7	3.6	1.7	2.2	−0.7	0.9
Nagpur	NA	0.8	1.3	1.3	6.7	0.24
Pune	NA	0.9	0.9	1.1	2.2	0.55
Sikkim	0.6	3.7	2.1	2.2	8.1	0.5
Thiruvananthapuram	NA	0.9	0.7	1.2	2.8	0.71

Taken from NCRP database, *P* value for APC estimated at 95% CI. AAR – Age-adjusted incidence rate per 100,000; NA – AAR not available; APC – Annual percentage change; NCRP – National Cancer Registry Programme; CI – Confidence interval; ^ – Statistically significant

Table 2: Age-adjusted incidence rates of gallbladder cancer with annual percentage change in Indian registries (females)

Cancer registry	Time period				APC	*P*
	2001-2004	2004-2008	2009-2011	2012-2014		
Aurangabad	NA	0.3	0.1	0.1	−13.1	0.09
Bengaluru	1.0	1.4	1.5	2.0	5.7^	0.04
Barshi rural	0.5	0.6	0.2	0.2	−10.6	0.13
Bhopal	4.4	4.2	5.0	6.4	3.4	0.11
Cachar	5.1	6.5	10.1	10.2	7.0^	0.02
Chennai	0.9	1.2	2.0	1.5	5.7	0.17
Delhi	7.4	8.1	9.2	11.8	4.0^	0.04
Dibrugarh	5.8	7.5	7.7	8.6	3.1	0.09
Kamrup	10.2	12.6	14.0	17.1	4.3^	0.02
Kolkata	NA	4.5	5.6	7.7	6.7	0.14
Kollam	NA	0.6	0.8	1.0	6.4	0.09
Manipur	5.6	5.2	3.6	3.8	−4.1	0.06
Mizoram	4.1	2.7	2.9	3.6	−0.7	0.82
Mumbai	2.1	2.75	2.2	4.1	4.3	0.31
Nagpur	NA	0.8	1.0	1.4	6.9	0.21
Pune	NA	1.0	1.2	1.3	3.4^	0.04
Sikkim	2.4	5.2	6.8	6.7	9	0.12
Thiruvananthapuram	NA	0.8	1.3	1.1	4.7	0.47

Taken from NCRP database, *P* value for APC estimated at 95% CI. AAR – Age-adjusted incidence rate per 100,000; NA – AAR not available; APC – Annual percentage change; NCRP – National Cancer Registry Programme; CI – Confidence interval; ^ – Statistically significant

Table 3: Within-state variation in age-adjusted incidence rates of gallbladder cancer

State	Registry	2004-2008		2009-2011		2012-2014	
		Male	Female	Male	Female	Male	Female
Maharashtra	Nagpur	0.8	0.8	1.3	0.96	1.3	1.4
	Pune	0.9	1.0	0.85	1.2	1.1	1.3
	Mumbai	3.6	2.75	1.68	2.18	2.2	4.1
	Barshi	1.7	0.6	1.03	0.17	1.0	0.2
Northeast	Manipur	1.7	5.2	1.82	3.64	1.7	3.8
	Mizoram	1.9	2.7	2.44	2.94	1.8	3.6
	Cachar	4.7	6.5	5.11	10.13	5.2	10.2
	Kamrup	7.3	12.6	7.41	14.01	8.8	17.1
	Dibrugarh	3.7	7.5	3.24	7.7	4.1	8.6
	Sikkim	3.7	5.2	2.06	6.77	2.2	6.7
South India	Chennai	3.2	1.2	2.05	1.95	1.8	1.5
	Kollam	0.9	0.6	1.08	0.77	1.4	1.0
	Thiruvananthapuram	0.9	0.8	0.72	1.32	1.2	1.1
	Bengaluru	2.6	1.4	0.27	1.53	1.2	2.0

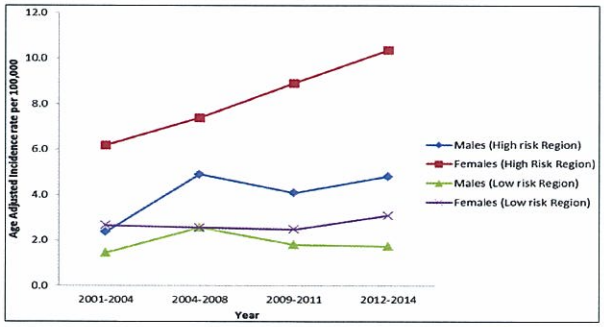


Figure 1: Trends in age-adjusted incidence rates of gallbladder cancer in the high- and low-risk regions. Data for Figure 1 are provided as supplementary table

from Northern states in India or there is a shift in lifestyle factors such as increase in obesity; consumption of fatty, spicy food; and reproductive factors for females which are contributing to develop GBC.

The time trend and pattern of GBC have striking differences even within state. For example, the rates are rising and are high only in Mumbai, Maharashtra, while among northeastern states, only Assam has high rates whereas Manipur, Mizoram, and Sikkim show low-to-medium rates of GBC. A detailed study about lifestyle patterns in Assam would thus be helpful to understand the reasons for high rates of GBC.

The risk factors of GBC are not widely studied. It has been estimated that the disease has high heritability component and risk loci surrounding ABCB4 and ABCB1 region on chromosome 7 has been identified.[6,7] The lifestyle factors which are generally considered to increase the risk of GBC are gallstone, obesity, and infection.[8-10] In addition, among females, high parity is a possible contributing factor for the increase in the risk of GBC.[9]

Conclusion

The time trend and pattern of GBC have striking differences within the country as well as in state. It would be important to conduct a large-scale study in the high- and low-risk regions of GBC to understand its etiology and to inform government regarding prevention strategies of this fatal disease.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Globocan 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide by International Agency for Research on Cancer. Available from: http://www.globocan.iarc.fr/. [Last accessed on 2018 Nov 01].
2. Mhatre SS, Nagrani RT, Budukh A, Chiplunkar S, Badwe R, Patil P, *et al.* Place of birth and risk of gallbladder cancer in India. Indian J Cancer 2016;53:304-8.
3. National Cancer Registry Programme by Indian Council of Medical Research. Available from: http://www.ncrpindia.org/. [Last accessed on 2018 Nov 01].
4. Joinpoint Trend Analysis Software (Version 4.6.0.0) by National Cancer Institute. Available from: https://www.surveillance.cancer.gov/joinpoint/. [Last accessed on 2018 Nov 01].
5. Kim HJ, Fay MP, Feuer EJ, Midthune DN. Permutation tests for joinpoint regression with applications to cancer rates. Stat Med 2000;19:335-51.
6. Mhatre S, Wang Z, Nagrani R, Badwe R, Chiplunkar S, Mittal B, *et al.* Common genetic variation and risk of gallbladder cancer in India: A case-control genome-wide association study. Lancet Oncol 2017;18:535-44.
7. Mhatre S, Chatterjee N, Dikshit R, Rajaraman P. Genetics of gallbladder cancer – Authors' reply. Lancet Oncol 2017;18:e297.
8. Misra S, Chaturvedi A, Misra NC, Sharma ID. Carcinoma of the gallbladder. Lancet Oncol 2003;4:167-76.
9. Randi G, Franceschi S, La Vecchia C. Gallbladder cancer worldwide: Geographical distribution and risk factors. Int J Cancer 2006;118:1591-602.
10. Hundal R, Shaffer EA. Gallbladder cancer: Epidemiology and outcome. Clin Epidemiol 2014;6:99-109.

Supplementary Table 1: Age-adjusted incidence rates of gallbladder cancer with annual percentage change in the high- and low-risk regions of India

Year	High-risk region		Low-risk region	
	Males (average AAR)	Females (average AAR)	Males (average AAR)	Females (average AAR)
2001-2004	2.4	6.2	1.4	2.7
2004-2008	4.9	7.4	2.6	2.6
2009-2011	4.1	8.9	1.8	2.5
2012-2014	4.8	10.4	1.7	3.1
APC	6.3	5.4^	1.1	1
P	0.2	0	0.8	0.5

AAR – Age-adjusted incidence rate; APC – Annual percentage change; ^ – Statistically significant