A Study on Correlation of Chronic *Salmonella enterica* serovar Typhi Infection with Gallbladder Disease

THESIS

Submitted in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

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Under the supervision of

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BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

2016

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

CERTIFICATE

This is to certify that the thesis entitled '**A Study on Correlation of Chronic *Salmonella enterica* serovar Typhi Infection with Gallbladder Disease**' and submitted by **Walawalkar Yogesh Dilip Dipti (ID No. 2012PHXF016G)** for award of Ph.D. of the Institute embodies original work done by him under my supervision.

Signature of the Supervisor

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Date: 27/08/2016

DEDICATION

To my parents **Mrs. Deepti D. Walawalkar** and

Late **Mr. Dileep Mahadeo Walawalkar**

You are my best example of life, strength and integrity.

To my loving brother **Mr. Dhanesh D. Walawalkar** and my dearest friend

Ms. Vishva Maru

Thank you for your limitless support.

To my grandparents Mr. Gajanan Walawalkar and Mrs. Annapurna Walawalkar,

Thank you for being with me in every difficulty regardless of the distance.

To my friends at BITS-Pilani University;

Ashu Sharma, Meghnath Prabhu, Omprakash Chouhan, Praveen Saini, Akansha Saini,
Angela Samantha, Priya Das
and Phalgun Soni

I do not conceive this journey without your loyalty.

ACKNOWLEDGEMENT

The present work would not have been possible without the support of many people during various phases of my graduate schooling at Birla Institute of Technology and Science – Pilani, KK Birla Goa Campus. I am grateful to them and would like to thank them from bottom of my heart.

First and foremost, I would like to thank my advisor Dr. Vijayashree Nayak for her unparalleled support and guidance. This piece of work would not have been accomplished without her knowledge, insight and wisdom. I acknowledge her patience through all the ups and downs faced during this journey. One simply could not wish for a better supervisor who made me learn some valuable lessons and gave me the right attitude to peruse my career in science.

I also take this opportunity to thank Prof. Sasikumar Punnekkat, Director, Birla Institute of Technology and Science Pilani (BITS Pilani) K K Birla Goa Campus, Prof. Srinivasan Ashwin, Deputy Director, BITS Pilani K K Birla Goa Campus, Prof. Souvik Bhattacharyya, Vice chancellor, BITS-Pilani, Prof. V. S. Rao acting Vice Chancellor, BITS Pilani, Prof. B. N. Jain, Ex-Vice Chancellor, BITS Pilani, Dr. Kumar Mangalam Birla, Chancellor, BITS Pilani, for giving me the opportunity to serve in this prestigious institute as a faculty while pursuing my research.

I wish to thank the Department Research Committee (DRC) members and the DRC Convener Dr. Utpal Roy. I also express my sincere thanks to Dr. Prasanta Kumar Das, Associate Dean, Academic Research, BITS Pilani K K Birla Goa Campus for providing the necessary facilities.

My sincere thanks also go to my Doctoral Advisory Committee (DAC) members Dr. Meenal Kowshik, Associate Professor. BITS Pilani K K Birla Goa Campus. and Dr. Anasuya Ganguly, Assistant Professor. BITS Pilani K K Birla Goa Campus. for preliminary assessment of the thesis and helpful suggestions during my progress seminars. I also thank all the faculty members and research scholars of the department of Biological Sciences, BITS Pilani K K Birla Goa Campus for their kind cooperation.

I am grateful to Dr. Rajni Gaind (VMMC and Safdarjung Hospital, Dr. Sunita Saxena (NIP-ICMR), Dr. Sudhir Krishna (NCBS-TIFR, Banglore) and Dr. Beena Pillai (IGIB) for their timely support and experimental expertise provided during the course of work. I am grateful to Dr. RS Mohil (Surgeon, Safdarjung Hospital), Dr. Vardan bhobe (Surgeon, RG stone hospital), Dr. Yatindra Vaidya (SMRC), Dr. Silvano CA Dias Sapeco (Forensic medicine, Goa Medical College), Dr. Jude Rodrigues (Goa medical college) and Dr. Madhu Ghodkirekar (Goa medical College) for providing us with the clinical samples, without which this work would not have been initiated. I also thank all the patients who volunteered for this study.

I heartily thank my friends for their prayers and constant encouragement to complete this work successfully. I am also thankful to my fellow colleagues and research scholars of BITS-Pilani Goa Campus whose challenges and productive criticism, especially at the progress seminars, has provided improvement in the presentation of work.

This thesis is heartily dedicated to my late father Mr. Dilip Walawalkar and my loving mother Mrs. Deepti Walawalkar who stood by me at all times.

Yogesh Dilip Walawalkar

PREFACE

Salmonella enterica serovar Typhi (*S.* Typhi), the etiological agent of typhoid fever, is a human-restricted pathogen causing exhibiting both acute and chronic phases of infection. Most of the studies focus on understanding the initial phases leading to systemic infection, with very few reports for chronic lifestyle adapted by the organism. The gallbladder is shown to be a primary site for chronic infection by *S.* Typhi from where the organisms disseminate into the environment via shedding through faeces or urine, thus spreading typhoid fever. This phase of chronic infection is generally asymptomatic and strongly associated with gallstones formed in the gallbladder. Furthermore, chronic persistence is also considered as one of the predisposing factors for gallbladder cancer. Many researchers associate findings such as the confinement of *S.* Typhi to gallbladder, their ability to resist antibiotic therapy and the intermittent shedding of organisms, to biofilm-forming ability of the organisms within the gallbladder. Cholesterol-gallstones are shown to be the best substrates for biofilm formation by *Salmonella* spp., and the bacteria produce various biofilm-related proteins that function in a highly coordinated manner to form successful matrix-encased biofilms.

In this study, we screen for *S.* Typhi persisting in the gallbladder from regions within the country which are mostly endemic for typhoid fever and reporting high incidences of gallbladder diseases. We further characterize these isolates *in vitro* based on their tolerance to bile, ability to form biofilms and resistance to antibiotics. These studies suggest that *S.* Typhi does persist in the gallbladder of patients, successfully adapting to bile and showing good ability to form biofilms on cholesterol-coated surfaces.

As a part of our efforts to partly understand mechanisms underlying bacterial adaptation to bile, we examine the implications of quorum sensing and persister cells as a response to bile generated oxidative stress in *S.* Typhi gallbladder isolates. Here we present the evidence that the quorum sensing system of *S.* Typhi aids in oxidative stress management via the regulation of antioxidant enzymes namely superoxide dismutase and catalase. As per our knowledge, this is the first study in *S.* Typhi to show the involvement of quorum-sensing (QS) in bile-generated oxidative stress. In addition, we also use various fluorescence based detection methods, including development of a

whole cell biosensor, to specifically detect the QS signal molecules secreted by *S.* Typhi and other organisms. This in turn helped us to understand their role during oxidative stress exerted by bile on the bacteria. In addition, we also show that the biofilm forming ability is dependent on the QS system but cannot restored by exogenous AI-2 molecules in mutants lacking the *luxS* gene (required for QS in bacteria)

Finally, using expression analysis we try to deduce the role of RhoA/NFκβ/MLCK (patway responsible for EMT in cells) as a prognostic marker for *S.* Typhi chronic infection in both, patients undergoing cholecystectomy and *in vitro* infection model comprising of gallbladder epithelial cells. Our finding reveals that alteration in the expression of these components may be attributed to the chronic inflammatory stages of the gallbladder, as presence of *S.* Typhi did not significantly bring about any changes in their expression. But *in vitro* infection of gallbladder epithelial cells showed converse results indicating role of *S.* Typhi in regulating epithelial to mesenchymal transition (EMT) cannot be neglected.

Taken together, this study supports the fact that *S.* Typhi can persist in the gallbladder, and subsequent chronic infection depends on various mechanisms adapted by the organism to host-generated responses or substances like the bile. The study reveals the complicated nature of mechanisms underlying chronic carriage including oxidative stress management, biofilm formation and cell-cell communication. Furthermore, the results reveal the significance of specific host mechanism as possible prognostic marker of chronic infection within the gallbladder. Understanding such chronic infections and mechanisms underlying their persistence is of utmost importance to develop effective strategies to detect and treat them.

Table of Contents

List of Figures

Chapter III

Chapter IV

List of Tables

List of Abbreviations and symbols

LB	Luria Bertani
SDS	Sodium dodecyl Sulfonate
PAGE	Polyacryl amide gel electrophoresis
PCR	Polymerase chain reaction
OD	Optical Density
nm	nanometer
MTCC	Microbial test culture centre
Amp	Ampicillin
AI-2	Autoinducer-2
µg	Microgram
mg	milligram
dl	decilitre
kDa	Kilo Dalton
QS	Quorum sensing
ROS	Reactive oxygen species
SOD	Superoxide dismutase
CFE	Cell free extract
sdiA	Suppressor of cell division inhibitor
rck	resistance to complement killing
P_{rck}	rck promoter
eGFP	enhanced green fluorescent protein
AHL	N-acyl homoserine lactone
Kan	Kanamycin
CFU	Colony forming unit
TTC	Triphenyl tetrazolium chloride
luxS	luminescence expression
mM	milli molar
NAD	nicotinamide adenine dinucleotide
a.u.	arbitary units
ORF	Open reading frame
°C	Degree celsius
RhoA	Ras homolog gene family, member A
NFkβ	Nuclear Factor Kappa Beta
MLCK	Myosin light chain kinase
GBEC	Gallbladder epithelial cells
H&E	Hemotoxylin and Eosin
CDH1	Cadherin 1
CK19	Cytokeratin 19
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal Bovine Serum
PBS	Phosphate buffered saline
SD	Standard deviation
SM	Standard mean
EMT	Epithelial to mesenchymal transition
mA	Milliampere
NaCl	Sodium Chloride

CHAPTER-I

INTRODUCTION

1. Introduction

1.1 The organism- *Salmonella enterica* serovar Typhi

Typhoid fever is an acute, systemic illness caused by an infection with *Salmonella enterica* serovar Typhi. The name typhoid is derived from a Greek word "*typhos*", meaning smoke, which refers to the smoke that was believed to cause it. In 1869 Wilson categorized typhoid fever under enteric fever and was described as a systemic illness characterized by abdominal pain and fever along with non specific symptoms like nausea, vomiting and headache (Parry et al., 2002). Enteric fever caused by *Salmonella enterica* serovar Typhi is known as Typhoid fever and that caused by *Salmonella enterica* serovar Paratyphi A, B, or C is called as paratyphoid fever, with subtle clinical difference between them. Typhoid fever is more common with a severe clinical course compared to paratyphoid fever (Ellermeier and Slauch, 2006).

1.1.1 Phylogeny and Classification

The genus *Salmonella* is named after an American scientist D.E. Salmon and belongs to Enterobacteriaceae family that includes *Klebsiella*, *Shigella*, *Escherichia* and *Yersinia*. The taxonomy is derived from Kauffman-White scheme and is updated by The World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* (Grimont and Weill, 2007). According to contemporary classification, *Salmonella* has two species namely *bongori*, *enteric* and *subterranean*. The *S.enterica* is further divided based on biochemical and genomic relatedness into six subspecies which are *enterica, salamae, arizonae, diarizonae, houtenae and indica* containing more than 2500 serovars (Ellermeier and Slauch, 2006) **(Figure 1.1)**. The genus *Salmonella* broadly causes food-borne salmonellosis in animals (usually vertebrates) having worldwide economic significance as it can be naturally transmitted to humans, referred to as zoonosis. They may be divided into three groups based on the specificity for their host. These include organism that are adapted to human host; animal host; and a third group containing un-adapted *Salmonella* that cause disease in humans and variety of animals **(Table 1.1)**. Furthermore, they may be classified based on the severity of disease they cause in human host i.e. typhoidal which causes enteric fever and non-typhoidal *Salmonella* (NTS) that is associated with gastroenteritis or enterocolitis/diarrheal disease (Galanis et al., 2006) **(Figure 1.1)**.

Host	Serovars
Human	*S.* Typhi *and S.* Paratyphi (cause typhoid and para-typhoid fever respectively), *S. schottmuelleri, S. hirschfeldii, S. sendai*
Animal	*S. dublin*(cattle), *S. pullorum* and *S. gallinarum* (Poultry), *S. abortus-ovis* (Sheep)
Both (non-typhoidal)	*S.* Typhimurium (most common), *S. enteritidis, S. javiana*

Table 1.1 Different serovars of *Salmonella enterica* and their respective hosts

1.1.2 Cultural characteristics of *S*. Typhi

S. Typhi was originally isolated by Karl J. Erberth in 1880 and is a multi-organ pathogen that inhabits the lympathic tissues of the liver, small intestine, spleen, and bloodstream of infected humans (August and Konert, 1993). The organism is a Gram-negative, motile, rod shaped bacillus which is 2–3 µm long and 0.4–0.6 µm in diameter. It is facultatively anerobic producing acid on sugar fermentation and reducing nitrates. They are non-spore-forming, flagellated (peritrichous helical flagella) bacillus that belong to the family of Enterobacteriaceae (Acosta et al., 2003; Ellermeier and Slauch, 2006). Selective media for isolation of this non-fastidious organism may be categorized as low selective which includes MacConkey agar and deoxycholate agar, intermediate selective that includes *Salmonella-Shigella* (SS) agar and highly selective media which includes Brilliant green and bismuth sulphite agar. The organism can also be enriched using Strontium selenite and selenite F broth. The minimum temperature for growth of the organism is 10°C and maximum is 45°C, with optimum being 37°C. The optimum pH for growth is 7-7.5 (Acosta et al., 2003). The most important fact is that the organism can survive in absence of host for weeks and even months. For instance it can survive in faeces for approximately 62 days and in ice for about 250 days. Besides, it can even undergo a transition to the VBNC (Viable but Non-Culturable Cells) state in water and other sources. This justifies epidemic nature of typhoid fever with no apparent cause of contamination (Gonzalez-Escobedo et al., 2011).

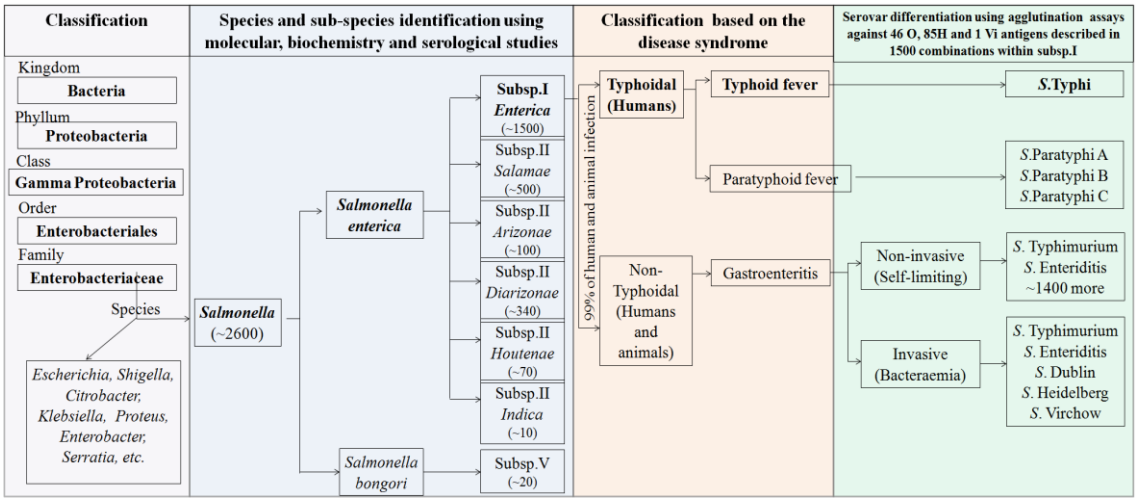


Figure 1.1 Overview of *Salmonella* classification

1.1.3 Serological characterization of *S.* Typhi

The genus *Salmonella* has three important antigens namely the somatic O antigen, flagellar H antigen and the surface Vi antigen. The O antigen (also called the bovine antigen) is a phospholipid-protein complex that forms an integral part of bacteria cell wall. It corresponds to the bacterial endotoxin and is involved in inducing fever. The O antigen shows variations in the type of sugars present in their arrangements making it highly polymorphic and antigenically diverse (large number of serovars) (Cooke et al., 2007; Ellermeier and Slauch, 2006). The H antigen is a protein structure which is immunogenic and heat labile. The production of H antigen is a diphasic process having the phase I (specific phase) and the phase II (the group phase) and the antigens discovered are designated with Arabic numerals. The Vi (virulence) antigen is a polysaccharide on the exterior cell wall and prevents O antibodies from binding to the O antigen (Cooke et al., 2007). These Vi antigens are poorly immunogenic and easily destroyed by heat. Thus boiling of drinking water and sufficient heating of foods is necessary to immediately kill *S.* Typhi and prevent the spread of typhoid fever. The Vi antigen was believed to be a necessary factor for *S.* Typhi virulence until recently, but Vi negative strains have also been shown to cause typhoid fever. Interestingly, Vi antibody titres correlate better with the carrier state than the O and H titres, and for this reason the Vi agglutination test for detection of carriers was introduced (Cooke et al., 2007; Ellermeier and Slauch, 2006). *S.* Typhi rarely exhibits serological variability compared to other *Salmonella*. Endemic areas show many strains in circulation but outbreaks are usually due to limited number of strains.

1.2 Epidemiology and Global incidence of Typhoid fever

Typhoid mainly occurs in developing countries especially in regions that lack proper sanitation and clean water. In the early 1980's, Edelman estimated the case fatality rate of nearly 4% that equates to 768,000 deaths per year globally due to typhoid (Crump et al., 2004). With a population of nearly 6.4 billion in 2004, the World Health Organisation estimated the global annual incidence of typhoid fever to be around 0.3% that equates to 19.2 million cases per year. Based on the global population in 2000, Crump et al. estimated the number of cases to be 21.7 million with 216,510 deaths per year (WHO, 2000; Parry et al., 2002; Crump and Mintz, 2010) **(Figure 1.2)**. The case fatality rate reported by them was around 1%. Also in the same study they report the global annual incidence of paratyphoid fever to be 5.4 million. The lack of effective treatment accounts for case fatality of 10%. Complications following acute phase of infection occur in about one third of untreated cases and accounts for 75% of all deaths. Interestingly, up to 5% of patients become chronic asymptomatic carriers of *S.* Typhi and pose a threat for intermittent dissemination of the bacteria in the environment (Ochiai et al., 2008; Gonzalez-Escobedo et al., 2011). Also around 94 million cases of gastroenteritis due to NTS occur worldwide, leading to 155,000 deaths each year. These estimates are of utmost importance as they account for improvement in water safety and hygiene within affected regions. For instance, from 1982-1993 the *S.* Typhi isolation rate decreased by 50% in Singapore because of their continuous efforts to improve hygienic conditions. Since some of these studies extrapolate from a relatively small number of incidences, it is necessary to carry out real-time analysis within populations to elicit the nearly exact burden of typhoid worldwide (Crump and Mintz, 2010).

A high proportion of typhoid occurs in developing countries like Africa, South America and Southeast Asia with higher case fatality rates compared with developed countries. Asia reports high incidences (>100/ 100 000 cases/ year) of typhoid, accounting for almost 80% of world's population. The crude annual typhoid incidence rate estimated in Asia was 274 per 100,000 persons in 2000. As per our survey, Nepal reported the highest incidence of 655 cases per 100,000 in 1987 (Ochiai et al., 2008). In developed countries sustained outbreaks are rare and sporadic infections have been reported in Europe and North America possibly due to immigrants or returning travelers. The later was observed commonly in UK, where a retrospective study from 1999 to 2009

reported 100 cases of enteric fever in both adults and children (Parry et al., 2002). Multi drug resistance is common in *Salmonella* and MDR *S*. Typhi is mostly endemic that can cause large epidemics in many parts of Southeast Asia including India, Bangladesh, China, Pakistan, Indonesia, Malaysia and Vietnam. There is also a pseudo-epidemic region that consists of the Middle East and Egypt, where MDR *S.* Typhi infection is generally related to migrant workers from the endemic zones who also have the potential to cause an epidemic. Since 1990, reports related to MDR *S*. Typhi outbreaks have consistently been on a rise. Such outbreaks are frequently reported from Asian and African countries, including some countries from Eastern Europe. High mortality rates due to typhoid fever have been reported in India, Indonesia and Nigeria ranging from 12-32% (Ochiai et al., 2008; Crump and Mintz, 2010; Gonzalez-Escobedo et al., 2011).

The Indian Scenario: Enteric fever is endemic in Northern part of the country with periodic outbreaks mainly water-borne or food-borne. Typhoid is the 5th most common communicable disease in India. In 1992, about 352980 cases with 735 deaths were reported due to typhoid, which then declined in consequent years that followed. A study conducted in Punjab in the mid 1960s examined 340 enteric fever cases out of which more than 98% were caused by *S*. Typhi and less than 2% were caused due to Paratyphi A (Crump et al., 2004; Tewari et al., 2010). But in 2003, an increasing occurrence of paratyphoid fever in Indian population was seen (Teh et al., 2014). Children constitute about 69% of hospitalized typhoid victims in India. Studies also reveal that majority of the cases occur in children that are aged above 5 years, with nearly 25% cases in children below 5 years of age. This age related bias was also seen in adults when recent studies showed mean age for paratyphoid fever was higher compared to typhoid fever. Thus, hospital-based studies and outbreak reports reveal that enteric fever is a major health concern in India (Ochiai et al., 2008; Crump and Mintz, 2010; Tewari et al., 2010). The common etiological agent is *S*. Typhi with apparently increasing number of cases due to *S*. Paratyphi A.

Figure 1.2 Worldwide epidemiology of typhoid fever

1.3 Pathogenesis of Typhoid fever

S. Typhi is a human-restricted obligate parasite that enters the body via ingestion of contaminated food or water. The organisms should be able to survive and pass the acidic environment of the stomach and thus must have a certain infectivity dose (usually 10^5 to 10^8) before they could set up a successful infection (Hornick et al., 1970a; Parry et al., 2002). In the distal ileum *S.* Typhi adheres to the intestinal mucosal layer and then invades the mucosa to get localized into the Payer's patches. An interesting study carried out by Hornick and colleagues in the early 1970's, showed that the organism does not enter through the pharyngeal mucosa, indicating its specificity towards intestinal mucosa (Hornick et al., 1970a). The M cells are specialized epithelial cells overlying Payer's patches that are probably the site for internalisation of *S.* Typhi and its subsequent transport to the underlying lymphoid tissue (Rescigno et al., 2001; Niess et al., 2005). After penetration, the organisms replicate within the macrophages of the Peyer's patches followed by translocation to the intestinal lymphoid follicles and the draining mesenteric lymph nodes (MLN). In case of gastroenteritis, the bacteria do not further penetrate the gut and the gut-associated lymphoid tissue (Zhao et al., 2006; Voedisch et al., 2009). The bacteria then spread via the thoracic duct into systemic circulation which takes around four to ten days finally resulting in transient primary bacteraemia. During this phase the organisms are shed in the liver, spleen, lymph nodes, gallbladder, lungs and the kidneys (Hornick et al., 1970a; Parry et al., 2002; Kaur and Jain, 2012). The circulating bacteria are then removed from the blood by mononuclear phagocytic cells present in the

reticuloendothelial system of liver and spleen. This in turn assists the bacteria to evade the deleterious effects of antibodies and simultaneously provides a good niche for survival and multiplication. *Salmonella* have been shown to survive and multiply within macrophages (House et al., 2001a) and they also infect the neutrophils and dendritic cells of the MLN (Cheminay et al., 2005; Geddes et al., 2007). Hence, phagocytic carriage of *Salmonella* is necessary for a systemic infection (Voedisch et al., 2009). Following this intracellular multiplication, the bacteria re-enter the blood stream to cause continuous secondary bacteraemia (Grant et al., 2008a). Simultaneously, the bacteria reach the gallbladder form where they are released into the intestine for a secondary invasion (the gallbladder also forms a main reservoir for chronic *S.* Typhi persistence). This results in inflammation, necrosis and sloughing of Peyer's patches to give rise to characteristic typhoid ulcers (Gordon, 2008; Kaur and Jain, 2012). Thus, it is not surprising that relapses may be seen during convalescence due to recrudescence of bacteria that lie quiescent within host tissues. Around 10% of the patients show relapses 2-3 weeks after fever resolution which is generally milder than the primary attack (Parry et al., 2002). Also, re-infection in the same patient can occur and is distinguished from relapse by molecular typing (Wain et al., 1999). The overall cycle of *S.* Typhi pathogenesis in humans is summarized in **Figure 1.3**.

Understanding the pathogenesis of *S.* Typhi in intestinal and systemic sites is important ant advances in use of animal models have greatly assisted the same. Being a human-restricted pathogen, *in vivo* studies of *S*. Typhi involve murine model of infection using *S.* Typhimurium that mimics pathological features similar to those in humans (Santos et al., 2001). Since intracellular replication is a key aspect in *S.* Typhi systemic infection, mice lacking the functional copy of the Nramp1 (natural resistance-associated macrophage protein one) gene are used. The Nramp1 scavenges the intracellular bacteria in macrophages (Forbes and Gros, 2001).

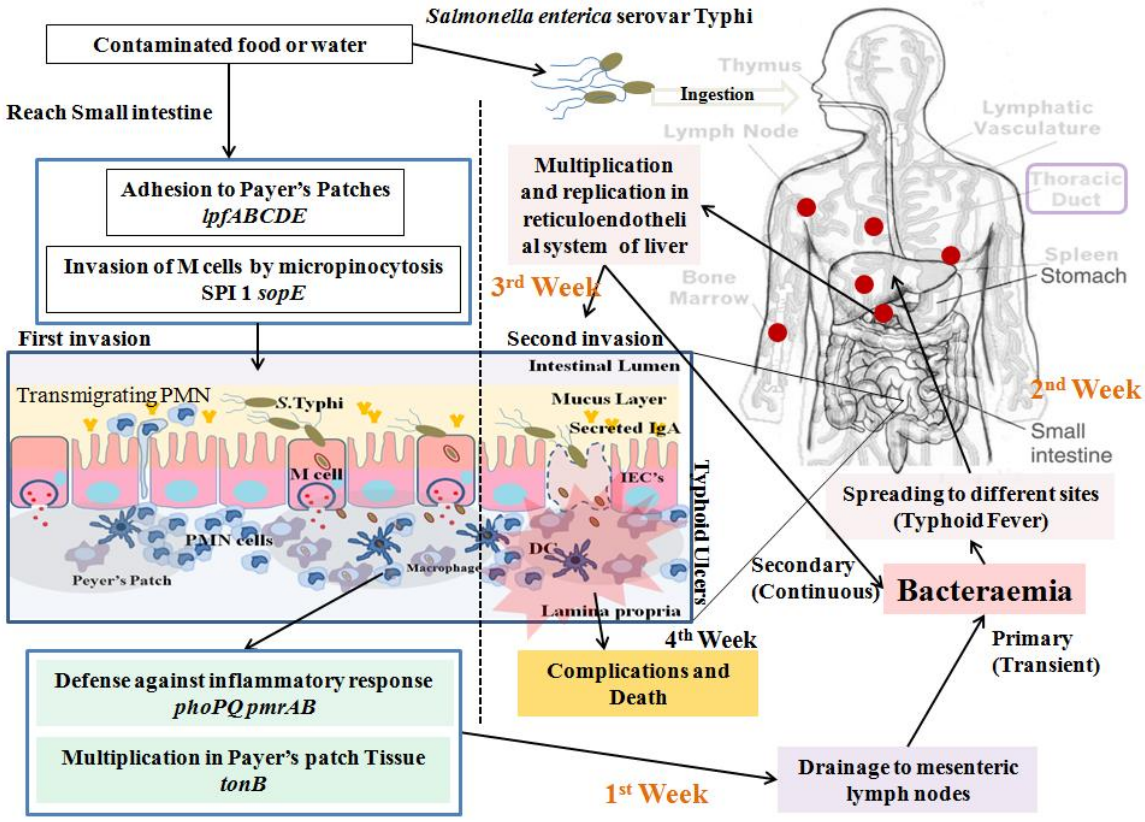


Figure 1.3 Pathogenesis of *Salmonella enterica* serovar Typhi in humans

Typhoid fever does not always present a distinct clinical picture with manifestations that are inconsistent and indistinguishable from other infections. General clinical presentation of untreated typhoid fever reveals a multi-stage disease characterized by fever and bacteraemia (may result in headache and diarrhea) in first week; abdominal pain, appearance of 'Rose spots' (5 to 30% of the cases) (Parry et al., 2002; Kuvandik et al., 2009), splenomegaly and relative bradycardia in second week; abdominal complications involving perforations and bleeding in the third week; and finally resolution or progression towards death after the third week (Mahmud et al., 2008; Patel et al., 2010). Around 10-15% of the patients in endemic regions manifest complications with highest incidences in immunocompromised and paediatric patients. But a number of exceptions are seen with respect to patterns of typhoid fever especially in the endemic regions of the disease (Gordon, 2008; Kaur and Jain, 2012). For instance, typhoid fever presents as pyrexia of unknown origin in some areas whereas in others as fulminating illness with acute renal failure or circulatory shock. In certain cases, typhoid infection often presents with features clinically indistinguishable from malaria or urinary tract infections or viral infection like influenza. Also the fever has an unpredictable onset with an asymptomatic period that lasts from 3 to 60 days.

Neutophilia and septic shock are not typical manifestations of typhoid, as seen in many other gram negative bacteraemia (Tsolis et al., 2008). Thus, the clinical diagnosis of Typhoid fever is mostly inaccurate. Typhoid fever during pregnancy can lead to neonatal typhoid through vertical intrauterine transmission route and in worst cases may even lead to miscarriage (Gordon, 2008; Kaur and Jain, 2012).

1.3.1 Molecular mechanisms underlying *S*. Typhi pathogenesis in the host

Various substances like small molecules, proteins and DNA are secreted by bacteria and released via specialized macromolecular nanomachines referred to as secretion systems. These substances play a crucial role during bacterial response to the environment and in several biological processes like pathogenicity, adhesion, adaptation and survival. The fate of these substances depends on the secretion system i.e. they remain associated with the bacteria or they are released into the host (Costa et al., 2015). In Gram negative bacteria these machinery can be divided into three categories; (1) Single membrane-spanning transporters in inner membrane; (2) those spanning both the inner and outer membrane; and (3) those that span outer membrane (Costa et al., 2015). The latter two are of particular importance for virulence and pathogenicity in Gram-negative infections and are summarized in **Figure 1.4**. None of these systems are constitutively active and secretion may be triggered by the recognition of host receptors by specialized molecules called adhesisns.

Both *S. enterica* and *E.coli* are believed to be derived from a common ancestor and differ by only 10% in their DNA sequences (Sabbagh et al., 2010). The variation in pathogenesis may be due to the horizontal gene acquisition and ability of *Salmonella* to lose certain genes so as to assist virulence. Though around 90% of genes in *S.* Typhimurium and *S.* Typhi are identical, *S.* Typhi specifically adapts to human host (McClelland et al., 2001). This may be attributed to the presence of 23 *S.* Typhimurium genes that are inactivated or disrupted in *S.* Typhi. The 11 pseudogenes present in NTS suggest a similar process of host-adapted microbes may occur in them.

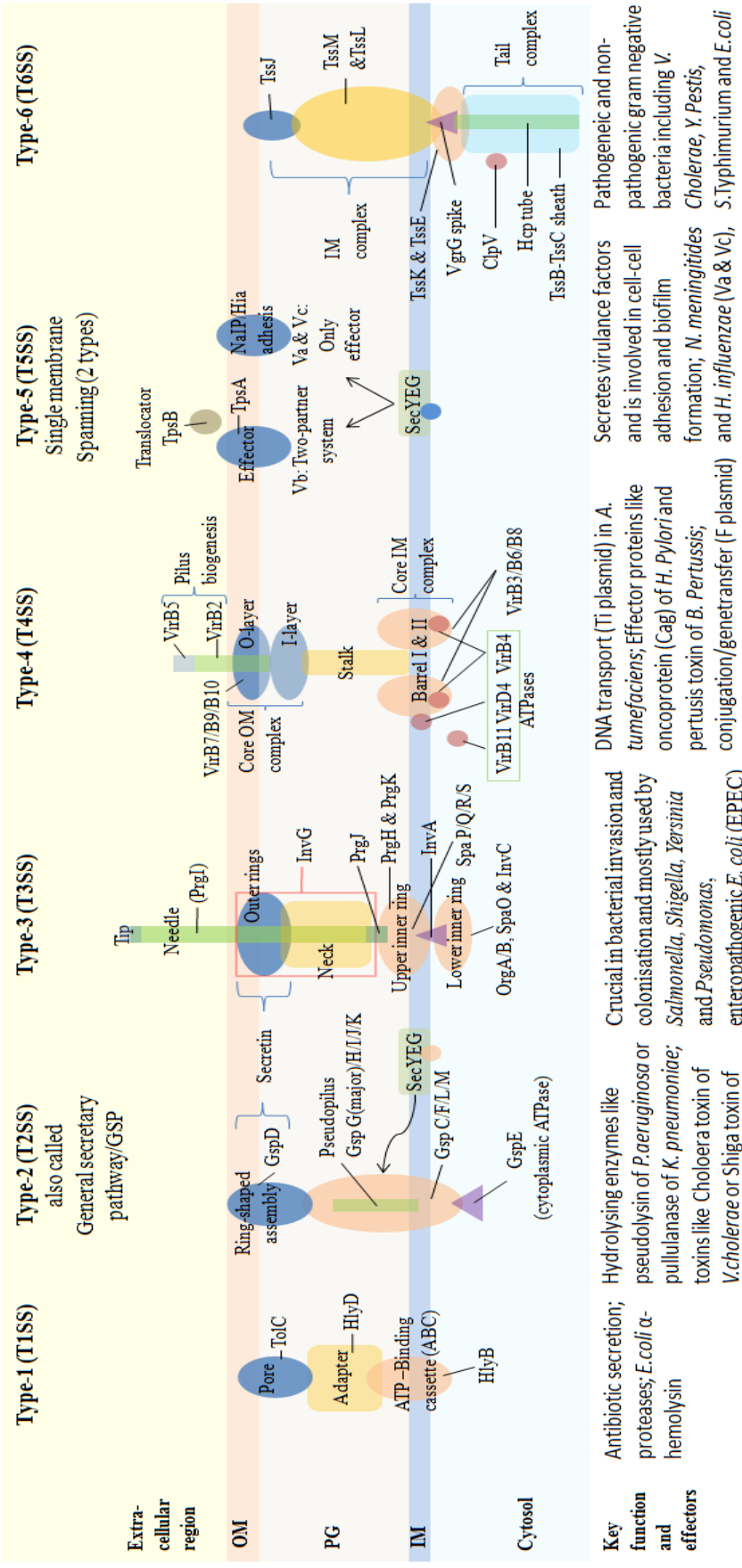


Figure 1.4 Different types of bacterial secretion systems and their components

At present, the virulence factors essential for intracellular life cycle of *S.* Typhi like the Vi antigen (*S.* Typhi only), LPS and other surface polysaccharides, and T3SS have been well characterized. Most virulence factors necessary for internalisation, invasion and survival within target cells are usually located within genomic locations called *Salmonella* Pathogenicity Islands (SPIs) which are tightly regulated by global regulators such as PhoP-PhoQ, RcsDBC, OmpR-EnvZ and RpoS (Groisman, 2001; Marathe et al., 2012; Garai et al., 2012). At present, 21 SPIs have been identified. *S.* Typhimurium and *S.* Typhi genomes share 11 common SPIs; four are specific to *S.* Typhi (SPI-7, 15, 17, and 18) and only one (SPI-14) to *S.* Typhimurium. Five major PAI's (SPI 1-5) are key regulators in *Salmonella* virulence (Schmidt and Hensel, 2004) and translocated as effectors by Type 3 secretion system (T3SS) (Figueira and Holden, 2012). T3SS-1, encoded by SPI-1, is required for SPI-1-dependant internalisation of epithelial cells (non-phagocytic cells) and induces membrane ruffles causing alteration in actin cytoskeleton that finally results in macropinocytosis (Raffatellu et al., 2005). SPI-1 plays an important role in both enteric fever and gastroenteritis (Hansen-Wester and Hensel, 2001). Although important in invasion of non phagocytic cells, SP-1 deficient *S.* Typhimurium is shown to cause systemic infection via direct uptake by CD-18 phagocytic cells (Rescigno et al., 2001). It also mediates the translocation of effector proteins such as SptP, SipA, SipB and AvrA into eukaryotic cells (McGhie et al., 2009). These SP-1 encoded protein effectors induce fluid accumulation and pro-inflammatory responses in *S.* Typhi (Bruno et al., 2009; Garai et al., 2012). Following internalisation *Salmonella* remains in a membrane bound compartment called the *Salmonella* containing vacuole (SCV) and translocates the effector proteins into the host cytoplasm endomembrane system (Bakowski et al., 2008). T3SS-2, encoded by SPI-2, is required for intracellular replication and immune evasion. They include SpiC, SseF and SseG, whereas SifA, SifB, PipB, PipB2, SseI, SseJ, SseL, SspH2, GogB are other effectors of T3SS-2 which are encoded on a different loci (Lavigne et al, 2008). These effectors control the formation and maintenance of SCV and arrest host endosomal pathway (Figueira and Holden, 2012), conferring protection against reactive oxygen species (ROS) inside macrophages (prevents phagocytic burst) (Haraga et al., 2008). Both T3SS1 and T3SS2 contribute in developing enterocolitis (Grassl and Finlay, 2008) and the tetrathionate generated during inflammation is exploited by *Salmonella* to outcompete the gut microflora (Winter et al., 2010). Most of these functions are true for *S.* Typhimurium, but in case of *S.* Typhi there is relatively limited

data concerning the role of T3SS. Also the functions may differ, for instance, the T3SS2 of *S.* Typhi is not required for survival in macrophages but may have a role in modulating host immune response to establish an asymptomatic long term infection (Forest et al., 2010). SPI-3 encodes virulence factors that are important in intestinal colonization and intracellular survival which include proteins like MgtC and MisL required for intramacrophage survival and intestinal colonisation respectively. SPI-4 encodes a type I secretion system (T1SS) and also a substrate of T1SS, SiiE, a large (600kDa) surface-associated non-fimbrial adhensin that contributes to invasion and adhension to eukaryotic cells. Many additional effectors of T3SS-1, including SopA, SopB, SopD, SopE, SopE2, Ssph1 and Slrp, are not encoded on SPI-1; rather they are encoded by other horizontally acquired elements, such as on SPI-5 (Lavigne et al, 2008). Some of these effectors, namely SopB and SopE/SopE2 induce dramatic rearrangement of the actin cytoskeleton in host cells, which results in large membrane ruffles and subsequent internalization of *Salmonella* (McGhie et al., 2009). The actions of SopB, SopE, SopE2 and SipA are likely to disturb the integrity in the epithelial barrier and result further in PMN transmigration whilst promoting fluid flux, thereby contributing to diarrhoea. SPI-7 is a large 134 kb segment that encodes the Vi capsular polysaccharide antigen, Type IV pili and SopE. The *viaB* locus (containing the tviA and tviB) is a 14Kb region within SPI-7 that prevents host recognition of *Salmonella* by TLR4 and TLR5 (Raffatellu et al., 2005). The locus contains genes that synthesize, regulate and export the Vi capsular antigen. The presence of the Vi antigen contributes greatly to the virulence in *S.* Typhi, which is required for intracellular survival in phagocytes and has been implicated in system dissemination. The Vi antigen is absent in *S.* Paratyphi A, *S.* Typhimurium and other serovars (Virlogeux et al., 1995). Four different types of fimbriae are known to be expressed by *Salmonella* that include type 1 fimbriae (fim), plasma-encoded fimbriae (pef), long polar fimbriae (lpf) and thin aggregative fimbriae (curli or csg), all of which seem to have specificities for different cell types (Humphries et al., 2001; Ledeboer et al., 2006). A recent study has shown fimbriae, namely type 1 fimbrial adhesion FimH, mediates *Salmonella* uptake into murine DCs in a T3SS-independent fashion. It is thought that 13 fimbrial loci exist in *Salmonella*, which are thought to aid the internalization process through biofilm formation, attachment to host cells and colonization (Guo et al., 2007; Gerlach et al., 2007b). BapA, a large cell-surface protein is also required for biofilm formation and subsequent evasion and colonization, similar to FimH (Latasa et al., 2005). The

significance of the biofilm stems from the extreme environmental conditions the bacteria are subjected to, and their successful survival lies in their ability to grow in surface-attached biofilms protected by an extracellular matrix. In *S.* Typhi, type IV B pilus is a major adhesion factor during entry of this pathogen into intestinal epithelial cells (Balakrishna et al., 2009).

1.4 Diagnosis of Typhoid fever

Excretion of bacteria in the stools usually begins in a week following onset of illness and continues through the convalescence. The bacteria are shed sporadically thereafter in chronic carriers. Thus, conventional microbiological methods involving recovery of the pathogen from blood or stool samples help to confirm typhoid fever in patients. These cultures have been shown to have low sensitivity (positive for only 30-60% of cases) (Vallenas et al., 1985) and thus aspirate cultures of bone marrow having sensitivity greater than 80% is considered to be gold standard method (Vallenas et al., 1985; Baker et al., 2010). Due to the difficulties involved in performing these methods, generally in typhoid endemic regions, serological tests have been extensively used in diagnosis of typhoid. The Widal test identifies antibodies present against *Salmonella* specific O and H antigens in the serum of patients (Pang and Puthucheary, 1983). But even these tests are associated frequently with inaccurate results showing false positives or negatives (Olopoenia and King, 2000). Thus, more sensitive and accurate methods for diagnosis of typhoid fever are needed, including those that detect the asymptomatic carriers. Recent advances include molecular methods like PCR assays that detect bacteria within 4-5days (Zhou and Pollard, 2010), and antigen based rapid diagnostic tests like Multi-Test Dip-S-Ticks and IDL® Tubex Test, Typhidot® (Olsen et al., 2004).

1.5 Treatment and Vaccination

The emergence of rapid multi-drug resistance (MDR) in *Salmonella* has become a major concern for treating typhoid fever (Mirza et al., 1996). Over the past decade, MDR has risen exponentially in endemic areas with strains resistant to nearly all first-line antibiotics. Furthermore, around 60% of the strains exhibit MDR in acute and chronic carriers (Harish and Menezes, 2011; Pratap et al., 2012). The MDR phenotype is generally plasmid-mediated (like incHI and *p*HCM1) (Mirza et al., 1996, Parry et al., 2002), but variants of *Salmonella* have developed MDR as a part of their genome

making them likely to retain drug-resistant genes even when antibiotics are no longer in use. The *Salmonella* genomic island-I in *S.* Typhimurium is an example of chromosomally-encoded gene complex which can be transferred to other serotypes (Mulvey et al., 2006). Besides various molecular mechanisms along with co-ordinated gene regulations contribute to diverse bacterial phenotypes that confer resistance to wide range of antibiotics.

Chloramphenicol was widely used to treat typhoid fever till early1970s, after which chloramphenicol-resistance became a major problem as reported during outbreaks in India, Mexico, Korea, Thialand, etc. The resistance was attributed to the self-transferrable, high molecular weight incHI plasmids present within the bacteria (Mirza et al., 1996). Ampicillin and trimethoprim-sulfamethoxazole (TMP-SMZ) then became drugs of choice for treating typhoid fever, but soon *S.* Typhi and Paratyphi strains developed similar plasmid-mediated resistance to these antibiotics. Thereafter, the use of fluorquinolones like ciprofloxacin, pefloxacin and ofloxacin became wide spread in treating MDR strains of *S.* Typhi. Third generation cephalosporin's such as cefotaxime and ceftriaxone are also effective in treating MDR *S.* Typhi infections (Zavala Trujillo et al., 1991).

The rate of fluoroquinolone resistance is high and rising in south and Southeast Asia and, to some extent, in East Asia. Susceptibility to chloramphenicol, TMP-SMZ, and ampicillin has highly decreased and some MDR strains in Southeast Asia have been shown to acquire resistance to fluoroquinolones (Harish and Menezes, 2011; Pratap et al., 2012). Recently, ciprofloxacin resistance has also been observed in India and Karchi where MDR *S.* Typhi strains were reported (Chau et al., 2007). Such fluoroquinolone resistance is attributed to mutations in gyrA and parC genes, the products of which are targets for the fluoroquinolone drugs. For instance, a single point mutation in gyrA gene confers partial resistance and a second point mutation further increases the resistance. However, single mutation in both gyrA and parC make the organism fully resistant to first-generation fluoroquinolones (Randall et al., 2005). In such cases, cephalosporins have been shown to be effective in treating clinical strains of *S.* Typhi (Capoor et al., 2007).

The Indian Association of Pediatrics (IAP) in 2006 issued the guideline for treatment of typhoid fever in south Asia. Although these guidelines were published for pediatric typhoid fever, the authors feel that they are also applicable to adult cases. For empiric

treatment of uncomplicated typhoid fever, the IAP recommends cefixime and, as a second-line agent, azithromycin. For complicated typhoid fever, they recommend ceftriaxone. Aztreonam and imipenem are second-line agents for complicated cases. The authors believe that the IAP recommendations apply to empiric treatments of typhoid fever in both adults and children (Kundu et al., 2006).

In highly prevalent areas other than those mentioned above, the rate of intermediate sensitivity or resistance to fluoroquinolones is 4.7% in sub-Saharan Africa, 10.8% in the Middle East and 3.7% in the Americas. Therefore, the WHO recommendations may still be valid for strains that originate outside of south or Southeast Asia, and that complicated typhoid fever from these regions should be treated with intravenous ciprofloxacin and uncomplicated disease should be treated empirically with oral ciprofloxacin (Acosta et al., 2003; Capoor et al., 2007).

Various non-commercialized vaccines including DNA vaccines for typhoid fever have been developed by researchers. At present, there are only two commercially available and licensed vaccines for typhoid fever. These are the live attenuated Ty21a vaccine and Vi polysaccharide (VI-PS) parenteral vaccine (WHO, 2000, 2008a). However, these vaccines are ineffective against children below 2-5years and do not confer complete protection against infection. Thus, research is still going on to develop potent and effective vaccines effective in all age groups. The most recent one is a complex of CRM197 to the Vi-PS of *S.* Typhi and O-polysaccharide of *S.* Paratyphi (Vi-CRM197) (Micoli et al., 2011)

1.6 The host responses to *S.* Typhi infection

Both, cell mediated and humoral immune responses are necessary for containment of *Salmonella* infection in humans (Mastroeni, 2002). But *S.* Typhi ensures its dissemination throughout the body by using cells of the immune system for its multiplication and survival. The attachment of the bacteria to the epithelium generates pro-inflammatory responses that recruit neutrophils, macrophages and dendritic cells. This subsequently activates the adaptive immune responses mediated by the B and T cells (Grassl and Finlay, 2008). The innate secretory antibody IgA is also present in the mucus lining which controls the spread of *Salmonella* (Wijburg et al., 2006). The containment of NTS to gastroenteritis is brought about by rapid infiltration of neutrophils (Tsolis et al., 2008), but in case of *S.* Typhi the cell infiltrates are

dominated by mononuclear cells with few neutrophils (Kraus et al., 1999). This justifies the lack of inflammatory response and longer incubation time associated with *S.* Typhi. Also, evasion from immune response by tightly regulated virulence gene expression helps the organism to establish a successful systemic infection within the host (Wangdi et al., 2012). Furthermore, to avoid the intra- and extracellular killing due to anti-microbial peptides secreted by the gut epithelia and macrophages, *Salmonella* undergo alterations in the LPS composition or synthesize proteins to export these peptides out of the bacterial cells (Gunn, 2001; Nizet, 2006). This resistance mechanism produced towards *Salmonella*-induced colitis provides fitness advantage in the lumen of inflamed gut (Fischbach et al., 2006; Liu et al., 2012).

Pattern recognition receptors are the first components of the immune system that detect pathogen invasion and initiate immune responses. They form a key link between the innate and adaptive immune responses in the host (Kawai and Akira, 2010). The detection of mucosal invasion by *Salmonella* begins with the recognition of pathogen-associated molecular patterns (PAMPs) present on the bacteria by the PRRs namely Toll-like receptors (TLRs) and Nod-like receptors (NLRs) (Kawai and Akira, 2010). The TLR 2, 4, 5 and 9 recognize the curli fimbriae, LPS (lipid A), flagella and bacterial DNA respectively (Tukel et al., 2010; de Jong et al., 2012), and the complement recognize the O antigen of the LPS. The bacterial effectors and flagellin have also been shown to be recognized by the NLRs present in the cytosol (like NLRC4) (Miao et al., 2006). These NLRC recognize bacteria within macrophages and release them through pyroptosis, thus making them exposed to neutrophils. This leads to containment and avoids dissemination, as seen in NTS infection (Bergsbaken et al., 2009; Miao et al., 2010). The pro-inflammatory responses involve the production of cytokines, mostly the tumour necrosis factor (TNF-α), Interleukins (IL-6,1β, 12, 18) and interferon-γ (Thompson et al., 2009). Cell death pathways like pyroptosis are induced early in infection by SP-1 to promote inflammation (Fink and Cookson, 2007). These responses recruit neutrophils and macrophages to the site of infection. *Salmonella* exploits the changes in osmolarity of the environment to prevent its recognition by the host cells. For instance in conditions of high osmolarity (like that found in the intestinal lumen) the bacteria suppress Vi antigen expression and activates genes encoding the flagella and T3SS-1, whereas in low-osmolarity conditions it is vice-versa (Zhao et al., 2001; Winter et al., 2010). The Vi antigen prevents the recognition of *S.* Typhi LPS by TLR4

and thus cannot be detected by the innate immune system (Wilson et al., 2008). Once the *Salmonella* reach the mysentric lymph nodes, liver and spleen, inflammatory macrophages are recruited to the site of infection. Intracellular adhesion molecules like ICAM1 and TNF-α confine the macrophages to localized foci that form pathological lesions surrounded by normal tissue. Failure to form these lesions causes bacterial dissemination due to uncontrolled growth (Mastroeni and Grant, 2011). In fact *S.* Typhi is shown to spread and colonize organs by inducing apoptosis in cells followed by infection of new phagocytes (cell-to-cell spread) (Grant et al., 2008b; Mastroeni and Grant, 2011). Interestingly, where macrophages are shown to be important in systemic dissemination of bacteria, studies also report that many intracellular bacteria do not replicate in macrophages and appear to be in a dormant-like state (Helaine et al., 2010). The O antigen confers resistance to complement (Holzer et al., 2009) whereas the modifications in the LPS prevent recognition by TLR-4 (Chow et al., 1999). The bacteria are also shown to inhibit dendritic cells from activating T cells, thus evading the adaptive immune responses of the host.

1.7 Chronic persistence of *S.* Typhi in the host

Chronic carriers are those individuals who carry disease causing bacteria without any symptoms and are possible reservoir for dissemination of the organisms into the environment which may lead to new infection in other or same individual. The concept of chronic carrier was best illustrated by Mary Mallon, or Typhoid Mary, a cook in New York City who infected at least 54 people. She was later subjected to involuntary lifelong quarantine on North Brother Island in 1907 and her autopsy revealed that the gallbladder was colonized by *S.* Typhi (Marr, 1999). Such cases lead doctors trace typhoid epidemics to human sources and identify carriers so as to continue the confinement practice. It emerged that around 43 female typhoid carriers were quarantined from 1907-1992 in England and some were held for more than 40 years. With increased research on typhoid fever carrier state and widespread use of antibiotic this practice was later discontinued (Gonzalez-Escobedo et al., 2011).

Following the acute phase of infection and adequate treatment, 3-5% of the individuals become chronic carriers of *Salmonella* Typhi and spread the disease through bacterial shedding in urine and faeces (Gonzalez-Escobedo et al., 2011). A study on chronic infection using 129x1/SvJ *Nramp1*$^{+/+}$ revealed that *S.* Typhi was detectable in faeces

and tissues for 1 year following oral infection. Being asymptomatic, identification of such infection is difficult and further complications arise when around 25% of chronic carriers never experience any clinical manifestation of acute phase of the disease (Parry et al., 2002). Also due to intermittent shedding of bacteria in their stools, it is not always possible to detect the bacteria efficiently in carriers (Gopinath et al., 2012). Thus such typhoid carriers are crucial targets for disease control and prevention. In addition reports also reveal incidence of chronic carriers in individuals infected with NTS (Boisrame-Gastrin et al., 2011).

Usually females and individuals above 45 years are more likely to become carriers. In endemic regions chronic carrier state is associated with pre-existing hepatobiliary diseases like cholelithiasis, biliary obstruction, echinococcal cysts, biliary cirrhosis, hepatic hematoma, intrahepatic cholestasis and amoebic abscesses (Gosbell et al., 1995). Studies indicate that approximately 90% of the chronic *S.* Typhi carriers have gallstones (Gonzalez-Escobedo et al., 2011). Complications of typhoid carriage include cholangitis, chronic diarrhoea, acute and chronic cholecystitis, chronic hepatitis and rarely pancreatitis (Vaishnavi et al., 2005). Most importantly recent findings indicate *S.* Typhi chronic infection as a pre-disposing factor in development of gallbladder cancers. Such carriers have 150-fold excess risk of developing hepatobiliary carcinoma and 10-15 fold excess risk of developing gallbladder carcinoma compared to non-carriers (Shukla et al., 2000; Dutta et al., 2000; Nath et al., 2008). It is hypothesized that bacterial metabolites and toxins, and bacterial degradation of bile salts could promote gallbladder carcinoma. For instance, *S.* Typhi produces bacterial glucuronidase enzyme that acts on primary bile acids to produce secondary bile acids which are carcinogenic in nature. The enzyme also leads to precipitation of calcium bilirubinate, thus leading to gallstone formation (Shukla, 1993; Begley et al., 2005). In addition, *S.* Typhi is shown to produce the cytolethal distending toxin (CDT)-a bacterial genotoxin that assists in bacterial internalisation and chromatin fragmentation in the host (Haghjoo and Galan, 2004). These substances are highly concentrated in the gallbladder thus bringing about molecular changes in the host.

The pathogenesis of chronic carriage is best studied in animal models that mostly involve BALB/c or C57BL/6 mice (Santos et al., 2001). Animals showing increased shedding of bacteria to rapidly transmit the disease are referred to as super-shedders. They have been shown to have increased innate pro-inflammatory responses. In

chronically infected mice with *S.* Typhimurium, the gastrointestinal tract microbiota influences the *Salmonella* level and play key role in transmission (Lawley et al., 2008). Treatment with antibiotics induces a super-shedder phenotype and carrier status in host. Also the O-antigen variation is necessary during faecal shedding. For instance, SPI-16 (contains gene responsible for O-antigen glycosylation) mutant is outcompeted by wild type bacteria in the gut but not in systemic sites (Bogomolnaya et al., 2008). Several adhesions (MisL and SdhA) and fimbrial operons (bcf, lpf, stb, stc, std and sth) also contribute to intestinal carriage and faecal shedding. Usually T3SS1 and T3SS2 such as SseI are shown to be necessary in *S.* Typhimurium for persistent infection in mice. They prevent presentation of *Salmonella* antigens to T cells by controlling macrophage and dendritic cell migration thus inhibiting adaptive immunity. But the Sse is absent in *S.* Typhi. Factors such as Mig-4, VirK, RscC, PgtE and YdeI that protect *Salmonella* against antimicrobial peptides and genes like sspJ, hmp and sodC1 that protect against RNS and ROS also contribute to long term typhoid carriage (Lawley et al., 2008). A study in 2005 also showed that an enzyme AceA which enables use of fatty acids is needed during chronic persistent infection (Fang et al., 2005). Though limited data is available for chronic and persistent *Salmonella* infections, the use of humanized mice models like TLR 11-/- and immunodeficient Rag2−/− γc−/− mice engrafted with human fetal liver hematopoietic stem and progenitor cells, paves a way for future studies on chronic infection with *S.* Typhi (Song et al., 2010).

Chronic *S.* Typhi carriers have high levels of circulating serum antibodies against flagella and Vi antigen. High levels of IgA antibody are also seen in such carriers (House et al., 2001). In mice, INF-γ plays a key role in persistent infection by controlling the extent of macrophage activation. Control of T-cell responses through limited antigen presentation and limited intracellular replication are key factors in establishing a persistent *S.* Typhi infection in the host. In fact a recent report reveals that progression of persistent infection is influenced by regulatory T cells (Tregs) that modulate Th1 responses (Ruby et al., 2012). Chronic bacterial infection is also associated with continuous release of mediators of inflammation (summarized in **Figure 1.5**) in their sites of infection that brings about specific alteration in host cell to cause diseases like cancer (Nath et al., 2010).

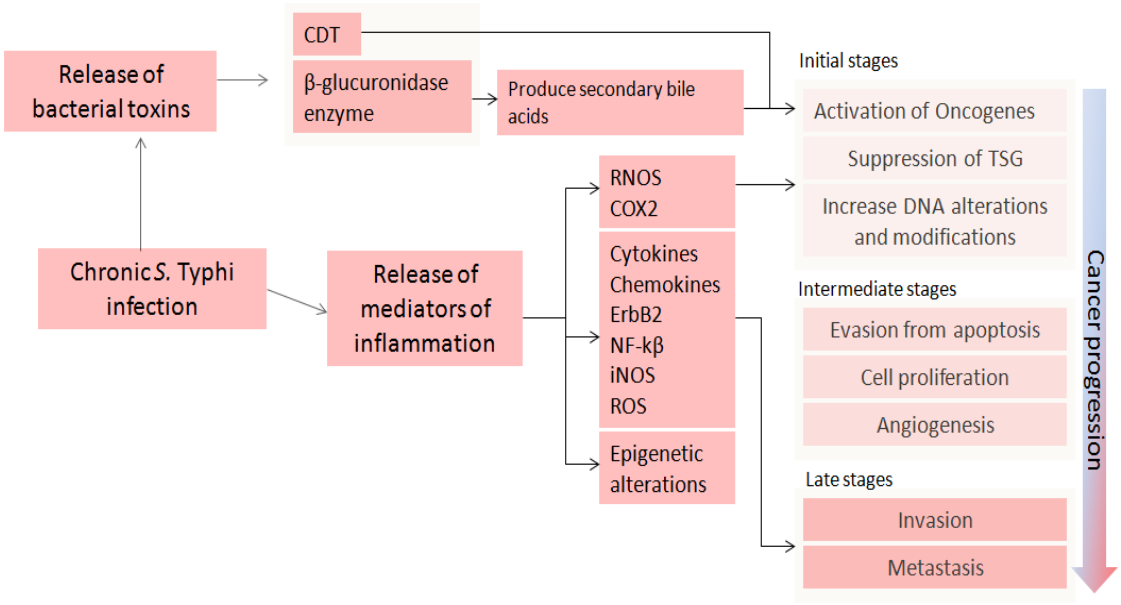


Figure 1.5 Role of *S.* Typhi in mediating host responses during chronic infection

1.7.1 Treatment of chronic carriers

Antibiotic treatment of chronic *S.* Typhi persisting in the gallbladder is difficult compared to treatment of acute infection. Less than two-thirds of the chronic infections are resolved with prolonged high–dose antibiotic therapy that is associated with side effects like gastric discomfort and gastrointestinal bleeding (Gonzalez-Escobedo et al., 2011). Furthermore, treatment with ampicillin is only effective in patients without gallstones. Currently, removal of the gallbladder is the only cure for chronic *S.* Typhi infection within the gallbladder with. But this does not ensure the removal of bacteria persisting in additional foci (like biliary tree, spleen, etc.) within the body. Due to continuous rise in the MDR *S.* Typhi strains even in carriers, it is difficult to treat such infections, with further research needed to understand the carrier state of the *S.* Typhi in the gallbladder (Song et al., 2010; Gonzalez-Escobedo et al., 2011).

1.8 The Gallbladder: Anatomy, physiology and clinical manifestations

The human biliary tract consists of the gallbladder, common bile ducts, cystic duct and intrahepatic ducts **(Figure 1.7)**.

1.8.1 Anatomy and histological classification

Gallbladder is pear shaped hollow organ located in a region on posterior surface of right hepatic lobe and is 7-10cm long with a width of around 3cm. It stores bile

produced by the liver and is divided into three regions: The fundus, the body and the neck **(Figure 1.7)** (Odze and Goldblum, 2009).

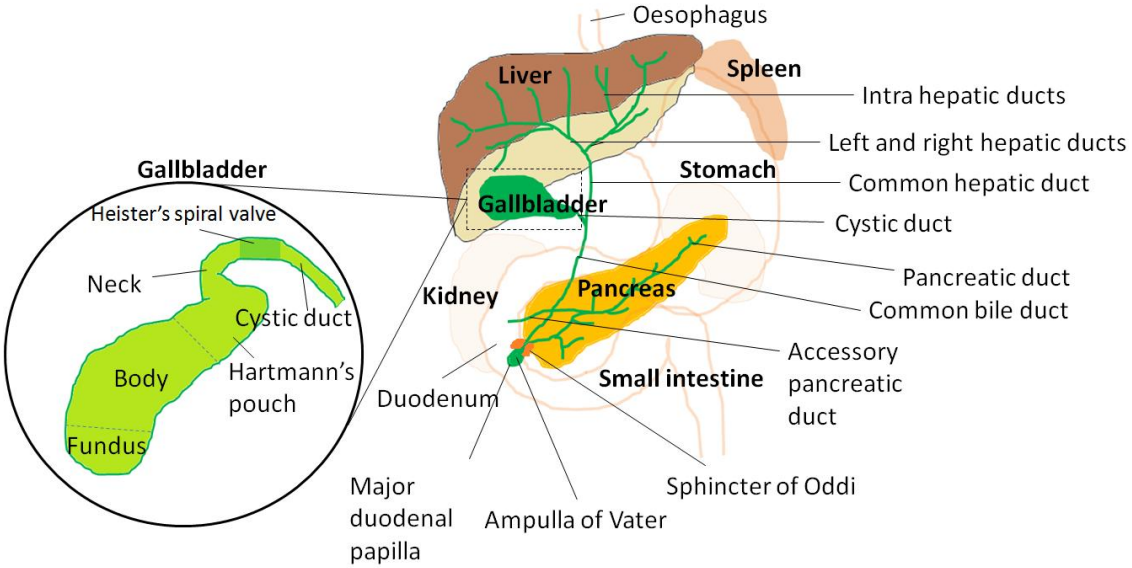


Figure 1.6 The hepato-biliary system and gallbladder in humans

Histopathological analysis reveals that gallbladder consists of three layers namely mucosa (innermost), muscularis and adventitia or serosa (outermost). The mucosa is made up of columnar epithelial cells and the submucosa is absent in the gallbladder. The underlying lamina propria contains blood vessels, connective tissues and diffused lymphatic tissues. Mucosal folds are generally present in the non-distended state of the gallbladder which disappears when gallbladder is distended following bile influx. These folds vary in shape and size with irregular arrangements and somewhat resemble the intestinal villi. The mucosa often shows deep notches referred to as the crypts or diverticula. The muscularis has randomly oriented smooth muscle bundles that contain numerous elastic and collagen fibres. This layer is responsible for bile expulsion by contraction of the gallbladder. A thick layer of connective tissue is present outside the muscularis layer that is rich in elastic fibres and adipose tissue. It also contains large lymph and blood vessels along with autonomic nerves (a division of peripheral nervous system). **Figure 1.6** represents the overall histology of the gallbladder tissue. Where the gallbladder attaches to the liver the connective tissue is covered by adventitia and gallbladder surface exposed to peritoneal cavity is covered by the serosa (Eroschenko, 2008; Rosai 2011; Kumar et al., 2013).

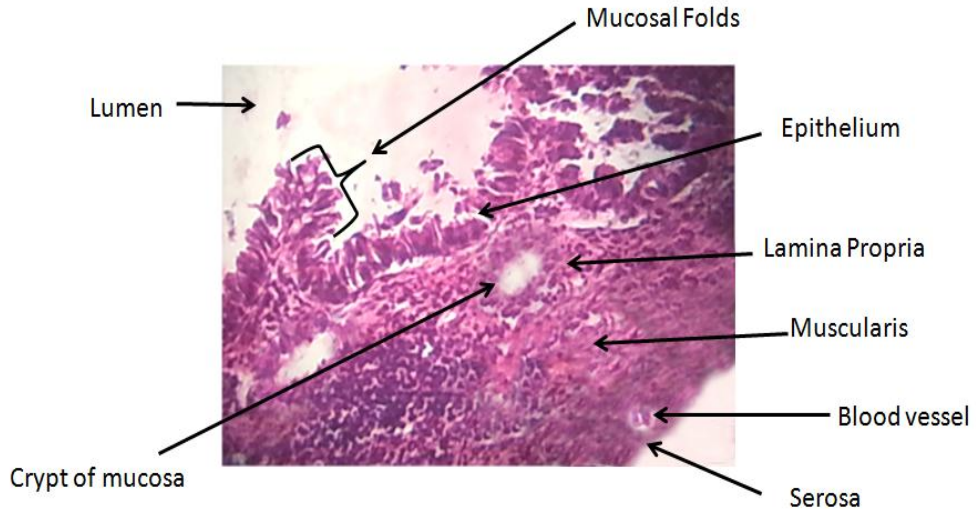


Figure 1.7 Histopathology of the human gallbladder (H&E staining)

Bile is continuously produced by liver hepatocytes and transported through the common hepatic duct which then joins the cystic duct(that originates from gall bladder) to form a common bile duct. The common bile duct then unites with the pancreatic duct towards the end into a common opening called the ampula of Vater or hepatopancreatic ampulla present in second part of the duodenum. Bile is needed for emulsification of lipids and digestion of fats, hence following ingestion of fatty meal stimulates production of bile. The stimulation is under hormonal control i.e. when the food reaches proximal duodenum, the entroendocrine cells of the intestinal mucosa secrete a hormone called cholecystokinin into the bloodstream. This hormone increases the bile release into the duodenum by three ways: promoting increased production of hepatic bile, causing smooth muscle contraction of the gallbladder wall for expulsion of bile and relaxation of the sphincter muscles (sphincter of Oddi) to release secreted bile. The bile is continuously produced by hepatocytes of the liver but as the sphincter of Oddi is closed the excess bile is stored within the gallbladder (Martini, 2005; Eroschenko, 2008; Odze and Goldblum, 2009). The gallbladder not only stores but also concentrates the bile by 5-10 times by allowing passive transport of water and chloride ions from the lumen into the intracellular spaces of epithelium. Along with digestion of fats, bile is also needed for inducing mucin secretion, absorption of fat-soluble vitamins and elimination of excess cholesterol and waste metabolites produced by the liver. Bile is a complex fluid made up of bile salts (derived from cholesterol and makes up two-third of bile dry weight), phospholipids, cholesterol, biliary pigments, billirubin, mucus and variety of other electrolytes and proteins. The liver produces around 1 litre of bile

everyday in adults and the gallbladder holds approximately 50-80mL of bile (Begley et al., 2005; Hofmann and Hagey, 2008).

1.8.2 Bacteriology of bile and gallbladder

The liver and the bile secreted by it is mostly sterile in healthy individuals with normal biliary tree. But, researchers have shown the presence of various bacteria in the gallbladder or hepato-biliary tree using different microbiological and molecular methods. The bacteria reach the gallbladder via the hematogenous route, or, exist due to certain pathology of the hepato-biliary or gastrointestinal system (Nath et al., 2010). Most studies report isolation rate of bacteria from the bile to be <50%. Furthermore, the type of organisms and their frequency also varies with different populations. For instance, a study in 2004 reported that enteric organisms were isolated more frequently (54% cases) than other non-enteric bacteria (24% cases) from the gallbladder (Hazrah et al., 2004). **Table 1.2** summarizes the type and frequency in occurrence of organisms within the gallbladder, shown by different studies. Along with single bacterial infection, these studies also report polymicrobial infections in the gallbladder. For example, Capoor et al. and Hazrah et al showed polymicrobial infections in bile (4.8% cases) and gallstones (3.8% cases) (Hazrah et al. 2004; Capoor et al., 2008).

Organisms	**Percentage (%) of cases**			
	Brook et al.	**Capoor et al**	**Hazrah et al.**	**Our Study**
***Escherichia* spp.**	32.9	29.7	15	6
***Enterococcus* spp.**	-	-	7.5	-
***Streptococci* spp.**	19.4	-	-	-
***Klebsiella* spp.**	15.3	27	17.5	4
***Enterobacter* spp.**	12.5	-	7.5	3
***Proteus* spp.**	6.9	-	5	-
***Citrobacter* spp.**	3.6	8.1	2.5	-
***Staphylococcus* spp.**	3.2	2.7	3.8	2
***Pseudomonas* spp.**	0.9	5.4	8.8	3
***Salmonella* spp.**	-	8.1	1.5	2.5
***Acinetobacter* spp.**	-	2.7	7.5	-

Table 1.2 Type and frequency of bacteria in gallbladder

The existence of such bacteria in the hepatobiliary system is asymptomatic, ensuring chronic persistence of the organism within the body leading to dissemination and spread into the environment. This may bring about alteration in the host and may possibly be associated with disease progression. As seen in **Table 1.3**, the presence of these organisms is strongly associated with diseased state of the organ. In addition, studies also reveal *S.* Typhi persistence considered to be one of the pre-disposing factors for gallbladder cancers. Nath et al used a very sensitive and specific nested PCR assay to show that 67.3% of the gallbladder cancer patients were typhoid carriers in a typhoid endemic area, compared to 8.3% of normal patients (without any gallbladder pathology) (Nath et al., 2008; Nath et al., 2010). Moreover, using Vi serology, Shukla et al revealed that typhoid carriers are at a 7.9 times higher risk of developing gallbladder cancer (Shukla et al., 2000).

Source	**Cases showing positive culture**		**Country**	**Year**	**Author**
	Cholelithiasis	**Gallbladder Cancer**			
Bile	41%	81%	China/Japan	1996	Csendes et al.
Bile	25%	13%	Chile/Japan	1999	Roa et al.
All stones	81%	77%	India	1999	Hazrah et al.
Bile, stones and tissue	21.80%	33%	India	2014	Present work

Table 1.3 Association of *S.* Typhi with diseased state of the gallbladder

1.8.3 The Gallbladder disease: Clinical manifestations

(a) Cholelithiasis: The presence of gallstones in the gallbladder or the bile duct leading to pain and discomfort in the abdomen is referred to as cholelithiasis. The incidences of gallstones ranges from 10-20% across the world and various factors contribute to their formation like obesity, age, gender (female gender bias), unknown genetic determinants (like the 'lithogenic' genes) and chronic bacterial colonisation of the gallbladder. These gallstones are classified based on their composition and may be categorized into three types: (1) Cholesterol gallstones made up of 70-80% cholesterol, (2) Pigment gallstone made up of 40-60% calcium bilirubinate and (3) Mixed gallstones having 30-70% cholesterol and calcium bilirubinate. The gallstones found in the gallbladder are generally cholesterol and mixed gallstones (80%) and pigment

stones (20%) generally develop in bile ducts (Kim et al., 2003; Wistuba and Gazdar, et al., 2004).

- Formation of cholesterol gallstones depend on various factors like super-saturation of bile with cholesterol, cholesterol monohydrate nucleation followed by crystal retention and stone growth, changes in gallbladder contractility and mucin hypersecretion. Cholesterol being water-insoluble is dispersed in aqueous medium as bile acid micelles or bile acid vesicles. Cholesterol saturation within the bile may occur due to metabolic defects in the biliary lipid secretion. These include bile acid hyposecretion or cholesterol hypersecretion. Excess cholesterol in relation to phospholipids (95% phosphatidylcholine) and bile acids because of any of the two defects forms unstable cholesterol rich vesicles (lecithin-cholesterol vesicles) which aggregate and fuse ultimately resulting in cholesterol monohydrate crystals (multi-lamellar vesicles). Further aggregation and compaction of cholesterol alone (for cholesterol gallstones), or aggregation of cholesterol and calcium bilirubinate together (for mixed gallstones), leads to formation of stones in the gallbladder. The excessive cholesterol may also be carried by phospholipid lamella to form discoid particles that lead to cholesterol crystal nucleation. Furthermore, excessive mucin production (indirectly stimulated by fatty acids and lysolecithins) by gallbladder wall provides suitable condition for cholesterol crystal growth to a gallbladder stone. In case of impaired gallbladder emptying the bile remains within the gallbladder (also called Bile stasis) leading to cholesterol precipitation and formation of gallstone (Tazuma et al., 2001; Wistuba and Gazdar, et al., 2004).
- Formation of pigment stones are a results from precipitation of increased amounts of unconjugated and insoluble bilirubin (product of haemoglobin degradation) followed by aggregation. Excessive bilirubin may results from chronic hemolysis or chronic liver damage and decrease in bile acid secretion that leads to decrease in bilirubin solubility causing precipitation (Kim et al., 2003; Wistuba and Gazdar, et al., 2004).

(b) Cholecystitis: The inflammation of the gallbladder caused by obstruction of the biliary tract due to presence of gallstones is referred to as cholecystitis. The obstruction causes bile stasis, distension and infection of the gallbladder. Different type of bacteria have been identified using culture or PCR methods in gallbladder of patients with

cholelithiasis and cholecystitis which include species *of Salmonella, Eschericheia, Klebsiella, Enterococcus, Enterobacter, Proteus, Pseudomonas, Helicobacter, Acinetobacter, Citrobacter*, etc (Hazrah et al. 2004; Capoor et al., 2008). Interestingly, Maurer et al reported that mice fed with lithogenic diet (induces gallstone formation) containing bacteria had higher frequency of gallstone formation than similar diet without the bacteria (Maurer et al., 2005a). But the exact role and mechanism of bacteria in gallstone formation in not yet understood and requires further studies. Inflammation of the gallbladder in absence of gallstones (also called Acalculous cholecystitis) is less common and occurs in approximately 10% of acute cholecystitis patients. Mostly, injured patients, or a complicated surgery, or patients critically ill show Acalculous cholecystitis (Huffman and Schenker, 2010).

(c) Gallbladder cancer (GBC): Gallbladder Cancer, one of the commonest biliary-tract carcinomas (BTC), is an aggressive disease with late diagnosis and dismal prognosis. GBC is rarely diagnosed before cholecystectomy (removal of gallbladder) because symptoms rarely suggest cancer and imaging techniques are mostly non-diagnostic. GBC has low survival rates (Five year survival rate of 0-12%) and neither conventional chemotherapy nor radiation can increase the survival of patients. The incidence rate of GBC following cholecystectomy is between 0.9-6% and varies with geographic location (Wistuba and Gazdar, et al., 2004). Thus urgent efforts are needed for identifying markers and methods for early detection of gallbladder cancers, and simultaneously develop novel therapeutic strategies for treatment of patients. Various risk factors are associated with GBC which include geographic variation and ethnicity (Uncommon neoplasm worldwide, but high incidences seen in Chile and northern India with highest mortality rates in South America), irritation, inflammation (due to gallstones) and infection of gallbladder, female gender bias (affects females 2-6 times more frequently than men), genetic predisposition (like Anomalous pancreatobiliary duct junction disorder) and carcinogens or environmental factors (like higher risk in miners exposed to radon, diet, etc (Roa et al., 1999; Wistuba and Gazdar, et al., 2004) **(Figure 1.8)**. There is a direct link between GBC and gallstones because 85% of the GBC's have gallstones (with frequency of both running in parallel) and risk of GBC is proportional to the size of the gallstone (For instance, patients with gallstones larger than 3cm in diameter are at higher risk compared to those with gallstones smaller than 1cm) (Tazuma et al., 2001; Diehl 1983).

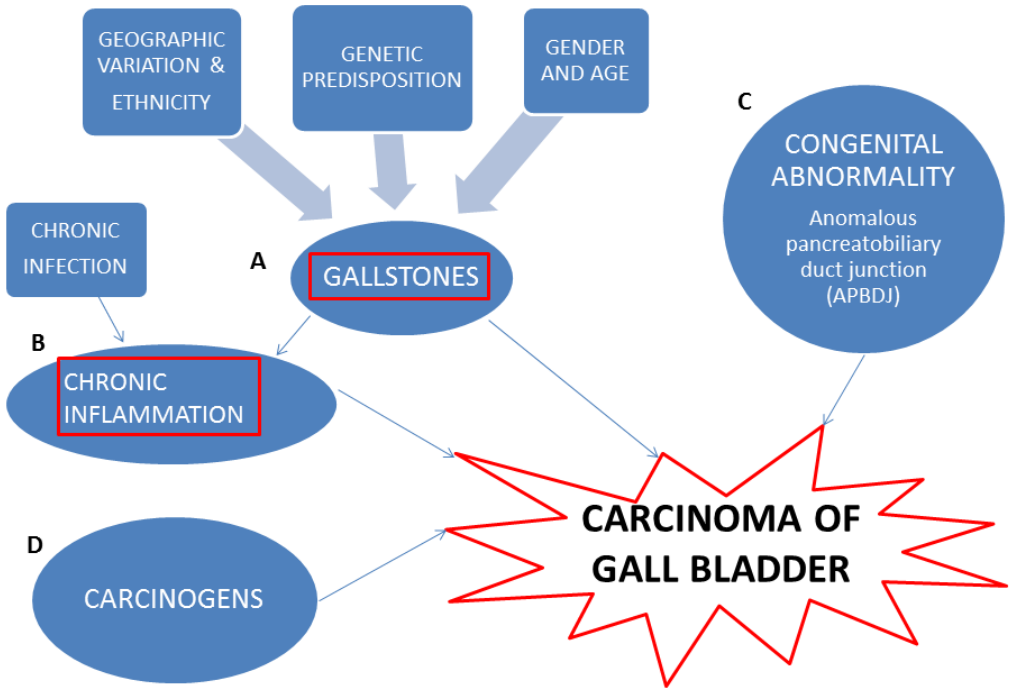


Figure 1.8 Factors contributing to carcinoma of gallbladder

Around 60% of the gallbladder cancers originate in the fundus of the gallbladder and more than 90% of the adenocarcinomas arise in glandular epithelium. Asymmetric thickening of gallbladder wall and infiltration of surrounding areas are common features of gallbladder cancers, and mostly they go undetected due to similar appearances as those shown by tissues affected with chronic cholelithiasis (Eroschenko, 2008). Through clinical, pathological and molecular data two pathways that lead to GBC have been hypothesized. The primary mechanism associated with majority of the cases across the globe involves cholelithiasis and resultant cholecystitis (inflammation) that alters molecular mechanism in the host leading to cancer (Wistuba et al., 1996). The major risk factors associated with this pathway are genetic predisposition, and female gender bias. The second mechanism involves Anomalous pancreatobiliary duct junction (APBDJ) abnormality which is rare congenital malformation (mostly genetic) and more frequent in Japan (17% patients with GBC have this disorder). In APBDJ, the sphincter of Oddi losses its control and causes reflux of pancreatic juice into the gallbladder. This leads to continuous irritation and bile stasis, followed by reactive and pre-malignant histopathological changes of the epithelium and finally causing cancer (Sasatomi et al., 2000).

A well defined sequence of pre-malignant changes has been identified in progression towards cancer that includes invasive GBC, dysplasia and carcinoma in situ.

Symptomatic cholecystitis rarely appears before the age of 40 and ideal age for dysplasia is 45 years and carcinoma in situ is 55 years. GBC develops usually 20 or more years after cholecystitis with intermediate steps of dysplasia developing after 5 years and carcinoma in situ after 15 years. Though these changes occur in inflammatory background they are not characteristics of cholecystitis. The GBC is usually spread by regional lymph node metastasis and by direct invasion of the tumour through gallbladder wall into the surrounding tissues. The tumour invasion is generally facilitated by the thin gallbladder wall and its underlying discontinuous muscular layer, thus resulting in local and regional disease being present during diagnosis. The pathological state including the extent of tumour invasion and metastasis to lymph nodes and other organs serve as the main prognostic factors for gallbladder cancer (Roa et al., 1999; Wistuba and Gazdar, et al., 2004).

Molecular changes in gallbladder disease progression: Genetic alterations have been well understood in many human tumours, but there is relatively a paucity of information related to molecular changes occurring in gallbladder disease progression and GBC. The dominant oncogenic changes involved in pathogenesis of GBC have been investigated and reported by few researchers. KRAS mutations (usually in codon 12 of the gene) have mostly been reported in Japan with incidences of 0 to 59%, with greater frequencies (50-80%) associated with APBDJ. This indicates that reflux of pancreatic juice has a role in bringing about these mutations (Hanada et al., 1996). Around 33-64% of GBC's have reported ERBB2 overexpression. Overexpression of this gene in basal mice biliary tract epithelium leads to GBC within 3 months in nearly all the subjects (Kim et al., 2001). Positive regulators of cell cycle like cyclin D1 and cyclin E have also been shown to have an important role in GBC pathogenesis as seen by their overexpression in 41% and 49% of GBC's respectively using immunostaining (Eguchi et al., 1999). The incidence of TP53 mutations in GBC has no apparent geographic variations are very common. These are generally misssense mutations that produce a non-functional protein detected by immunostaining. Most studies report TP53 mutation frequency of greater than 50% which varies widely across different regions (ranging from 35-90%). Nearly 31-70% of the GBC's demonstrate mutations in exon 5-8 of the TP53 gene. Though there are no specific 'hot-spots' identified, studies in high-prevalence areas i.e. Chile and Japan report mutation rates in exon 5 with a frequency of 59-60% respectively. The G:C>A:T transitions were the commonest form

of mutation in both these locations with G:C>C:G and G:C>T:A transversions restricted to Japanese cases. Also allelic loses at TP53 locus i.e. 17p13 has frequently been reported in GBCs (Hanada et al., 1996; Kim et al., 2001; Wistuba and Gazdar, et al., 2004). The *CDKN2A* is inactivated in over half of GBCs and it has been shown to have a key role in development of GBC associated with gallstones. Inactivation of *CDKN2A* results from combination of deletions, mutations and abnormal hypermethylations in the gene and is correlated with findings of relatively low incidences of RB. Around 20% of cases show allelic loss at the *CDKN2A* gene locus with 4-14% showing a loss in protein expression. Also CDKN1A has been shown to have a reduced expression in nearly 50% cases of GBCs (Wistuba and Gazdar, et al., 2004). A varying number of polymorphic microsatellite markers have been used for allelotyping (identifying chromosomal regions with frequent rates of allelic losses/loss in heterozygosity-LOH). In GBC, a low density genome wide allelotyping from Chilean population using 169 markers showed 18 different chromosomal arms with frequent allelic loss. This allelic loss pattern was different for patients showing APBDJ. Other chromosomal locations showing frequent losses in 1p, 3p, 8p, 9p, 13q, 16q, 17p and 22q regions have been reported in GBC. In particular, numerous and frequent allelic losses have been shown in the short arm of chromosome 3 that harbours several putative or known tumour suppressor genes. Most GBC in Chilean patients also show allelic loss at the FHIT locus with 75% of GBCs showing FHIT abnormalities (Wistuba and Gazdar, et al., 2004). Recent studies report mutations in mtDNA310 sequence at the displacement loop, which is relatively frequent and occurs as an early event during GBC pathogenesis. It is usually detected in normal-appearing epithelia of cases with chronic cholecystitis, thus indicating that inflammatory condition of gallbladder is a precursor to gallbladder cancer progression (Wistuba and Gazdar, et al., 2004). Cyclooxygenase COX2 in highly overexpressed in GBC and adjacent preneoplastic lesions, including cases with APBDJ. Recent reports show that COX2 overexpression leads to synthesis of prostanoids that have significant effect on gallbladder pathophysiology by mediating inflammatory responses in gallbladder (Kawamoto et al., 2002). Other molecular abnormalities including alterations in WNT signalling pathway, genetic instability, loss of expression of DCP4 and abnormal methylation pattern of tumour associated genes have been studied and associated with GBCs. **Figure 1.9** summarizes the sequential molecular events that occurs during progression towards Gallbladder cancers.

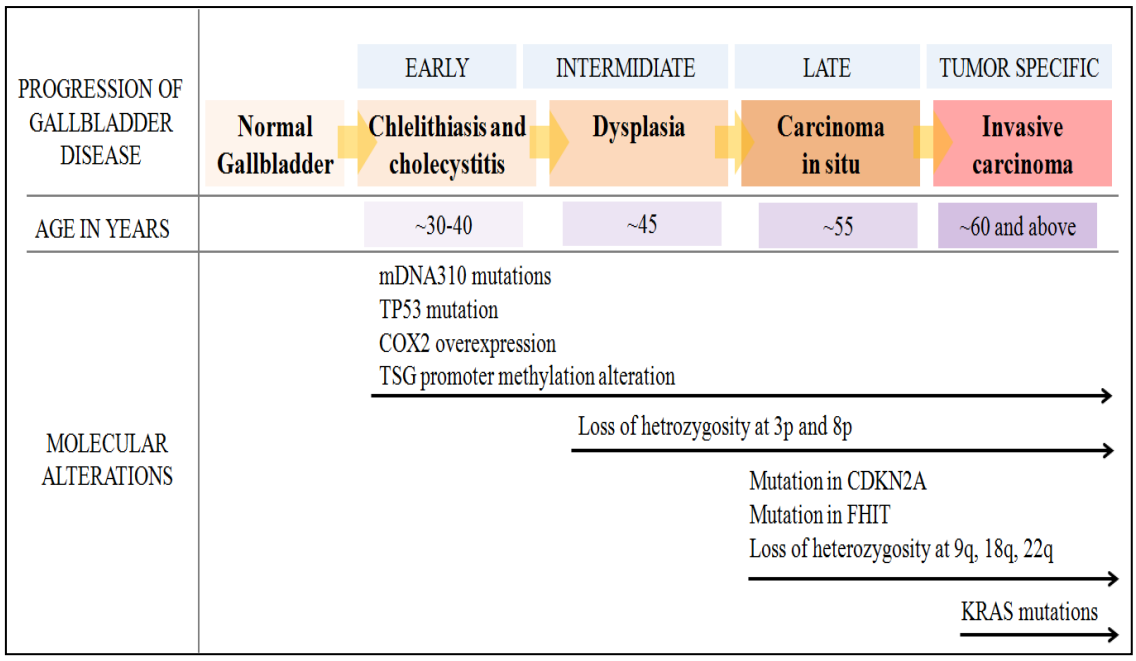


Figure 1.9 Molecular alterations during gallbladder disease progression

1.9 Interaction of *S. Typhi* with the gallbladder

The human bile is considered to be an antimicrobial agent due to its detergent like properties along with its role in aiding fat digestion. Bile salts have been shown to disrupt membrane architecture of bacteria by acting upon the phospholipids and integral membrane proteins. Within the cells, the bile salts cause protein misfolding and denaturation, and bring about chelation of iron and calcium. Furthermore, bile salts affect bacterial DNA by inducing DNA rearrangements and even cause plasmid curing. Thus bile prevents the growth of invading pathogens within the gastrointestinal tract acting as a defence barrier (Gonzalez-Escobedo et al., 2011). However certain enteric bacteria have evolved resistance to antibacterial effects bile and thus can be selectively grown on media containing bile salts (*Salmonella-Shigella* agar and MacConkeys agar). Interestingly, bile is also shown to regulate the expression of certain bacterial genes necessary for bile resistance and pathogenesis. Thus bile salts have contrasting roles in bacteria, where they inhibit growth in some while assist survival and persistence in others (Prouty et al., 2004b; Hernandez et al., 2012).

As discussed earlier, *Salmonella* pathogenesis involves its passage through the GI tract from where it spreads to liver and gallbladder, all of which have varying concentration of bile (highly concentrated in gallbladder). The changes in bacteria due to the effect of both physiological and commercial bile have been identified using transcriptomic and

proteomic analysis. In addition, a genome-wide screening to identify genes needed for bile resistance revealed that around 169 genes are altered as a response to bile in *S.* Typhi. These genes are associated mostly to factors such as envelope barriers (like LPS, efflux pump systems such as AcrAB-TolC that expel bile), the PhoPQ regulon, antibiotic resistance genes (mar) and DNA repair mechanisms (Prouty et al., 2004b; Hernandez et al., 2012; Tsai et al., 2012). For instance, regulating the length distribution of the O-antigen enhances resistance to bile as seen in NTS so as to confer increased fitness in the inflamed intestine (Crawford et al., 2012). Similarly, *S.* Typhi undergoes remodelling of the periplasmic space and alteration in membrane protein TolA to increase its resistance to bile. Interestingly, bile also has an effect on several known global gene regulatory pathways other than that implicated in general stress responses like RpoS and CsgD (Gunn, 2000; Prouty et al., 2002b). Furthermore, it is shown that bile is not only responsible for epigenetic changes in *Salmonella*, but also induces mutations important for adaptation of the organism in gallbladder niche. Interestingly, *Salmonella* is not only shown to resist bile but thrive and grow in it with growth rates comparable to those achieved in culture medium (Menendez et al., 2009). Also following *Salmonella* infection, the chemical composition analysis of murine bile revealed that the organism grows in bile by utilising the glycerophospholipids as carbon and energy source (Antunes et al., 2011). Also bile is shown to down-regulate SPI-1 and motility gene expression, both of which do not significantly affect the *S.* Typhi pathogenesis in the host (Gunn, 2000; Prouty et al., 2002b; Gonzalez-Escobedo et al., 2011).

1.9.1 The *Salmonella* biofilm-associated disease in gallbladder

Biofilms may be defined as a structured community of organisms that adhere to live or inert substrates in a self-produced polymeric matrix which help organisms persist in conditions like stress, antibiotic exposures, encounter with host immune responses, etc. Biofilms formation is a sequential and highly regulated process that requires regulation through a number of genes (Costerton et al., 1995). The multi-stage process of biofilms is depicted in **Figure 1.10** which begins with attachment of planktonic bacteria to a surface, followed by maturation through multiplication and production of extra-polymeric matrix encasement (composed of exopolysaccharides, nucleic acids and proteins), and finally shedding of organisms into the environment from the matrix-bound population of cells. Thus during biofilms formation the bacteria initially undergo

a motile to sessile phase with a fate resulting in vice versa, which helps the organisms to survive, multiply and disseminate (Costerton et al., 1995).

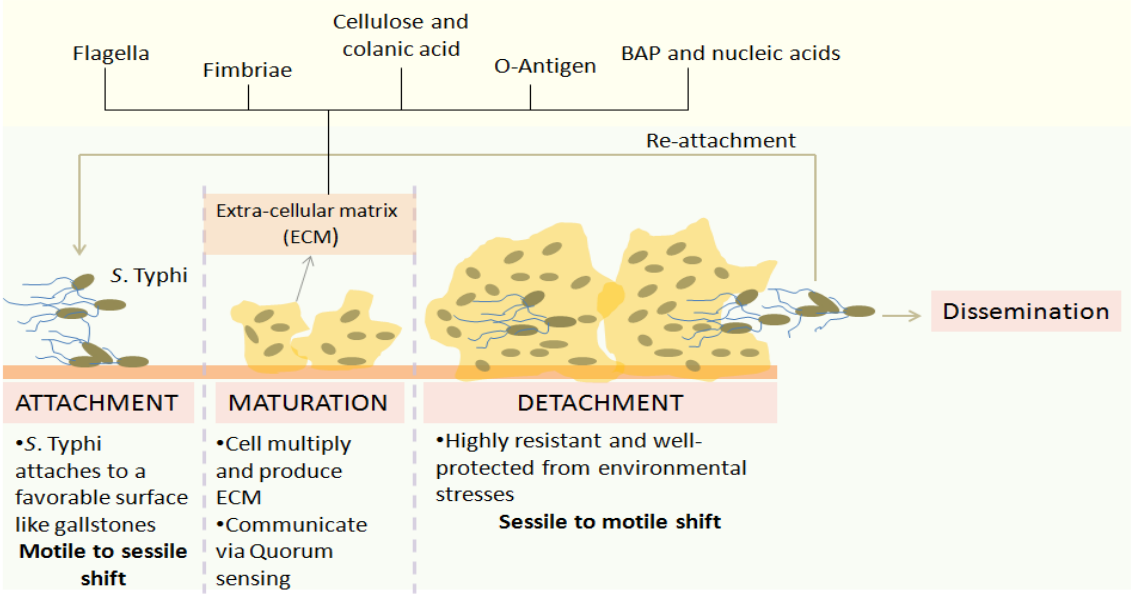


Figure 1.10 The multi-stage process of bacterial biofilm formation

Typically biofilms are considered as a response to stress, but in instances they have been implicated in much acute and chronic infection in hosts. Biofilms formation is highly associated with contamination of foods and nearly 80% of all bacterial infections are related to biofilms. *Salmonella* spp. is known to form biofilms on both biotic (epithelial cells and gallstones) and abiotic (plastic, glass, metal surfaces, etc) surfaces (Hall-Stoodley and Stoodley, 2009; Steenackers et al., 2012). *S.* Typhimurium, *S.* Typhi and *S.* Enteritidis form dense matrix-encased biofilms on gallstones in 7-14 days, but not on alternate substrates of similar shape and size (Prouty et al., 2002a). The gallstones for these studies were obtained from patients undergoing cholecystectomy. In 2008, Crawford et al. developed an alternative for gallstones where they introduced the tube biofilm assay (TBA) for studying biofilm formation on cholesterol. Besides confirming the specific binding to cholesterol, the method also revealed the significance of bile in enhancement of biofilm formation *in vitro*. Studies using TBA also showed poor biofilm formation by *Salmonella* spp. on calcium bilirubinate (usually present in pigment stones) compared to cholesterol (Crawford et al. 2008). Though TBA is reproducible and economical without the need for gallstones, the flow-through system is best representative of gallbladder niche. In these systems, media with

bile is made to flow at specific flow rates through chambers containing cholesterol–coated glass surfaces (Crawford et al. 2008).

Successful colonisation of gallbladder prior to biofilm initiation is assisted by bile stasis irrespective of gallstones, but biofilm formation on gallstones is assisted by various substances produced by the bacteria (Swidsinski and Lee, 2001; Crawford et al., 2010b). For instance, deletion of outer membrane protein OmpC negatively affected binding to cholesterol and thus biofilm formation (Crawford et al., 2012). Attachment of *Salmonella* to a surface is preceded by microcolony formation and biofilm maturation (Gonzalez-Escobedo et al., 2011). Cell-cell communication and production of ECM are the key features of these stages, and various components of the ECM have been studied during *Salmonella* biofilm formation (Prouty and Gunn, 2003). This includes the curli-fimbriae, flagella, O-antigen capsule, colonic acid, cellulose, biofilms-related proteins and nucleic acids which have some key roles as follows:

- Fimbriae: These mediate adherence and cell to cell interactions during initial attachment and biofilms maturation respectively (Prouty et al., 2002a). As mentioned earlier, there are 13 putative fimbrial operons (all of which are not expressed *in vitro*), its ability to regulate biofilms formation varies depending on the substrate as seen previously in case of flagella (Barnhart and Chapman, 2006; Ledeboer et al., 2006).. Though, the type I fimbriae encoded by the fimAICDHF gene cluster are not necessary for biofilm formation on gallstones incubated with bile, they have been shown to assist biofilms formation on murine intestinal epithelium and HEp-2 cultured cells (Boddicker et al., 2002). Furthermore, insertions in the FimW gene (a negative regulator of type 1 fimbriae) lead to a hyper-fimbriate phenotype that inhibited initial stages of biofilm formation on cholesterol but not on plastic or glass. Curli-fimbriae (encoded by csgBAC-csgDEFG) are thin aggregative fimbriae that mediate biofilms formation on plant surfaces and intestinal epithelium, but not on glass surfaces (Barnhart and Chapman, 2006). Other fimbriae like long polar fimbriae, plasmid encoded fimbriae, sth and bovine colonization fimbriae are also necessary in biofilm formation on intestinal tissue and Hep-2 cells. Recently, an *in vitro* study by Raza et al has shown that the type-IV pilus can be correlated to biofilms formation (Raza et al., 2011).

- Flagella: Flagella promote surface binding during initial phases and have been shown to mediate adherence of *S.* Derby and *S.* Enteritidis to cultured HEp-2 monolayers and epithelial cells respectively (Dibb-Fuller et al., 1999). *S.* Typhimurium phenotype that has the presence of flagella but is unable to rotate it (by mutation in motA), was able to form biofilms on gallstones, indicating that the flagellar filament is necessary and not motility (Prouty and Gunn, 2003). In contrast motility was necessary for biofilm formation on different substrate like glass and plastic. In addition, the conserved flagellar regulon gene flhE (switch that regulates flagellar type III secretion) assists in biofilms formation on microtitre well-plates rather than flagellar production. Mutants with transposon insertions in flagellar biosynthetic genes were unable to adhere to cholesterol-coated surface, and specifically, the FliC subunit was important for initial binding. Thus, the role of flagella varies with binding substrates. Also, the flagella-mediated phenotypes like chemotaxis and motility depends on the environment in which the bacteria are present. For instance, bile downregulates motility and enhances tumbling frequency in *S. enteric* (Crawford et al., 2012).
- O-capsular antigen and Vi antigen: O antigen capsule (encoded by two divergent operons yihU-yshA and yihVW) consists of more than 2300 repeating tetrasaccharide units and is linked to the membrane by 2-hydroxymyristic acid residue. They differ from O-antigen of the LPS in size, substitution pattern, charge and immunoreactivity. It plays a role in environmental persistence by assisting desiccation tolerance and enhanced plant colonization (Gibson et al., 2006). In *S.* Typhimurium, the O antigen capsule is shown to be transcriptionally induced during murine infection, suggesting its role in systemic infection and development of typhoid. This exopolysaccharides is not necessary for binding of *Salmonella* to plastic or glass surfaces, but is important in biofilms on gallstones and cholesterol surfaces (White et al., 2008). Rezende et al. showed that the O antigen from MDR *S.* Typhimurium DT104 (lacks rhamnose) was necessary for biofilms maturation. Organisms that do not express cellulose or curli rely on O antigen for biofilms formation (de Rezende et al., 2005). Two genes are involved in synthesis of LPS O antigen namely galE and rfaD. While the mutations in the former gene negatively affects

biofilms formation, mutations in latter does not affect the biofilms forming ability of the bacteria (Gibson et al., 2006). Bile has been shown to regulate expression of O antigen in a csgD-independent manner.The Vi antigen is not present in *S*. Typhimurium or *S.* Enteritidis, yet both are able to form robust biofilms on cholesterol. Also deletion of the genes encoding Vi antigen in *S*. Typhi does not affect its biofilms forming ability (Prouty et al., 2002a; Raza et al., 2011).

- Cellulose and colanic acid: Cellulose (encoded by bcsABZC-bcsEGF) is important for biofilms formation in *Salmonella* on epithelial surfaces, glass and plant surfaces but is not necessary during biofilms formation on gallstones (Prouty and Gunn, 2003). Similarly, *S*. Typhimurium isolated from biofilms matrix of feed industry, and *S*. Enteritidis biofilms on polystyrene, do not show cellulose as a necessary component of the ECM (Vestby et al., 2009). Atomic force microscopy analysis also demonstrate that cellulose and curli both are important for EPS production and both are co-regulated during biofilms formation in *S.* Typhimurium. Similarly, colanic acid-an extracellular polysaccharide (encoded by wcaM, wcaA, wza) plays a role in biofilms formation and is shown to create extensive three-dimensional structures on epithelial surfaces, but is not required for biofilms formation on abiotic surfaces like gallstones (Ledeboer et al., 2006). Furthermore, *S*. Typhimurium double mutant for colonic acid and cellulose was unable to form biofilms on glass and plastic, but formed biofilms on gallstones.
- Biofilms associated proteins (Bap) and extracellular DNA (eDNA): The Bap's usually contribute to EPS production in *E. faecalis* and *S. aureus*, and in *Salmonella* their function varies depending on the species. For instance, bapA encoded cell surface protein is necessary for biofilms formation in *S.* Enteritidis but not in *S*. Typhimurium (Latasa et al., 2006). *Salmonella* also expresses an auto-transporter adhesion on its cell surface called the SadA that is necessary during biofilm formation (Raghunathan et al., 2011). Nucleic acids are important chelating agents that confer resistance to antimicrobial peptides in biofilms by activating PhoPQ/PmrAB systems. These eDNA thus allow immune evasion and chronic persistence of *Salmonella* biofilms during intestinal infection or on gallstones (Johnson et al., 2013).

The co-ordinated regulation in *Salmonella* biofim formation: The production of ECM components is majorly controlled by the CsgD (AgfD) transcriptional regulator which in turn is regulated by seven global regulatory proteins namely H-NS, RpoS, OmpR, MlrA, Crl, IHF and CpxR. A recent study by Latasa et al. identified the RcsB phosphorelay-system also regulates CsgD depending on phosphorylated state of RcsB (i.e. phosphorylated RcsB represses CsgD expression and unphosphorylated RcsB is positive regulator of CsgD) (Latasa et al., 2006). CsgD directly regulates the expression of O-antigen, curli and BapA. It also controls cellulose level indirectly by activating AdrA (a GGDEF containing protein) that alters cellular levels of c-di-GMP to activate cellulose expression (Ahmad et al., 2011). *Salmonella* usually adapts to highly variable microenvironments with heterogenous biofilms populations. This can be attributed to the bistable nature of CsgD which is determined by its phosphorylation state. For instance, CsgD regulates the cellulose and curli formation only in the unphosphorylated state (Latasa et al., 2006). Such bacteria, expressing the curli and cellulose, when grown on Congo red agar plates have a red, dry and rough (rdar) morphotype. Such morphotype has been shown to aid survival and transmission of the bacteria in unfavourable environments via biofilms formation, and also affects cellulose and curli production to some extent (de Rezende et al., 2005).

1.9.2 *In vivo* studies for *S.* Typhi chronic infection

For *in vivo* studies in animals, Monack et al. in 2004 adapted the persistence model of *Salmonella* chronic infection using mice (Monack et al., 2004). Basically, they induced gallstone formation by a lithogenic diet, followed by infection with *S.* Typhimurium which revealed that presence of cholesterol gallstones enhanced *S.* Typhi colonisation of gallbladder. Also the mice with gallstones exhibited 3-log increase in faecal shedding compared to the ones lacking the gallstones (Crawford et al., 2010b). Interestingly, the gallstones removed from these mice were studied using electron microscopy which revealed that a dense *Salmonella* biofilm covered more than 50% of the stone surface. Earlier hypothesis of *Salmonella*-biofilm formation on gallstones was strongly supported by these studies which also revealed the importance *Salmonella* shedding that leads to environmental dissemination and spread of typhoid fever (Monack et al., 2004).

In humans, various studies in endemic regions using highly specific and sensitive molecular methods show a strong link between *S.* Typhi chronic carriage and presence of gallstone (Crawford et al., 2010b). Furthermore, these studies also reveal that chronic *S.* Typhi persistence can be considered as one of the pre-disposing factors for gallbladder cancers (Shukla et al., 2000; Dutta et al., 2000; Nath et al., 2008). For instance, multiplex PCR and traditional culture methods showed the presence of *S.* Typhi in approximately 5% (5 of 103 patients) of the patients with cholelithiasis in Mexico City in 2010. Interestingly, all the five patients showed *S.* Typhi in gallstones specimens and further microscopic analysis revealed that 3 patients exhibited 80-90% coverage of gallstone surface with dense bacterial biofilm (Crawford et al., 2010b). Furthermore, the gallstones from patients lacking the biofilm were pigment stones which supports previous *in vitro* findings that calcium bilirubinate is not a preferred surface compared to cholesterol for *Salmonella* biofilm formation. In addition, 13% of patients who showed *E.coli* (a common inhabitant of poorly functioning gallbladder) in gallstones in absence of *S.* Typhi exhibited no such biofilms (Crawford et al., 2010b; Gonzalez-Escobedo et al., 2011). Recent studies also report correlations between shedding duration and biofilm formation in *S.* Typhi isolated from stool samples of patients. Also the best biofilm producers obtained in these studies had increased antibiotic resistance as they harboured multiple antibiotic resistance cassettes (Raza et al., 2011).

These findings demonstrate that *S.* Typhi can chronically persist in the gallbladder, and highlight the need for development of specific detection and treatment methods. Also, clinical data reveals that many coordinated bacterial mechanisms function during chronic persistence of *S.* Typhi in gallbladder that contributes to phenotypes like biofilm on specific substrates, and yet remain to be completely understood.

1.10 Gaps in existing research

As mentioned earlier, majority of the research deals with the acute and systemic phase of *S.* Typhi infection with few studies reporting some significant findings related to chronic carriage. There still exist many gaps in the existing research related to studies on chronic infection of gallbladder by *S.* Typhi and their subsequent role in regulating host mechanisms leading to diseases like cancer.

Firstly, as *S.* Typhi chronic carriage is shown to be in a close relation to biliary diseases like gallstones and gallbladder cancer, rapid and early detection of carriers amongst large populations in typhoid endemic regions is necessary. Though the serological tests using anti-Vi antigen screening are still used for identifying carriers, the methods used (like heamagglutination and ELISA) have certain limitations (like less sensitivity and poor binding of antigen to plates, respectively) and are inconvenient to screen large populations. Furthermore, the expression in *S.* Typhi varies and Vi negative mutants of *S.* Typhi have been shown to accumulate from previously Vi positive strains. Also, the asymptomatic nature of chronic infection makes it difficult to identify chronic carriers, indicating a need to develop strategies for identifying such carriers.

Secondly, *Salmonella* spp. are shown to be highly variable in nature and adapts efficiently based on changes in environmental conditions for possible survival and spread. This is best demonstrated in acute phase of *S.* Typhi infection, where the organisms shows coordinated regulation of various bacterial components to trick the host immune responses and establish a successful infection. Somewhat similar hypothesis is also proposed during the chronic infection of the gallbladder without any strong evidence showing how the organism is able to persist in gallbladder with many mechanisms yet to be understood.

Thirdly, organisms like HPV and *H. Pylori* have been directly implicated in altering host mechanisms leading to transformation and cancer, but there is lack of such evidence in case of *S.* Typhi chronic persistence. Though the organisms is hypothesized to be a pre-disposing factor for GBCs based on its presence in gallbladder of cancer patients, no reports indicate significant role of the organism in regulating the host mechanisms. Also, *S.* Typhi is shown to produce certain effector proteins and toxins that have been shown to assist bacterial persistence and regulate the host, but their implications in transforming the gallbladder are yet to be well-understood.

Though current research utilizes different *in vivo* (involving mice) and *in vitro* infection models to better understand the carrier state, further knowledge and experimentation is necessary to prove the direct involvement of *S.* Typhi chronic infection in GBC development.

Finally, though various drugs and their combinations are used to treat the *S.* Typhi acute infections, no therapy ensures the removal of chronically persisting bacteria in the gallbladder. In fact, studies report that organ removal is the only best possible way to treat such long-term carriers. Depending on the projected implication of such chronic infection in humans, there is an urgent need to develop effective and affordable treatment strategies. This would further demand the need to understand the bacterial behaviour (like biofilm formation) and mechanisms (like drug-resistance) during its chronic persistence in gallbladder.

1.11 Hypothesis and Goals

The specific pathogen and host factors that facilitate chronic *S.* Typhi persistence in the gallbladder are poorly understood. The main objective of this work was to understand the mechanisms underlying *S.* Typhi persistence in the gallbladder which involved the use of various *in vitro* model systems to mimic the gallstone and gallbladder niche.

It is shown that *S.* Typhi chronically persists in gallbladder of patients undergoing cholecystectomy for gallbladder diseases. Researchers use various microbiological and molecular based techniques to detect *S.* Typhi and other organisms in the gallbladder tissue, bile or gallstones. Thus, in our first objective we screen for *S.* Typhi in gallbladder of patients with and without gallbladder disease. This would give an overview of the chronic carrier state within these populations along with recovery of any persisting *S.* Typhi, which will be characterized further for bile tolerance, biofilm formation and antibiotic resistance.

It has been demonstrated that chronic persistence of *S.* Typhi is mainly attributed to the biofilm forming ability of the organism, especially on cholesterol gallstones. Bile is shown to be a key regulator in bacterial persistence and various bacterial components function co-ordinately to establish a successful chronic infection in the gallbladder. Thus our second objective was to understand the role of bile in regulating specific bacterial mechanisms like adaptation, quorum sensing and persister cell formation. This

would in turn assist development of new therapeutic approaches to eliminate the carrier state in humans. Taking into account the significance of QS molecules during adaptation and biofilm formation, we additionally develop a novel fluorescence based method to specifically detect these molecules. Also, considering the fact that co-infection with bacteria is possible within the gallbladder; we determine significance of *S.* Typhi co-existence with other bacteria by engineering whole cell biosensor.

Various molecular regulations occur within the host as response to chronic bacterial infections and products secreted by these bacteria. These include the mediators of chronic inflammation the continuous release of which may lead to transformation of gallbladder cells causing cancer. Thus, our third objective focuses on the RhoA/ MLCK and NFkβ signaling which generally has implications in epithelial to mesenchymal (EMT) transition and carcinogenesis in eukaryotic cells. We hypothesize *S.* Typhi induces the Ras homolog gene family member RhoA, with increased expression of NFκβ, resulting in changes in MLCK expression that may lead to EMT. We determine the prognostic significance of these components during the gallbladder disease progression in patients, and also develop an *in vitro* infection model to understand their expression in gallbladder epithelium.

In summary, this work is focused on determining the mechanisms used by *S.* Typhi to allow chronic carriage in the gallbladder. We also try to reveal the role of *S.* Typhi in regulating a host mechanism implicated in carcinogenic transformation of cells. Investigating such mechanisms could lead to identification of novel drug targets and biomarkers for *S.* Typhi chronic carriage during the gallbladder disease progression. This would further result in developing new approaches to prevent and treat such infections. Ultimately, these studies would eliminate *S.* Typhi carriage and prevent the spread of typhoid fever and gallbladder-associated diseases like gallstones and gallbladder cancer.

CHAPTER-II

Screening and Characterization of *S.* Typhi Persisting in Gallbladder of Patients Undergoing Cholecystectomy

2. Screening and Characterization of *S.* Typhi Persisting in Gallbladder of Patients Undergoing Cholecystectomy

2.1 Abstract

Salmonella enteric serovar Typhi chronically persists within the gallbladder and is considered as a predisposing factor for gallbladder cancers. Thus, it is necessary to screen such carriers and understand their distribution within a population with high incidences of gallbladder diseases. We screen for *S.* Typhi isolates present in the gallbladder of 200 patients undergoing cholecystectomy for gallbladder disease along with 45 patients with normal gallbladder histology Along with microbiological screening of specimens, we develop a sensitive and specific PCR method for *S.* Typhi detection in samples. *S.* Typhi isolated on culture was further characterised by determining its tolerance to bile, quantifying its biofilm- forming ability using crystal violet assays and estimating its drug susceptibility using disc diffusion tests. Our findings reveal that nearly 22% of the entire population shows the presence of both enteric as well non-enteric organisms on culture. *E. coli* made up major portion of the Enterobacterial isolates obtained on culture followed by *K. pneumoniae, Enterobacter* spp. and *S.* Typhi. Also some patients (7.5%) showed co-infection in the gallbladder, with 1% harbouring *S.* Typhi and *E.coli*. The culture method was positive for *S.* Typhi in only 5% of the total population, whereas PCR assay showed positive results for 11.5% of the patients, indicating low efficiency of culture based method to screen organisms in gallbladder. Though a very few cancer samples were obtained in this study, none of the three patients suffering from gallbladder carcinoma were positive for *S.* Typhi. Also specimens from normal gallbladder did not show the presence of *S.* Typhi on culture or PCR, indicating a strong association of *S.* Typhi presence with gallbladder diseased state. Upon further characterization of these isolates it was seen that the MIC to bile was 3%, and the isolates showed significant biofilm formation in presence of bile. The SDS-PAGE analysis also revealed significant alteration in total protein profiles of intracellular and membrane proteins, in the presence of bile. The rdar (Red, dry and rough) - morphotype shown by all the isolates revealed the significance of fimbriae and cellulose in biofilm formation. Also, significant increase in biofilm mass was observed on cholesterol coated surfaces. The isolates obtained from

gallbladder were resistant to first–line drugs like cholarmphenicol and TMP-SMZ, but were susceptible to ampicillin, fluoroquinolones and cephalosporins.

2.2 Introduction

The human gallbladder is a niche for persistence of different kinds of organisms and intestinal flora had frequently been recovered followed intervention on the biliary tree. *Salmonella enterica* serovar Typhi is able to survive and persist within the gallbladder following acute phase of infection and has been proposed as a predisposing factor for gallbladder cancers. Several studies implicate contaminated foods as vital transmission route, infecting 21.7 million people annually (Crump et al., 2004). The organism has also shown variability in its biofilm forming ability and multi-drug resistant patterns becoming a major concern for eradication and treatment, especially within typhoid endemic regions (Prouty and Gunn, 2003; Crawford et al., 2012).

Salmonella survival in the mammalian gall bladder seems to involve several strategies including invasion of gallbladder epithelium, biofilm formation on gallstones, regulation of drug efflux mechanisms (conferring antibiotic resistance) and alteration in virulence (regulation of pathogenicity islands) (Pratap et al., 2012). Surprisingly, bile is shown to be one of the key mediators for these mechanisms by altering genetic material or bringing about changes in protein expressions (Crawford et al., 2010b; Hernandez et al., 2012).

At epidemiological level, North India is endemic for typhoid fever, and reports an increasing incidence of gallbladder cancers. A correlation between these has been established based on the fact that researchers were able to detect the organism or it's DNA in gallbladder of patients suffering from gallbladder cancer. Though Vi antigen testing is of great value for screening chronic carriers, various PCR methods for different genes are used to specifically detect *S.* Typhi within the gallbladder following cholecystectomy for direct evidence (Shukla et al., 2000; Dutta et al., 2000; Nath et al., 2008). Besides, a variety of *in vitro* models have been developed to understand chronic carrier state, which is frequently associated with biofilm formation on gallstones (Gonzalez-Escobedo et al., 2011).

S. Typhi chronic carriers are asymptomatic and mostly confined to gallbladder with gallstones. They are usually resistant to antibiotic treatment with gallbladder removal

being the only successful therapy. All these observations are strongly correlated with biofilm-forming ability of the organism (Crawford et al., 2012). Early studies have investigated various components of *S.enterica* (like the O-antigen capsule, curli, fimbriae, etc), along with bile and gallstones composition, are necessary for successful biofilm formation within gallbladder (Prouty et al., 2002a; Crawford et al. 2008). An in vivo study in *S.* Typhimurium infected mice revealed a dense bacterial biofilm covering the gallstones (Monack et al., 2004). Prolonged treatment with antibiotics like ampicillin resolves less than two-thirds of such chronic infections, but clinically administered antibiotics are ineffective in patients carrying gallstones and *S.* Typhi. It has also been difficult to apply any previous findings to current *S.* Typhi isolates because of rapid acquisition and emergence of multi-drug resistant strains (Gonzalez-Escobedo et al., 2011). This also needs to be investigated in regions where chronic *S.* Typhi infections are prevalent.

In this study we screen for *S.* Typhi chronically persisting in gallbladder of patients undergoing cholesystectomy, by using standard culture methods and a highly specific and sensitive PCR assay. We further characterize the isolate for its biofilm forming ability and antibiotic susceptibility *in vitro*. Furthermore, this would give us an idea of the incidence of chronic *S.* Typhi carriers within the population and show drugs effective against such persisting organisms.

2.3 Materials and methods

(A) Screening of *S.* Typhi in clinical specimens

2.3.1 Ethics statement and approval by institutional ethics committee

Consent was obtained from all the patients enrolled for this study. The study was approved by the Institutional Human Ethics Committee (IHEC) bearing the Ethics statement no. IHEC-31/13-14.

2.3.2 Clinical specimen, Bacterial strains and culture conditions

A total of 200 patients undergoing cholecystectomy for gallbladder disease from February 2012 to April 2014 at Safdarjung Hospital (New Delhi, India) and SMRC Hospital (Goa, India) were enrolled for this study. The bile was aspirated before the surgery by needle puncture, and the tissue and gallstone specimens were obtained post-

surgery. For normal specimens, we consider 45 human cadavers (without any gallbladder disease) from the Department of Forensic Science-Goa Medical College and Hospital (Goa, India). The samples were submerged in RNA*later*® solution for storage and DNA/RNA related studies (Florell et al., 2001).

Bacterial strains used for this study include *Salmonella enterica* serovar Typhi str. Ty2 and CT18 serve as standard control strains. For checking the specificity of the PCR-based screening method, we use Enterobacteriaceae other than *Salmonella* that include *E. coli* MTCC 723, *Klebsiella pneumoniae* MTCC 101, *Shigella* spp. (lab isolate), *Proteus* MTCC 1441, *Staphylococcus aureus* MTCC 737, *Streptococcus pyogenes* MTCC 442, *Pseudomonas aeruginosa* MTTC 741, *Enterobacter* and *Bacillus* (lab isolates). All the strains were grown at 37^{O}C in Lurai-Bertani (LB) media (Himedia).

2.3.3 Histopathlogical analysis

Previous research on RNA preservation studies demonstrates that RNA*later*® (Thermo Fisher Scientific) solution does preserve the histology of tissues. The preservation in RNA*later*® solution causes no damage to cellular epitopes (Florell et al., 2001). Gallbladder specimens were fixed in 10% buffered formalin. Transverse multiple sections of the wall including mucosa and serosa were taken, embedded in paraffin, sectioned and stained with haematoxylin and eosin for routine histology (These procedures were carried out at the respective hospitals from where the samples were obtained). (Rosai 2011; Kumar et al., 2013)

Cholesterol estimation of specimen: The estimation of cholesterol in gallstone specimens was carried out using Cholesterol estimation kit (Medsource Ozone Biomedicals, India) as per manufacturer's instruction. Briefly, the gallstones were crushed and mixed with preheated distilled water. The mixture was diluted (dilution factor 2), and 10µl of the diluted mixture was used for estimation of cholesterol. This was done by adding the reagent 1 and 2 followed by incubation for 20 minutes with tubes being left open for oxidation. The standard used for the experiment was 200mg/dl of cholesterol provided in the kit. The reddish-pink colour developed was then analysed using spectrophotometer at a wavelength of 525nm. The concentration in the test samples was reported in mg/dl.

2.3.4 Microbiological screening of specimens

The samples obtained (bile, tissue, gallstones) were plated onto Nutrient agar, MacConkeys agar and Blood agar. For enrichment selenite F broth (Himedia) was used and incubated for 48hrs at 37^{o}C with first sub-culture done on blood agar and MacConkeys agar plates. Non-lactose fermenting colonies were subjected to gram staining and motility. Gram negative motile bacilli were put for different biochemical tests (Indole, Voges-Proskauer, Methyl red, glucose, lactose, sucrose, urease and Triple sugar iron) for identification and characterisation of any isolated *S*. Typhi. The colonial morphology for *S*. Typhi isolates obtained was studied on Congo red agar plates (LB media without salt and containing 40µg/mL of Congo red dye) (Acosta et al., 2003).

2.3.5 PCR assay for screening of *S*. Typhi in clinical specimens

DNA extraction from specimen: (a) Tissue and gallstone samples once thawed at room temperature were removed from RNA later and minced using as sterile scalpel and pestle. The DNA was then extracted from 25–30 mg of sample using the NucleoSpin® Tissue (Macherey-Nagel-GmbH & Co. KG, Duren, Germany) Kit as per manufacturer's instructions. For gallstone samples, initial incubation with lysis buffer was standardized to 24 hours. The isolated DNA was stored at −80°C. For bile samples, 1 mL of each specimen was diluted with distilled water, centrifuged at 13000 g for 20 minutes, and the supernatant was discarded. This has the effect of concentrating any bacterial cells in the pellet. The pellet was subsequently mixed with 150µL of sterile distilled water and DNA extracted using the kit. (b) For bacteria, the isolated organisms were picked up from MacConkeys agar plate and dispensed in 100 µl of autoclaved distilled water. These were then were kept in boiling water bath for 9 minutes followed by centrifugation at 9000 x g for 1 minute. The supernatant was transferred to a clean eppendorf and DNA quantified using Nanodrop 2000 UV-Vis Spectrophotometer (Thermo scientific). (Sambrook and Russell, 2001; Hazrah et al., 2004; Nath et al., 2008)

DNA amplification using PCR: Template DNA was subjected to PCR using specific primers FP: 5'- TCG TTT GAG GAT AAA AAC GGT A -3' and RP: 5'- CAG TTT GAG CAA CGC CAG TA - 3', to flagellin gene H-1 d region of *S*. Typhi (GenBank: X16406.1), which amplify a 151-base pair segment (Fig. 2). The PCR mix (50 µL) contained 0.2 mM of each of the four deoxynucleotide triphosphates (dNTPs), 0.2 µM

of each of the two primers, 2mM $MgCl_2$, 2 U Taq DNA polymerase and 1X Taq buffer with KCl (both from Fermentas-Gene Taq) and 5.5 µl of template DNA. A gradient PCR was set up to optimize the T_m for primers (Fig. 3) and subsequent PCR reactions were conducted in a Gene Amp PCR system 2700 (Eppendorf Mastercycler® nexus) under the following conditions: 94°C for 5 min; 30 cycles at 94°C for 45 sec, 52.9°C for 30 sec, 72°C for 30 sec and a final elongation at 72°C for 10 min. The PCR products were separated in a 1.5 % agarose gel containing ethidium bromide. The expected PCR products were visualized with a gel documentation system referenced with a Genedirex 50bp DNA ladder. The sensitivity of the method was determined by amplifying serially diluted *S.* Typhi Ty2 DNA. (Sambrook and Russell, 2001; Nath et al., 2008)

TA cloning of the flagellin gene fragment: The flagellin gene fragment was extracted from agarose gel using the Qiagen QiaQuick Gel Extraction Kit. The gel portion containing the gene fragment was cut using a sterile scalpel and DNA extracted as per the manufacturer's instructions[#]. The eluted DNA was quantified using the nanodrop and stored at -20°C. The gene fragment was then cloned in the pGEM-T Easy vector system (Promega, USA)) with a vector to insert ratio of 1:3. The ligation reaction was set up according to the manufacturer's protocol as follows: 5µl of 2X rapid ligation buffer, 1µl of pGEM-T Easy vector, 2µl of PCR product, 1µl of T4 DNA ligase and 1µl of nuclease free water. The tubes were incubated at 4°C overnight for maximum number of transformants. The plasmid was transformed into competent *E.coli DH5α* cell and selection of transformants was done using ampicillin resistance. The plasmids were then extracted using GeneJet Plasmid DNA purification kit*****, visualized on agarose and subsequent PCR carried out to confirm the presence of the flagellin gene fragment in the plasmid (Fig. 4 (b)).

[[#]PCR cleanup kit: The DNA fragment was excised from the gel using a clean sharp scalpel and transferred to an eppendorf tube weighed previously. The weight of the gel slice was determined and 100µl of QG buffer was added per 100mg gel slice (maximum amount of gel per column is 400mg). The tube was incubated at 50°C for 12 minutes, ensuring the gel slice was dissolved completely (The tube was vortexed ever 3 minutes). 100µl of isopropanol was then added and mixed. The contents of the tubes transferred to QIAquick column and centrifuged for 1 minute at 13000 rpm at 4°C. The flow through was discarded and the column was placed back onto the

collection tube. For sequencing studies, 500µl of QG buffer was added to the column and centrifuged again for 1 minute. This was followed by washing in 750µl of buffer PE and centrifugation for 1 minute at 13000rpm at 4°C (after adding PE the column was allowed to stand for 3 minutes). The centrifugation step was repeated without any solution in the column to remove any residual wash buffer. The column was then placed into a new eppendorf tube followed by addition of buffer EB (15µl). The column was allowed to stand for 5 minutes and then centrifuged at 13000rpm for 3 minutes (This elution step was repeated with another 10µl of buffer EB). The purified DNA was visualized on agarose gel and stored at -20°C for further application.

*Miniprep extraction of Plasmid using kit: The bacterial cells containing the plasmid were grown in LB media till an OD of ~0.6. The cells were harvested by centrifugation at 6500 X g for 2 minutes at RT. All the centrifugation steps hereafter were carried out at 12000 x g at 4°C. Briefly, the cell suspension was resuspended in 250µl of resuspension solution and vortexed. The lysis solution (250µl) was then added and the tubes were inverted 5-6 times gently. 250µl of neutralisation solution was then added followed by mixing (invert 4-5 times) and centrifugation for 5 minutes. The supernatant was transferred to GeneJet spin column and centrifuged for 1 minute. 500µl of wash solution was added to the column and centrifuged for 1 minute. The washing protocol was repeated twice to remove any interfering substances. The final flow through was discarded and additional 1 minute centrifugation of empty column was carried out. The column was then transferred to a new RNase free eppendorf tube followed by addition of pre-heated (70°C for 5 minutes) elution buffer (10µl). The column was centrifuged for 3 minutes and the elution step was repeated once more with 10 µl of elution buffer. This increased the yield of DNA obtained. The final flow through was stored at -20°C till further analysis.]

DNA sequencing analysis: Cloned PCR amplicons were sent for sequencing to Chromus Biotech Pvt. Ltd., Bangalore, India. Sequences were assembled and analyzed using nucleotide Basic Local Alignment Search Tool (BLAST) and ChromasPro 1.7.5 provided by vendor.

(B) Characterising *S*. Typhi isolates obtained on culture

2.3.6 Growth analysis, MIC and MBC assays in bile

Overnight grown *S.* Typhi cultures were transferred to fresh LB media. The log phase cultures were challenged with bile, and assayed for Minimum inhibitory concentrations (MICs) in microtitre well plates. All assay plates were incubated at 37°C on orbital shaker with growth monitored at specific time interval using the spectrophotometer. The MBCs (Minimum bactericidal concentration) were determined by plating the well exhibiting no apparent growth in the MIC assay. The sub-lethal concentration of bile was defined as the concentration below MIC value (below lethality) (Hernandez et al., 2012).

2.3.7 Biofilm quantification

Bacterial attachment assays were performed in microtitre well plates by adding 0.01ml of overnight grown (OD_{600nm} ~ 0.4-0.6) culture to growth media without bile and media containing sub-lethal bile concentration. Every well had enough glass coverslips required for time based analysis. All the coverslips were coated with 1mg standard cholesterol (Medsource Ozone Biomedicals) by evaporating 100µl of 10mg/ml stock solution using hot air oven. Also, biofilm formation on glass coverslips in absence of cholesterol-coating was determined. The plates were incubated at 37°C, and media was replaced with fresh LB (with or without 3% bile) every 24 hrs for 18 days. Coverslips removed for biofilm mass estimation were washed in LB media and incubated at 60°C for 45 minutes to fix the cells. The biofilms were then stained using a solution of 0.1% crystal violet for 10 minutes at room temperature followed by a thorough wash using 1X PBS. The dye was extracted using 30% acetic acid and quantified at 570nm using spectrophotometer. (Prouty et al., 2002a)

To examine the biofilm formation on gallstones, stones primarily made up of cholesterol and retrieved from single patient were used. Overnight grown culture of *S.* Typhi in LB (with and without 3% bile) was diluted 1:10 and added to test tube containing gallstone and LB media (with and without bile). The tubes were incubated at 37°C and the media was changed every 24 hours with three washes and slight vortexing for 12 days. (Prouty et al., 2002a)

2.3.8 *In vitro* drug susceptibility testing

For antibiotic susceptibility testing, antibiotic sensitivity discs (Himedia) for chloramphenicol (30µg), ampicillin (10µg), trimethoprem- sulfamethoxazole (TMP-SMZ) (5µg), ceftriaxone (30µg), ciprofloxacin (5µg), ofloxacin (5µg) and nalidixic acid (30µg) were used. The sensitivity of gallbladder *S.* Typhi isolates to these antibiotics was determined using a standard protocol published by the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards or NCCLS). Briefly, *S.* Typhi culture containing 10^8 CFU/ml was spread evenly using a sterile swab on Mueller-Hinton (MH) agar and allowed to dry for 10 minutes under aseptic conditions. The drug impregnated discs were then applied using sterile forceps at a distance 15mm away from the edge of the petridish with centres of two discs being at least 30mm apart. The agar plates were incubated in inverted position at 37°C for 16 to 18 hours after which the zones of inhibition were identified by gross visual inspection and the diameters were recorded to the nearest mm.

2.3.9 Total protein expression profiles using SDS-PAGE

Any changes in intracellular and membrane proteins were determined by total protein profiles using SDS-PAGE. The membrane proteins were isolated as described by in literature previously with few modifications (Hamid et al., 2008). Briefly, for isolating membrane proteins bacterial cells were grown in LB (OD ~0.6) and harvested at 10000rpm for 15 minutes. The cells were kept on ice and resuspended in 300µl of 0.1M Tris/HCL (pH 7.5). The cells were then incubated for 12 minutes in lysis solution (500µl 0.2M Tris/HCl, pH 8.0, 1M sucrose; 60µl 10mM EDTA, pH 8.0; 40µl lysozyme, 2mg/ml; 1.6ml autoclaved double distilled water) followed by addition of the extraction buffer (1.5% triton X100, 10mM $MgCl_2$, 50mM Tris/HCl, pH 8.0 and 40µl DNase, 1mg/ml). The mixture was incubated at room temperature till a clear solution was obtained and dialysed against distilled water for 72 hours. The dialysed material was centrifuged at 10000rpm for 60 minutes and the supernatant containing the outer membrane protein was lyophilized using a lyophilizer. The crude OMP fraction was stored at -20°C till further use.

For intracellular protein extracts harvested cells were suspended in 250µl of autoclaved distilled water and sonicated on ice. The sonicated mixture was centrifuged at

10000rpm for 15 minutes at 4°C. The supernatant obtained was stored at -20°C till further use. (Sambrook and Russell, 2001)

Protein estimation and SDS-PAGE analysis: The concentration of protein in the samples was determined by using a NanoDrop lite spectrophotometer (Thermo Scientific). Samples were denatured by boiling them with the 10µl 5X loading dye (0.125M Tris, pH 6.8; 20% glycerol; 4% SDS; 10% β-mercaptoethanol; 0.1% bromophenol blue dye) for 5 minutes. A total of 5µg protein was loaded in the wells followed by SDS-polyacrylamide gel electrophoresis (PAGE) on 12% separating gel. The gels were stained using silver staining[#] and bands analysed with ImageJ 1.46r software. (Sambrook and Russell, 2001)

[#][Silver staining protocol: The gels were fixed in fixing solution (50% methanol and 10% acetic acid) for 1 hour followed by washing in distilled water overnight. The water was then discarded and sensitizer (0.02% Sodium thiosulphate) was added. This was followed by 2 washes of 1 minute each using distilled water. Cold silver nitrate solution was added and allowed to react for 20 minutes on gel rocker, followed by a rinse with distilled water for less than 60 seconds. The developer (6g Na_2CO_3, 50µl formaldehyde, 2ml $Na_2S_2O_3$ for 100ml in distilled water) was then added and allowed to react for 10 – 15 minutes on gel rocker. The stop solution (10% acetic acid in distilled water) was then added for 10 minutes followed by a wash in distilled water.] (Sambrook and Russell, 2001)

2.3.10 Statistical Analysis

All the values obtained are representative of at least three independent replicates with the Standard mean (SM), Standard deviation (SD), and Standard error (SE) values. The statistical significance for independent variables was determined using the chi-square test with p-values < 0.05 considered to be significant.

2.4 Results and Discussion

2.4.1 Histopathological classification of clinical specimens of gallbladder

On histological observation of gallbladder tissue obtained from patients undergoing cholecystectomy, 98.5% showed the presence of chronic cholelithiasis with 1.5% suffering from gallbladder cancer. The specimens obtained were classified based on

certain features summarized in **Figure 2.1** (Rosai 2011; Kumar et al., 2013). Majority of the patients (98.5%) enrolled for this study suffered from chronic cholelithiasis with different degrees of inflammatory responses and manifestations **(Figure 2.2)**. Only 1.5% patients were characterized to have gallbladder cancer with a very low survival rate following surgery. Interestingly, no specific histopathological changes were observed in patients harbouring chronic *S.* Typhi infection in their gallbladder.

On analysing cholesterol composition of the gallstones, it was seen that majority of the population (93.9%) in this study harboured cholesterol gallstones **(Figure 2.3)** with amounts varying between ~750mg/dl to ~1800mg/dl. The suspected pigment stones showed concentration of cholesterol below 45mg/dl. This was on par with some of the previous clinical findings which report that 60% of the gallstones recovered from patients are cholesterol gallstones and the composition varies as per geographic location.

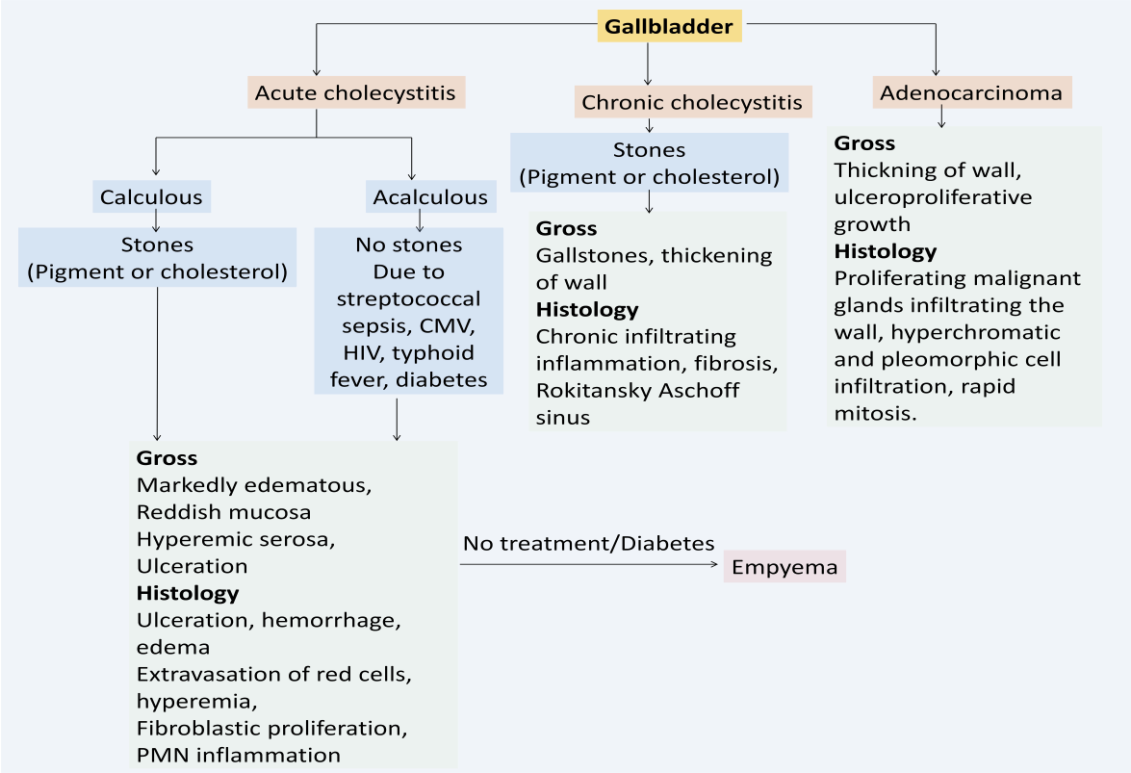


Figure 2.1 Classification of gallbladder disease based on histology

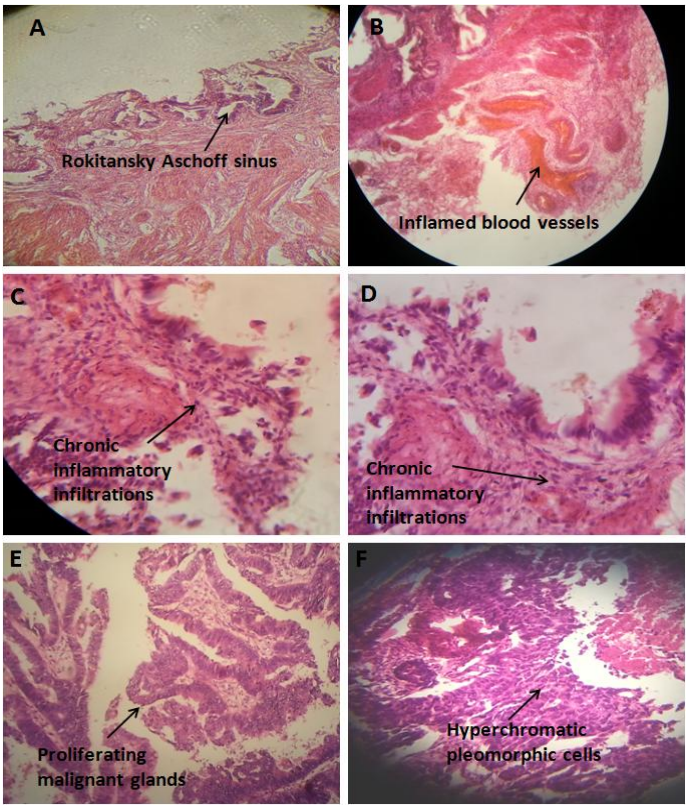


Figure 2.2 Histopathological features of gallbladder disease using H&E staining. **A-D** represents characteristic features of chronic inflammation of the gallbladder in patients undergoing cholecystectomy for chronic cholelithiasis. These include inflammatory infliltration, Rokitansky Aschoff sinus, inflamed blood vessels. **E & F** represent characteristics of adenocarcinoma of the gallbladder that include proliferating malignant glands with hyperchromatic pleomorphic cell infiltration.

Figure 2.3 Gallstone types in patients suffering from cholelithiasis. Majority of the patients show that the gallstones are made up of cholesterol with concentrations varying between ~750mg/dl to ~1800mg/dl, whereas pigment stones having values of less than ~50mg/dl.

2.4.2 Occurrence of Enterobacteriaceae in gallbladder

There is a considerable amount of research that supports the findings that bacteria, both enteric and non-enteric, can persist within the gallbladder. These have been identified on culture or by PCR and are mostly predominated by *Escherichia coli*, *Klebsiella*

pneumoniae, *Salmonella* Typhi, *Pseudomonas aeruginosa, Staphylococcus aureus, Helicobacter* spp. *and Enterobacter* spp. (Hazrah et al. 2004; Capoor et al., 2008). Upon thorough microbiological screening of the clinical samples (which included bile, gallbladder tissue and gallstones) obtained from 200 patients undergoing cholecystectomy for gallbladder disease (Table 2.1), it was observed that 16.5% of the patients harboured Enterobacteriaceae ($p<0.001$) in their gallbladder with 5% of the patients showing non-enteric bacteria (*P. aeruginosa* and *S. aureus*) being present ($p<0.001$) **(Table 2.2)**. These organisms obtained on culture were speciated using biochemical characterisation as shown in **Figure 2.2.**

Number of Patients	**Control**	**Diseased**
Enrolled for this study	45	200
Showing Enterobacteriaceae spp. on culture	2	33 (15.5%)*
• *Escherichia coli*	2	12*
• *Klebsiella pneumoniae*	0	8
• *Enterobacter* spp.	0	6
• *Salmonella* Typhi	0	5
Showing organisms other than Enterobacteriaceae	0	10(5%)*
• *Pseudomonas aeruginosa*	0	6
• *Staphylococcus aureus*	0	4
Showing more than one (co-infection) bacterial spp. on culture	0	15(7.5%)*
• *P. aeruginosa* and *E. coli*	0	3
• *S.* Typhi and *E. coli*	0	2
• *E. coli* and *Enterobacter* spp.	0	6
• *K. pneumoniae and E .coli*	0	4
Showing *S.* Typhi positive on PCR assay	0	23(11.5%)*

Table 2.1 Microbiological analyses of patients undergoing cholecystectomy. Values show the number of patients positive for different organisms in normal and diseased samples. The differences were considered to be significant with a *$p<0.001$.

Organisms	Source	Biochemical characterisation																		Molecular characterisation
						Carbohydrate Metabolism					IMViC				N_2 Metabolism	O_2 Metabolism				
		Gram stain	Morphology	Motility	Gas Production	Glucose	Mannitol	Maltose	Sucrose	Lactose	Indole	Methyl Red	Voges-Proskauer	Citrate	H_2S	Urea	Catalase	Oxidase	TSI (Slant/Butt)	Coagulase	PCR assay for *S* .Typhi Identification
S.Typhi	Gallbladder	-	B	M	-	A	A	A	-	-	-	+	-	+	+	-	+	-	AL/A	NA	+
Klebsiella spp	Gallbladder	-	B	NM	+	A	A	A	A	A	-	-	+	+	-	+	+	-	A/A	NA	-
E.coli	Gallbladder	-	B	M	+	A	A	A	A	A	+	+	-	-	-	-	+	-	A/A	NA	-
Enterobacter spp	Gallbladder	-	B	M	+	A	A	A	A	A	-	-	+	+	-	-	+	-	A/A	NA	-
S.aureus	Gallbladder	+	C	NM	+	A	A	A	A	A	-	+	+	+	-	+	+	-	A/A	+	-
Pseudomonas	Gallbladder	-	B	M	-	-	A	-	-	-	-	-	-	+	-	-	+	+	-	-	-

Table 2.2 Biochemical identification of isolates obtained on culture.

E. coli made up major portion of the Enterobacterial isolates obtained on culture followed by *K. pneumoniae, Enterobacter* spp. and *S.* Typhi. Also some patients (7.5%) showed co-infection in the gallbladder with more than one bacterial isolate obtained on culture ($p<0.001$). The presence of such chronically persisting organisms in the gallbladder (asymptomatic infection) has been associated with formation of gallstones, and certain bacteria like *H. pylori* and *S.* Typhi is now considered as a pre-disposing factor for gallbladder cancers (Nath et al., 2008; Nath et al., 2010).. Since very few gallbladder cancer specimens were obtained in this study, only one patient showed the presence of *E. coli*, with other two negative for culture and PCR. Furthermore, specimens from nearly all patients (except for two patients who were positive for *E. coli*) with normal gallbladder pathobiology were sterile and did not show presence of any organisms observed in the diseased state. Thus, in our study bacterial presence can be associated to the diseased state of the gallbladder.

Higher percentage (11.1%) of patients showed bacteria in their bile, with few patients showing bacteria in gallstones (5.58%) **(Table 2.3)**. Both, *S. aureus* and *S.* Typhi were mostly present in the gallbladder tissue of patients. This was also confirmed for *S.* Typhi using PCR with nearly 10 patients being positive for tissue compared to 6 and 2 for bile and gallstone, respectively. As reported in some of the earlier literature (Dutta et al., 2000; Nath et al., 2008), our findings also support the fact that the PCR was able to detect higher number of *S.* Typhi present within the gallbladder (with >2-fold increase in detection efficiency in bile, tissue and gallstones compared to culture), indicating low efficiency of culture methods. This may be attributed to bacteria being present in relatively few numbers or in a viable but non-culturable state (VBNC).

Specimen	Organism isolated	Method	No. of patients		
			Normal	CC	Cancer
Number of patients			**45**	**197***	**3**
Bile	*E. coli*	Culture	2 (4.4%)	5 (2.53%)	NA
	K. pneumoniae	Culture	0	4 (2.03%)	NA
	Enterobacter spp.	Culture	0	3(1.52%)	NA
	S. aureus	Culture	0	1(0.5%)	NA
	P. aeruginosa	Culture	0	3(1.52%)	NA
	***S.* Typhi**	Culture	0	1 (0.5%)	NA
		PCR	0	6 (3.04%)	NA
Gallbladder tissue	*E. coli*	Culture	0	3(1.52%)	1 (0.5%)
	K. pneumoniae	Culture	0	2 (1.02%)	0
	Enterobacter spp.	Culture	0	1(0.5%)	0
	S. aureus	Culture	0	3(1.52%)	0
	P. aeruginosa	Culture	0	1(0.5%)	0
	***S.* Typhi**	Culture	0	4(2.03%)	0
		PCR	0	10 (5.07%)	0
Gallstone	*E. coli*	Culture	0	3(1.52%)	NA
	K. pneumoniae	Culture	0	2 (1.02%)	NA
	Enterobacter spp.	Culture	0	2(1.02%)	NA
	S. aureus	Culture	0	0	NA
	P. aeruginosa	Culture	0	2(1.02%)	NA
	***S.* Typhi**	Culture	0	0	NA
		PCR	0	2(1.02%)	NA

Table 2.3 Percentage of organisms occurring in different specimens. *p<0.001

Hence, the values for other Enterobacteriaceae in the **Table 2.3** may not depict the exact picture of bacterial load in the gallbladder niche. Recovery of bacterial isolates from these specimens may be due to the following reasons: (1) Bacteria tend to overcome the deleterious effects of bile by temporary adaptation and escape out deep into the gallbladder tissue as a possible hideout to prevent long term exposure to bile and (2) Similarly, bile and gallstones have been shown to alter various bacterial persistence mechanisms and regulate biofilm formation respectively, as mentioned earlier (Crawford et al., 2012). Though the type of organisms present in gallbladder may be similar, their incidence may vary depending on the population under study as described in the previous chapter (Prouty and Gunn, 2003; Gonzalez-Escobedo et al., 2011).

2.4.3 PCR assay for screening *S*. Typhi in clinical specimens

Most of the previous findings associate culture-based methods with low isolation rates and false negatives due to relatively low number of bacteria present in the gallbladder niche. We thus use a molecular method involving a PCR assay to specifically detect *S*. Typhi in clinical samples. As shown in **(Figure 2.4 A&B),** the PCR successfully detected the presence of *S.* Typhi in gallbladder specimens. This method is able to detect DNA as low as 4ng/µl (10^6 organisms) **(Figure 2.4C)** and is highly specific for *S*. Typhi (**Figure 2.4D)**. Also, the culture method was able to detect *S*. Typhi in only 5% of the total population whereas PCR assay revealed *S*. Typhi to be present in 11.5% of the patients. This was only possible because the flagellin gene of *S*. Typhi has unique nucleotide sequences in the hyper-variable region of the gene that differentiates it from other *Salmonella* species and organisms **(Table 2.4)**. Interestingly, the nucleotide sequence and predicted amino acid sequence of *S*. Typhi also differs from that present in *S. muenchen* which also has the H-1d gene with highly homologous sequences (Frankel et al., 1989). Genes that encode the bacterial flagellin are highly conserved at their 5' end (region that codes for backbone of flagellary filament), but the central region is quite variable and encodes surface-exposed antigenically variable portion of the filament accounting for approximately 280 flagellin alleles. As seen in **Figure 2.5**, the sequencing studies revealed that the gene amplified was similar to the flagellin H-1 d DNA sequence of S. Typhi without any variations or mutations observed in different samples.

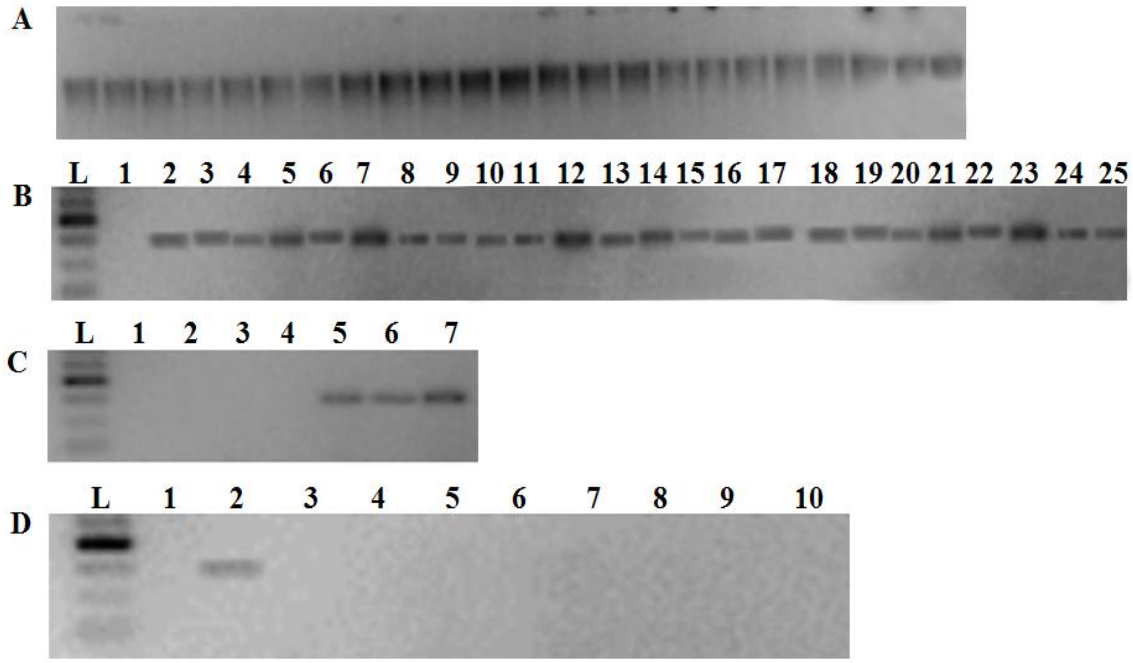


Figure 2.4 PCR assay for detection of *S*. Typhi in clinical specimens. A represents the genomic DNA isolated form specimens. **B** represents the amplified H-1d region of the flagellin (FliC) gene fragment using PCR. Lane 1 represents negative control; Lane 2-25 represents all positive samples for *S*. Typhi. **C** represents sensitivity of the assay towards presence of *S.* Typhi (Lane 2-7: 1-6ng DNA respectively). Lane 1: Negative control; Lane 2-7: Increasing concentrations of DNA molecules (1-6ng/µl). **D** represents the specificity of the assay to different organisms. Lane 1: Negative control; Lane 2: Positive control (*S.* Typhi Ty2 DNA); Lane 3: *E. coli* MTCC 723, *K. pneumoniae* MTCC 101, *Shigella* spp. (lab isolate), *P. vulgaris* MTCC 1441, *S. aureus* MTCC 737, *S. pyogenes* MTCC 442, *P. aeruginosa* MTTC 741, *Enterobacter* and *Bacillus* (lab isolates). L: 50bp GeneDirex DNA ladder.

Salmonellae	**Flagellar Antigens**
S. Typhi	**d:**
S. Paratyphi	a:
S.Typhimurium	i:1, 2, 3
S. Enteritidis	gom:
S .muenchen	**d:**1, 2

Table 2.4 Variation in flagellar antigens across *Salmonella* spp.

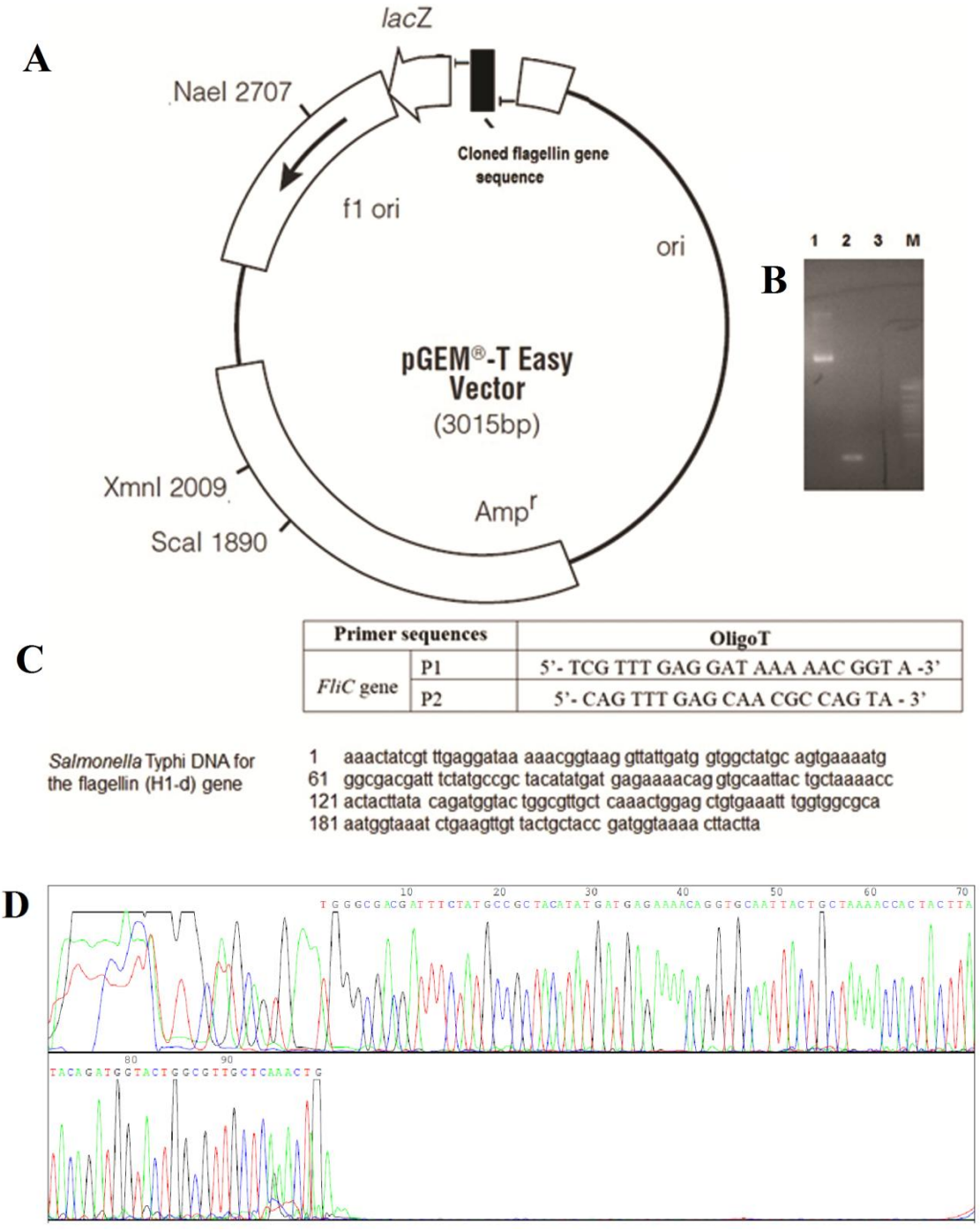


Figure 2.5. TA cloning and sequencing of the flagellin gene fragment. A represents the pGEM-T Easy vector used for cloning of the flagellin gene fragment. **B** represents the visualisation of plasmid and insert. Lane 1: pGEM-T Easy vector with cloned fragment; Lane 2: PCR amplification of cloned flagellin gene; Lane 3: Negative control; Lane 4: Genedirex 50 bp DNA ladder. **C** shows the primer pairs used for this study and the H-1d DNA sequence. **D** depicts the sequencing analysis of the cloned flagellin gene fragment (Chromas Biotech.).

2.4.4 Tolerance to bile and variance in biofilm forming ability

As depicted in the **Figure 2.6**, *S.* Typhi isolates obtained from gallbladder had an MIC of 4% to human bile with an MBC that varied between 4-6%. Hence a sub-lethal bile concentration (The concentration below lethality) of 3% was used for all other experiments. Earlier studies also report a similar MIC of *Salmonella* to bile salts. As mentioned earlier, the lower MIC may be attributed to the temporary adaption of *Salmonella* to bile. This adaptation process may begin when the organism encounters lower bile concentration in the intestine, finally getting exposed to concentrated bile in the gallbladder. Such isolates when grown in absence of bile regain their sensitivity to higher bile concentrations *in vitro*.

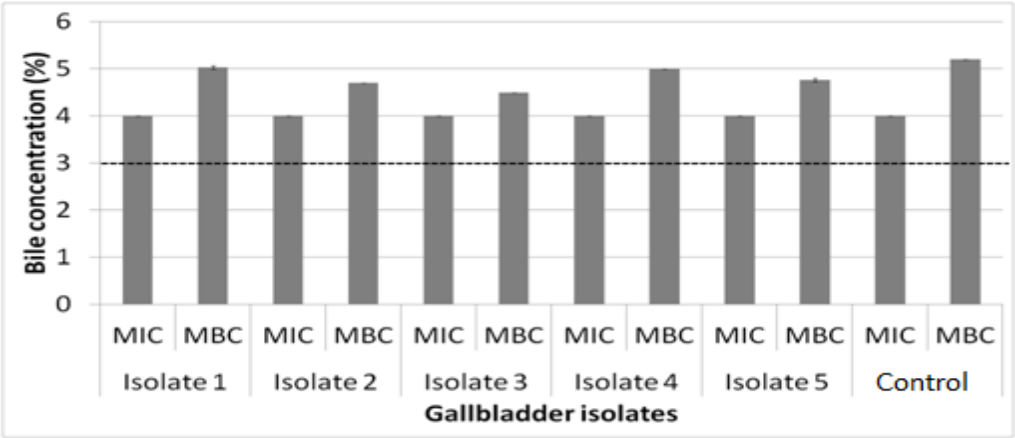


Figure 2.6 MIC and MBC of *S.* Typhi to bile. *S.* Typhi gallbladder isolates were grown in presence of varying bile concentrations to determine the MIC and MBC to bile. The dotted line represents the sub-lethal bile concentration (concentration below lethality) used for *in vitro* experimentations. The control strain used was *S.* Typhi CT18. The results are mean of at least three independent biological replicates with SD and SE.

Biofilm formation is a sequential process involving coordination of highly regulated genes and pathways. It begins with adherence of planktonic bacteria to a surface followed by subsequent maturation of the biofilm that is characterized by production of extracellular matrix composed of proteins, nucleic acids and exopolysaccharides. These biofilms are generally a result of stress environment, adapted by organisms for their survival and chronic persistence with a particular niche. In presence of bile, which is shown to induce stress, *S.* Typhi prefers to undergo a motile to sessile shift in order to overcome any further damage exerted by bile on the bacterial populations. (Prouty and Gunn, 2003; Gonzalez-Escobedo et al., 2011).

In our study **(Figure 2.7A)** we show that the bacteria form microcolonies-a characteristic feature of bacteria showed previously by Sriramulu et al. in P. *aeruginosa* (Sriramulu et al., 2005), followed by formation of complete biofilm mass. All the strains showed rdar (Red, dry and rough) morphotye on Congo red agar plates **(Figure 2.7B)** indicating expression of two ECM components namely curli and cellulose. Such morphotye aids in biofilm formation and affects production of both cellulose and fimbriae. Crystal violet staining assays revealed variation in biofilm forming ability across the gallbladder isolates as seen in **Figure 2.8** on cholesterol-coated surface. It was further observed that cholesterol was necessary for significant biofilm-formation ($p<0.01$) on glass surface as depicted in **Figure 2.8 C and D**. All the isolates showed an increase in biofilm mass with time, with significant biofilm formation by 18 days ($p<0.001$). Furthermore, in presence of bile **(Figure 2.8B)**, *S.* Typhi showed a nearly 1.5-fold increase in biofilm mass ($p<0.05$) compared to bacteria grown in absence of bile **(Figure 2.8A)**. Thus, sub-lethal bile concentration induced quicker and better biofilm masses *in vitro* in *S.* Typhi **(Figure 2.8B)**. Even when compared to the control strain CT18, the biofilm mass in presence and absence of bile was more, but not significantly higher with $p>0.05$. The TGA assay showed that *S.* Typhi successfully formed biofilms on cholesterol gallstones *in vitro*.

Thus, such a phenotype is exhibited by *S.* Typhi in presence of specific substrates and is accelerated in presence of bile to assist persistence and dissemination. Interestingly, previous reports indicate that bile reduces motility in bacteria and induces biofilm formation by regulating various biofilm and stress-associated mediators like CsgD and RpoS. These finding further support the hypothesis of bacterial persistence within gallbladder via gallstones (Crawford et al., 2012; Gonzalez-Escobedo et al., 2011).

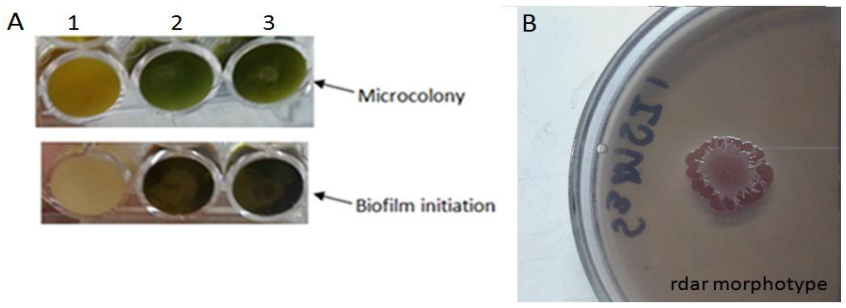


Figure 2.7 *In vitro* biofilm formation and RDAR morphotype shown by *S.* Typhi isolates. A represents the initiation, and successful biofilm formation of *S.* Typhi in 24-well microtitre plates. Lane 1: media without bile Lane 2 & 3: *S.* Typhi grown in presence of 2% & 3% bile. **B** The rdar morphotype shown by *S.* Typhi isolate on Congo red agar plate.

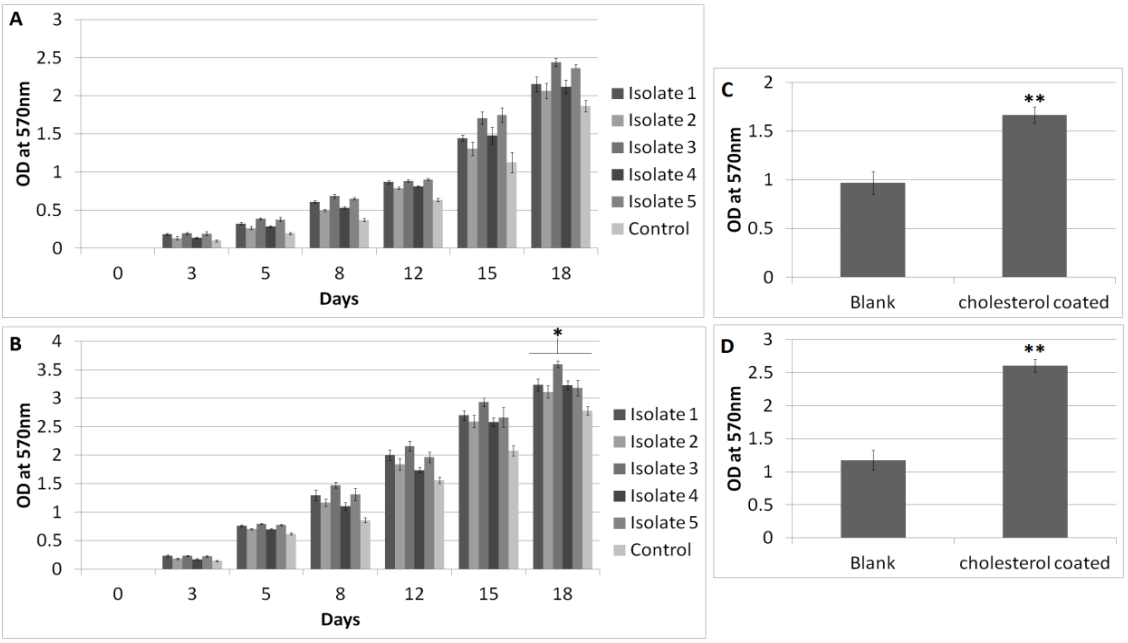


Figure 2.8 Quantifying the biofilm mass using crystal violet assay. To understand the role of bile in assisting biofilm formation, we grow *S.* Typhi isolates in absence (**A & C**) and presence (**B & D**) of sub-lethal (3%) bile. **A and B** represents biofilm formation on cholesterol coated surfaces. **C and D** represents significance of cholesterol coated surface for biofilm formation following 15 days of incubation with *S.* Typhi. The experiments were performed in triplicates with a p-value of *<0.05 and ** <0.01 considered to be significant.

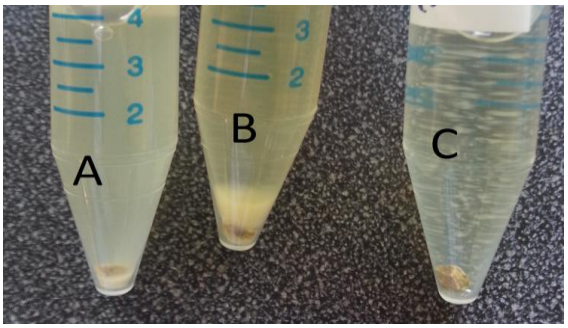


Figure 2.9 *In vitro* biofilm-formation on gallstones. A & **B** represent biofilm formation of *S.* Typhi on cholesterol gallstones in absence and presence of 3% bile respectively. **C** represents a negative control for the experiment.

2.4.5 Multi drug resistance and antibiotic susceptibility

The resistance to common antibiotics used in treatment of S. Typhi infections were tested *in vitro* using the standard CLSI/NCCLS guidelines **(Figure 2.10 A&B)**. All the chronically persisting isolates of *S.* Typhi obtained from the gallbladder showed resistance to common first-line antibiotics except for ampicillin (Pratap et al. 2012). This was on par with previous studies that ampicillin can be used to treat chronic *S.* Typhi infections. All the isolates were susceptible to second generation

fluoroquinolones and also to third generation cephalosporin. The degree of susceptibility varied and was classified as susceptible, intermediate and resistant based on the CLSI/NCCLS guidelines as shown in **Table 2.5**. The susceptibility to most of the antibiotics was significantly lower compared to the control CT18 strain, except for ciprofloxacin and ampicillin which showed similar susceptibilities as the control strain. In addition, only ciprofloxacin and ampicillin were classified as susceptible towards *S.* Typhi isolates.

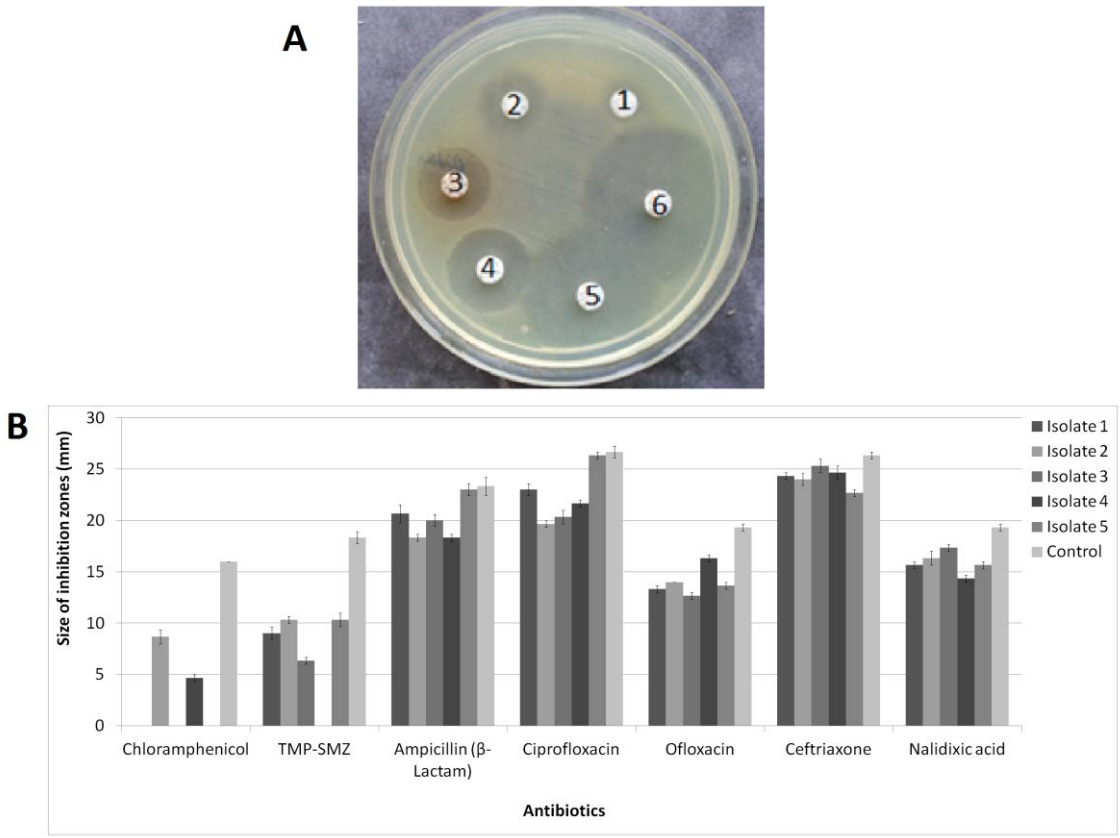


Figure 2.10 Antibiotic susceptibility of *S.* Typhi isolates to different drugs. The disc diffusion method was used to determine the susceptibility of S. Typhi gallbladder isolates to antibiotics. **A** depicts the zones of inhibition to different drugs used in this study. 1- 6: chloramphenicol, trimethoprem-sulfamethoxazole (TMP-SMZ), ofloxacin, nalidixic acid, ampicillin and ciprofloxacin respectively. **B** represents differences in drug susceptibility across all the isolates. The control strain used was CT18. The data represents mean of at least three independent replicates with p-values of *<0.01 and **<0.05.

Antibiotics	Disc potency	Zones in mm		
		Susceptible	Intermediate	Resistant
First-line drugs				
Chloramphenicol	30µg	≥18	18-20	≤17
TMP-SMZ	5µg	≥16	11-15	≤14
Ampicillin	30µg	≥17	14-16	≤13
Fluoroquinolones				
Ciprofloxacin	5µg	≥21	16-20	≤15
Ofloxacin	5µg	≥16	13-15	≤12
Cephalosporin				
Ceftriaxone	30µg	≥26	25-26	≤24
Synthetic quinolones				
Nalidixic acid	30µg	≥19	14-18	≤13

Table 2.5 CLSI standard chart for susceptibility of Enterobacteriaceae.

	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Control
Chloramphenicol	R	R	R	R	R	S
TMP-SMZ	R	R	R	R	R	S
Ampicillin (β-Lactam)	**S**	**S**	**S**	**S**	**S**	**S**
Ciprofloxacin	**S**	**S**	**S**	**S**	**S**	**S**
Ofloxacin	I	I	I	I	I	S
Ceftriaxone	I	I	I	I	I	S
Nalidixic acid	I	I	I	I	I	S

Table 2.6 Classification of isolates based on antibiotic susceptibility as per CLSI guidelines.

2.4.6 Changes in total protein profiles on exposure to bile

A 12% SDS PAGE analysis revealed no alteration in total protein profiles (Intracellular and membrane) when the organisms were grown in absence of bile (figure 5). As depicted from **Figure 2.11**, exposure to sub-lethal bile concentrations showed similar changes in total protein profiles of all *S.* Typhi isolates (marked with arrows). Bile-induced overexpression was seen in positions corresponding to ~66kDa for intracellular

proteins and ~43kDa for membrane proteins. Bile is shown to alter the expression of number of genes and simultaneously regulate multiple mechanisms that assist bacterial adaptation. The major outer membrane proteins (OMPs), drug efflux mechanisms (like ArcAB and TolC), stress related responses (like RpoS and csgD) and biofilm associated factors (motility and EPS components) are shown to be directly or indirectly regulated by presence of bile in the environment. Still many mechanisms remain unexplained during bile stress and understanding such molecular alteration is of utmost importance to understand chronic persistence in gallbladder (Prouty and Gunn, 2003; Crawford et al., 2012).

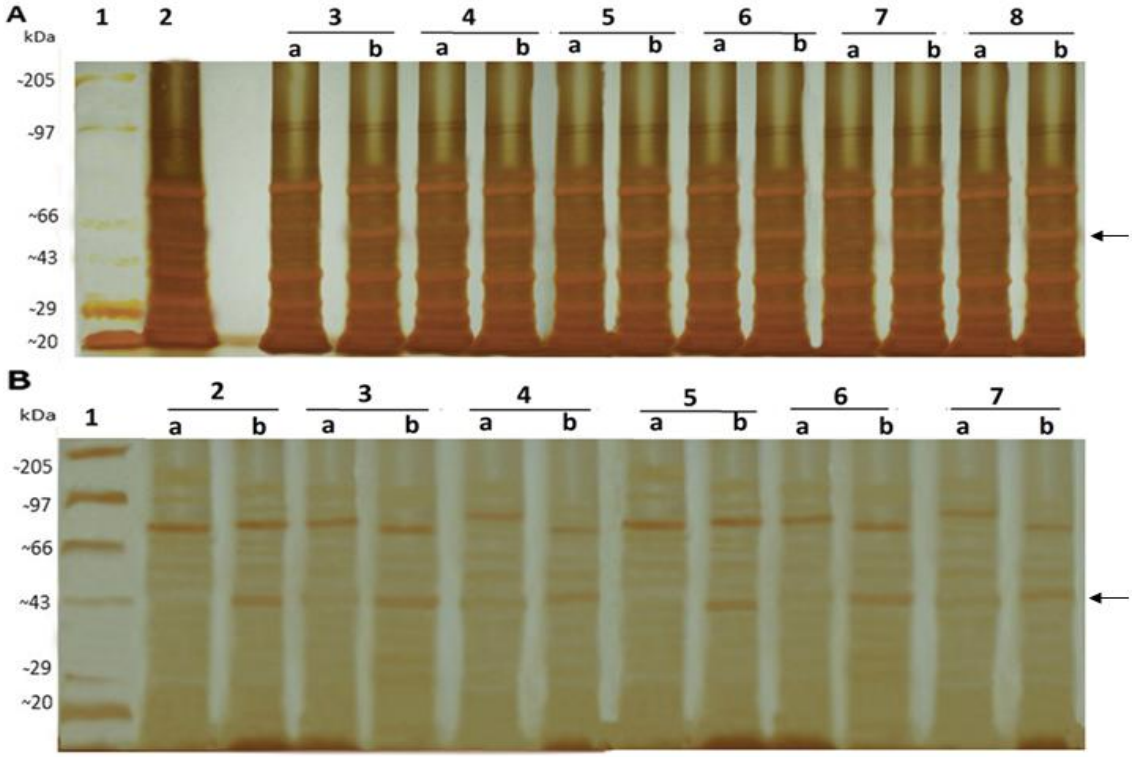


Figure 2.11 Changes in total protein profiles in response to bile. 12% SDS PAGE analysis was performed for intracellular and membrane proteins of *S*.Typhi strains in (a) absence and (b) presence of sub-lethal bile concentration. (**A**) Intracellular protein profiles. Lane 1: Broad range protein molecular weight marker; Lane 2: positive control; Lane 3-7: Gallbladder isolates; Lane 8: *S.* Typhi CT18 (**B**) Membrane proteins profiles. Lane 1: Broad range protein molecular weight marker; Lane 2 - 6: Gallbladder isolates; Lane 8: *S.* Typhi CT18. The arrows represent the changes observed in the profiles.

2.5 Conclusion

In conclusion, *Salmonella enterica* serovar Typhi is shown to chronically persist within the gallbladder of patients undergoing cholecystectomy for gallbladder disease. Due to relatively low gallbladder samples obtained in this study we could not establish the link of *S.* Typhi chronic persistence with gallbladder cancers as reported in previous literature. Hence, all the patients' positive for *S.* Typhi suffered from chronic cholelithiasis, indicating a strong link of such chronic infections with gallstone disease. Gallstones have been shown to assist chronic persistence by acting as suitable substrates for initiation and development of bacterial biofilms. Though we could not recover any *S.* Typhi isolates from gallstones on culture, our *in vitro* studies showed that the gallbladder isolates could significantly form biofilms on gallstones and cholesterol coated surfaces. In addition, bile was shown to be a key mediator of this phenotype in *S.* Typhi. Thus, the role of bile is complex in a way that it is shown to have bactericidal properties and can simultaneously regulate bacterial mechanisms for persistence within the gallbladder. On further analysis of the drug susceptibilities of the isolates obtained from gallbladder, it was seen that the organisms were susceptible only to ampicillin and ciprofloxacin. For other drugs used in treatment of typhoid fever, the organisms were either resistant or had intermediate susceptibility.

Some of our findings like bile regulating bacterial phenotypes and presence of polymicrobial populations within the gallbladder lead us to further investigate role of quorum sensing and persister cells in mediating *S.* Typhi persistence within the gallbladder, described in Chapter 3.

CHAPTER-III

Understanding Mechanisms Underlying Chronic *S.* Typhi Persistence in Gallbladder: Implication of Quorum Sensing and Persisters

3. Understanding Mechanisms Underlying Chronic *S.* Typhi Persistence in Gallbladder: Implication of Quorum Sensing and Persisters

3.1 Abstract

Salmonella Typhi can chronically persist within gallbladder of patients suffering from gallbladder diseases. This study was intended to improve our understanding on significance of quorum sensing and persister cell generation as a response to bile-stress encountered during colonisation of gallbladder. Bile led to the generation of reactive oxygen species (ROS) in *S.* Typhi, which in response showed a significant increase in the production of anti-oxidative enzymes namely superoxide dismutase (SOD) and catalase. The quorum sensing mutant of *S.* Typhi was found to be sensitive to bile with significantly lower levels of antioxidant enzymes compared to other clinical isolates. Furthermore the addition of exogenous cell-free extracts (CFE) of *S.* Typhi containing the quorum sensing signalling molecule significantly increased the levels of these enzymes within the mutant. Interestingly the CFE addition did not significantly restore the biofilm forming ability of the mutant strain when compared with the wild-type. The work for the first time reports that the AI-2 mediated quorum-sensing system of *S.* Typhi plays a key role in regulating the level of these enzymes during oxidative stress. In addition, *S.* Typhi is also shown to sense quorum sensing signals produced by other organism via the *sdiA*. On this basis we develop a whole cell biosensor (P_{rck}-eGFP transcriptional fusion) to detect the AHL molecules produced by other organisms in mixed cultures, and try to find out the behaviour response of *S.* Typhi in presence of other organism. Though our findings show that *sdiA* is upregulated as a response to AHL molecules and bile, it did not confer any significant advantage to *S.* Typhi growth and biofilm formation. Interestingly, *sdiA* was found to be necessary in enhancing the resistance to certain drugs like ofloxacin, ceftriaxone and nalidixic acid. Furthermore, in the presence of ciprofloxacin and ampicillin, *S.* Typhi formed persister cells which increased > 3 fold for both when media was supplemented with bile. These findings show that *S.* Typhi adapts a protective mechanism and shows increased persistence against antibiotics in the presence of bile which could be of clinical significance in the case of chronic bacterial persistence within gallbladder.

3.2 Introduction

Salmonella enterica serovar Typhi chronically persists within the gallbladder of patients following an acute phase of infection, and is proposed to be one of the predisposing factors for gallbladder cancers (Nath et al., 2008; Tewari et al., 2010). The organism is shown to tolerate bile-a bactericidal agent, which it encounters during its passage through the intestine into the gallbladder-a site for bile storage. Furthermore, bile salts are shown to enter the bacterial cells and regulate several gene loci involved in oxidative stress, cell and membrane protein synthesis, efflux mechanisms and other survival mechanisms (Gunn, 2000; Begley et al., 2005). Transcriptome studies in *S. enterica* also report up-regulated levels of superoxide dismutase enzymes in response to bile salt stress (Herna´ndez et al., 2012). Thus, the resistance to the oxidative stress exerted by bile may rely on the ability of the organism to produce antioxidant enzymes mainly the superoxide dismutase (SOD) and catalase (Tsolis et al., 1995; Farr and Kogoma, 1991). Interestingly, expression of SOD and catalase is shown to be under the control of a quorum sensing system in *Pseudomonas aeruginosa* and *Acinitobacter baumannii* (Hassett et al., 1999; Bhargava et al., 2014).

Quorum sensing usually involves communications within a same bacterial population (For e.g. *Vibrio fisheri* senses its population densities using their own QS signal molecules) or between different bacterial species. Variations in the QS signal molecules and receptors of these signals ensure that bacteria respond to their own signals even in a diverse microbial community (Bassler, 2002). Besides, some species just 'listen' to signals produced by other organisms and function accordingly. For instance, *Escherichia* and *Salmonella* use *sdiA* to sense AHL produced by other organism (including some of the intestinal pathogens) and respond by regulating various mechanisms like biofilm formation, alteration in drug efflux mechanisms, etc. (Lee et al., 2009). Similarly, *Burkholderia cepacia* sense AHL produced by *P. aeruginosa* and increase their production of lipases, proteases and siderophores. Thus, these communications assist the bacteria to either co-exist or out-compete with other species within the same niche. Previous studies report that bacteria like *Pseudomonas, Klebsiella, E.coli, Enterococcus, Enterobacter, Proteus* and *Staphylococcus* can be successfully isolated from the gallbladder with varying frequencies depending on the population being studied. Interestingly, co-infection with more than one organism has also been reported (Hazrah et al. 2004; Capoor et al., 2008). Our findings also support

the fact that bacteria can coexists in the gallbladder, where even *S*. Typhi is shown to co-exist with *E. coli*. Thus, it is important to understand if one bacterial species actually has a role in the persistence of other bacteria within such extreme niches, and if this could possibly lead to any severe health impact in the course of time.

In most cases, bacteria alter population densities by producing inter- or intra-species signalling molecules referred to as *N*-acylhomoserine lactones or AHLs, that regulate host interaction genes or secondary metabolism by a process termed as quorum sensing (Bassler, 2002). But organisms like *Escherichia*, *Salmonella*, and *Klebsiella* fail to produce these AHLs though they house a putative AHL receptor. This is because the bacteria don't encode the AHL synthase enzymes as they lack the *luxI* family member, but each species carries single *luxR* homolog named *sdiA* that function as an AHL receptor (Inter-species signalling mechanism). In *Salmonella*, the *sdiA* effectively responds to AHLs produced by other bacterial species and up-regulates two loci the *rck* (resistance to complement killing) operon and srgE (Lee et al., 2009). Thus, in this study we develop a whole cell biosensor using the P_{rck}-eGFP transcriptional fusion which indirectly responds to external AHL signals (via the *sdiA* molecules) to give fluorescence that is assumed to be proportional to the concentration of AHL in the environment.

For intra-species cell communication, *Salmonella* harbours a quorum sensing system activated by autoinducer-2 (AI-2) which aids in bacterial pathogenesis and virulence. The *luxS* gene of *Salmonella* encodes AI-2 synthase which catalyses the biosynthesis of 4, 5-dihydroxy-2, 3-pentanedione (DPD) from S-adenylmethionine. The AI-2 signalling molecule (2R, 4S)-2-methyl-2, 3, 3, 4 tetrahydroxytetrahydro- furan is then derived from DPD. AI-2 regulates a number of genes with different functions, including genes having role in virulence, motility, antibiotic production, cell division, biofilm production, carbohydrate metabolism and regulation of an ATP binding cassette (ABC)-type AI-2 transporter (Surette et al., 1999; Choi et al., 2007; Jesudhasan et al., 2010). In this study we report for the first time the role of this *S.* Typhi intra-species quorum-sensing system in regulating SOD and catalase levels, as a response to oxidative stress generated by bile.

Studies on mechanisms underlying *Salmonella* survival within the mammalian gallbladder are emerging as a response to bacterial persistence and resistance to bile (Herna´ndez et al., 2012). *S. enterica* is shown to produce persister cells in response to

oxidative and osmotic stress. These persisters are phenotypic variants which may be pre-adapted to a selective agent by mutation in specific loci or they may activate resistance-associated responses (towards antibiotics) within a bacterial subpopulation to impose an adaptive nature (Hassett et al., 1999; Wood et al., 2013). Very little is known about these variants in *S.* Typhi and their role during chronic persistence. We report that the bile-induced stress in *S.* Typhi gallbladder isolates increases the number of the persister cells formed during antibiotic challenge.

3.3 Material and Methods

3.3.1 Bacterial strains, primers and growth conditions

All the strains and primer pairs used for quorum sensing related studies are listed in **Table 3.1 and Table 3.2** respectively. Clinical isolates of *S.* Typhi were isolated from gallbladder of patient undergoing cholecystectomy at SMRC hospital-Goa and characterized as described in earlier chapter. Bile was aspirated from patients prior to surgery. For propagation of the plasmids the host strain used was *E. coli* DH5α. For AI-2 related studies in *S.* Typhi, we use AI-2 producers i.e. *E. coli* MTCC 723 and *S. aureus* MTCC 737 and non-producer i.e. *P. aeruginosa* MTCC 741. All strains were grown at 37°C in LB with constant aeration and agitation. For AI-2 synthesis by bacteria, we use peptone water media (PW media) comprising of 1% peptone, 0.5% NaCl and 0.5% glucose.

Strain	Characteristic(s)	Reference or source
T1-T5	Clinical isolates from patients	Gallbladder of patients undergoing cholecystectomy
CT 18	Standard strain	Dr. Rajni Gaind, Safdarjung hospital-New Delhi (India)
Δ*luxS*	Luminescence expression (*luxS*) mutant of T1; Kan^r	This study
Δ*sdiA*	supressor of cell division inhibitor (*sdiA*) mutant of T1; Kan^r	This study

Table 3.1: *S.* Typhi strains used in this study

Gene	Sequence
***luxS*::km**	FP:5'CCGGAACAAAGAGTTCAGTTTATTTTTAAAAAATTATCG GAGGTGACTAAGTGTAGGCTGGAGCTGCTTC 3'
	RP:5'CGGCCATAAACCGGGGTTAATTTAAATACTGGAACCGC TTACCAATAAGACATATGAATATCCTCCTT 3'
	FP:5' CCGGAACAAAGAGTTCAGTTTATTT 3'
	RP:5' CGGCCATAAACCGGGGTTAATTTAA 3'
***sdiA::*km**	FP:5'TAAGTCATTCATAATAATGATTATCAATATCAAAGGCGT GACCATAAAATGTGTAGGCTGGAGCTGCTTC 3'
	RP:5'AGCATCGCTTTCAGCCGGTTTCGCGTCTGGCGCGAAGCA TCGTCAGCACGCATATGAATATCCTCCTT 3'
	FP: 5' TAAGTCATTCATAATAATGATTATC 3'
	RP: 5' AGCATCGCTTTCAGCCGGTTTCGCG 3'
sodB	FP: 5' ACCGTATGCCAAAGATGCTC 3'
	RP: 5' TATTAAAGATGCCGCCTTCG 3'
katG	FP: 5' CGGTATTAGAAGCGCTCCAG 3'
	RP: 5' GCGCACGATAGTTACGGAAT 3'
sdiA	FP: 5' GGTTCGGCAACATCACACAC 3'
	RP: 5' GTTCGTCATCCCGTTCCCTT 3'
P_{rck}-eGFP	FP:5'TGCAGGAGGTACGACAAAAA 3'
	RP:5' GTACCAGGACGACCTCAAGCAC 3'
Orf6-pefI region	FP: 5' TAAGCAGAATTCTGCAGGAGGTACGACAAAAA 3'
	RP: 5'ATATCGCGGGTTTTAACTGGGGATCCTAAGCA 3'

Table 3.2: Primer pairs used in this study

3.3.2 Gene disruption assay using lambda red mutagenesis

The pKD46 plasmid was extracted from *E.coli* DH5α using miniprep kit and transformed into the target strain i.e. *S.* Typhi using $CaCl_2$ method. The resulting transformants were selected by plating them on LB containing 50µg of ampicillin. The colonies obtained were then picked and grown at 30°C in 2ml LB containing ampicillin (OD_{600}=0.1). This was followed by the addition of 20µl 1M L-arabinose (10mM) to induce λ-red expression. The bacteria were allowed to grow at 30°C till a final OD_{600}=0.4-0.5 and 1mL was aliquoted in two separate eppendorfs (One served as the test whereas other as negative control). The cells were chilled on ice for 10 minutes

followed by centrifugation at 4°C for 10 minutes. The supernatant was discarded and cells were resuspended in 50µl ice cold water (this step was performed twice). Simultaneously, a linear fragment containing the kanamycin cassette was PCR amplified from the plasmid pKD4 using the oligo's A & B as shown in **Figure 3.2.** 0.5µg of this fragment was transformed into the λ-red expressing cells present in test tube followed by plating on LB plates containing 50µg of Kanamycin. For confirmation of the gene disruption in organism, a PCR verification step was carried out using the oligo's C and D (**Figure 3.2)**.

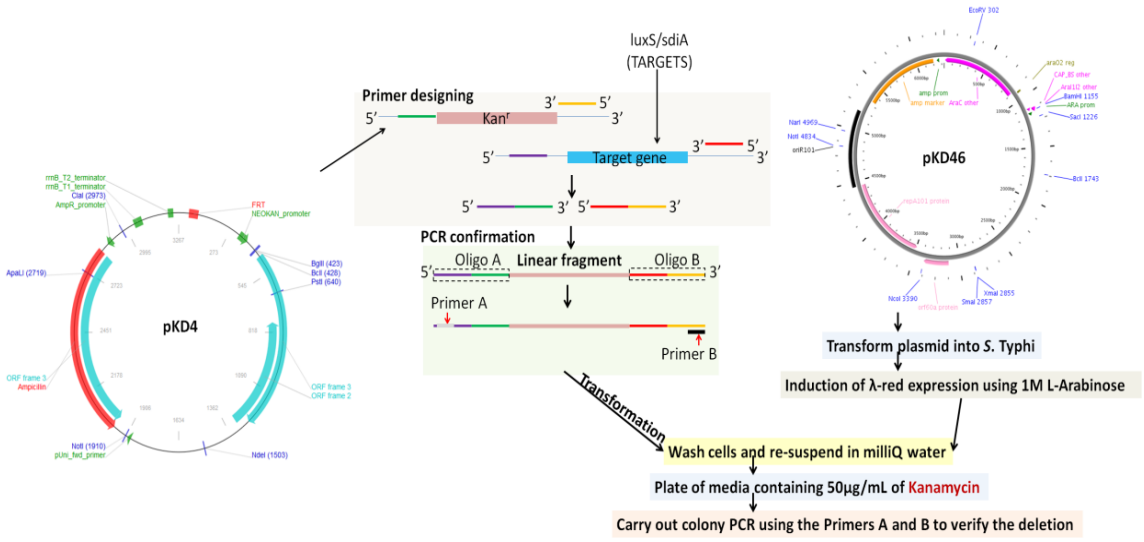


Figure 3.1 Target gene disruption using λ-Red recombinase system in *S.* Typhi.

PCR condition for amplification of linear fragment: The PCR mix (50µl) contained 0.5µl (45ng/µl) of pKD4 template, 2.5µl (10µM) of each of the two primers, 5µl (2mM each) of dNTP, 2µl (25mM) of $MgSO_4$, 5µl of 10X KOD buffer, 1µl of KOD polymerase enzyme and 31.5µl of Millipore water. The PCR conditions were as follows: 95°C for 7 min; 35 cycles at 94°C for 15 sec, 50°C for 30 sec, 72°C for 90 sec and a final elongation at 72°C for 10 min. The PCR products were separated in a 1.5 % agarose gel containing ethidium bromide. The expected PCR products were visualized with a gel documentation system referenced with a Genedirex 50 bp DNA ladder.

For complementation assay, we adapt a method previously described by Karavolos et al (Karavolos *et al*. 2008). Briefly, the *luxS* gene was amplified from wild-type *S.*Typhi using the primers P1-GCGAAGCTTACCGAGCCGTTTGCCGCGTGG and P2-GCGGGATCCATTTAA CAGGCCAGGCATTAC containing the HindIII and BamHI sites respectively. The amplified fragment was cloned into the pBR322 plasmid

digested with HindIII and BamHI to obtain the pBR*luxS* plasmid. Similarly, the *sdiA* gene was amplified from wild-type S. Typhi using the primers P3-CGAAGCTTACGG TTCGGCAACATCACACAC and P-4 CGGGATCCATGTTCGTCATCCCGTTCCC TT and cloned into pBR322 plasmid to obtain pBR*sdiA* plasmid. These recombinant plasmids were the used to complement the mutant strains generated in this study.

3.3.3 Synthesis and detection of *S*. Typhi AI-2 molecules for QS studies

Colorimetric estimation: The bacteria were grown in PW media followed by centrifugation at 13000 x g for 5 minutes. The supernatant was filtered through a 0.2µm filter to obtain a cell-free extract (CFE) which was used for AI-2 detection. We use a method previously described by Wattanavanitchakorn et al (Wattanavanitchakorn et al., 2014). Briefly, working solution of 10 mM 1, 10-phenanthroline/3.32 mM Fe (III) was prepared. For standardisation a fresh 0.5mM stock solution of ascorbic acid was used as molecular structure of AI-2 is almost similar to ascorbic acid. For assay, 1mL of the sample was mixed with 1mL Fe (III)-1, 10-phenanthroline reagent and left to stand for 1min. The solution was diluted to 5mL using deionized water and filtered through 0.2µm filter. The filtrate was scanned within 3 min spectrophotometrically using Shimadzu UV-Vis Spectrophotometer and the absorbance at 510 nm was monitored. The positive control used was PW media with 20µM ascorbic acid whereas PW media alone served as blank. Since *Pseudomonas* does not produce any AI-2 molecules, it served as the negative control.

Detection using a fluorescent chemodosimeter: The optimum cell density for AI-2 production by the organisms was determined using the colorimetric assay at different time intervals. We use this data to determine the specific detection capablility of an emission based ‘‘turn-on’’ fluorescent chemodosimeter previously developed by Chatterjee and group for selective detection of ascorbate ions (Khandare et al., 2013). They show that TPE (tetraphenylethylene) derivatives 1 and 2 which have alkyne and azide functionalities that undergo a “click” reaction in the presence of Cu^{+} to form a TPE based polymer (with several TPE moieties in a chain joined by triazole units) which emits blue fluorescence **(Figure 3.2)**. The sensor exploits the redox potential of copper where in the ascorbate ions facilitates the conversion of Cu^{2+} to Cu^{+}, and the Cu^{+} then consequently increases the rate of TPE-based polymer formation with increasing concentrations of ascorbate ions **(Figure 3.2)**. This approach was on par to

the one used by Wattanavanitchakorn et al. (Wattanavanitchakorn et al., 2014) to detect the bacterial AI-2 molecules which have a nearly similar structure to ascorbic acid and hence similar reducing property. The process involves the use of probe 1 and 2 which was synthesized as described in a previous study. 15 µL (10 mM) of each of probe 1 and probe 2 were taken in deionized water (2.7 mL), followed by addition of 15 µL cell-free supernatant and 15 µL (10 mM) of Cu^{2+} respectively. The volume was made up to 3ml using THF and water to get a 93% H_2O-THF solution. This solution was then stirred at room temperature for 2 hour followed by fluorescence measurement using fluorimeter.

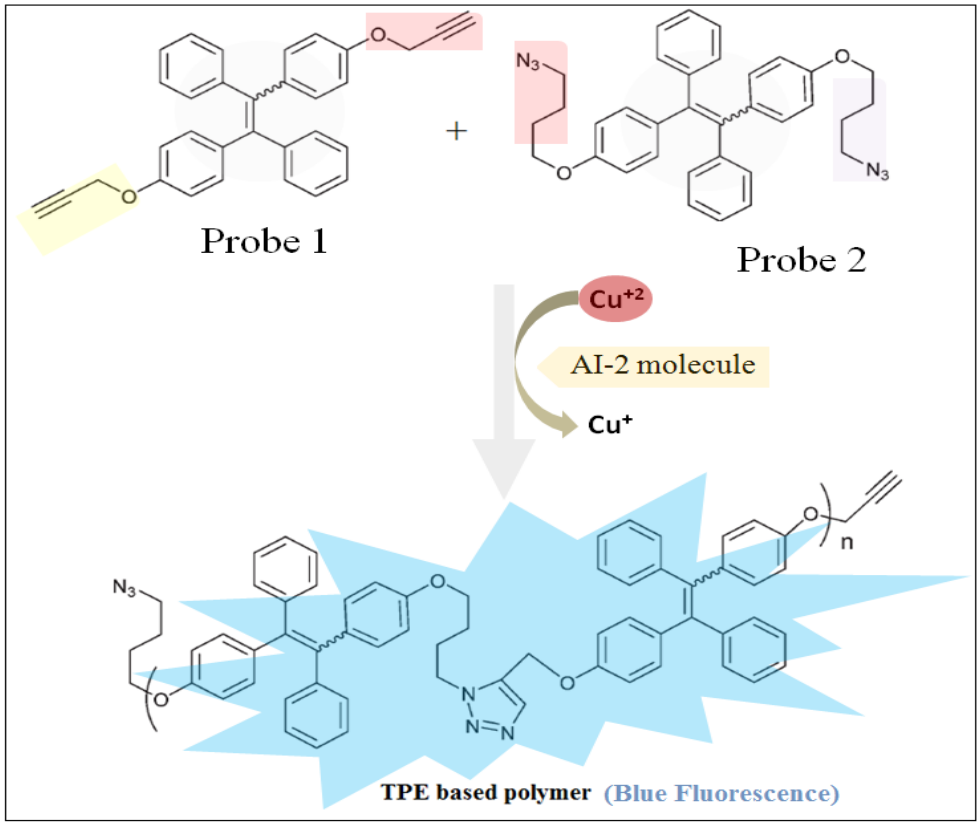


Figure 3.2 Detection of AI-2 molecules using the fluorescent chemodosimeter

(a) Adaptation and pre-adaptation of *S.* Typhi to human bile

3.3.4 Growth characteristics and Adaptation assays for *S.* Typhi isolates

The log phase cultures were challenged with bile, and assayed for MICs in microtitre well plates (Himedia, India). The MBCs were determined by plating the well exhibiting no apparent growth in the MIC assay. A fluctuation analysis was performed as described by Luria-Delbruk (Luria & Delbruck, 1943). Adaptation of *S.* Typhi to bile was carried using a method described by Hernández et al with few modifications

(Hernández et al, 2012), *S.* Typhi isolates were grown in LB broth+ 5% bile and these were plated on LB media containing 20% bile. The isolates obtained were transferred to LB broth and grown overnight. The MIC of bile for these isolates was determined. Isolates still tolerating higher concentration of bile were characterized as stable bile resistant mutants **(Figure 3.3)**. The data is representative of at least three independent replicates with standard errors for all experiments.

(b) Implication of quorum sensing and persister cell populations

3.3.5 Growth of *S*. Typhi in presence of bile

The *S.* Typhi clinical isolates were challenged with varying exogenous bile concentrations (ranging from 0.5%-20% bile) to determine the MIC in vitro. The viable cells that survived bile stress were quantified by growing *S.* Typhi at sub-lethal bile concentration (concentration below lethality) followed by a tetrazolium assay. The OD at 560nm (treated and untreated) was used to determine the percent survival of *S.*Typhi (Herna´ndez *et al*., 2012; Moussa *et al*., 2013).

3.3.6 Detection of Reactive oxygen species (ROS) in bile-treated *S*. Typhi

S. Typhi was grown in presence of sub-lethal bile concentration followed by centrifugation at 10000g for 15 minutes at 4°C. The cell pellet was resuspended in PBS (pH 7.2) containing 10µM concentration of 2', 7'- Dichlorofluorescin diacetate (DCFDA) and incubated at 37°C for 45 minutes in the dark. The cells were then pelleted by centrifugation at 10000g for 10 minutes at 4°C and re-suspended in PBS to obtain a cell density of 10^5 CFU/ml. The resulting DCF fluorescence of bacterial suspension was quantified using fluorimeter (JASCO FP-6300) with excitation at 495nm and emission at 510nm. The relative fluorescence level was measured by taking the mean ratio of treated to non-treated cells (Bhargava *et al.*, 2014).

3.3.7 Estimating the catalase and SOD activity in bile-treated *S*. Typhi

The gene expression studies were carried out by extracting total RNA from bacterial pellets using RiboZol™ (Amresco, USA), cDNA synthesis using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, India), followed by PCR (Eppendorf Mastercycler®) using gene specific primers to sodB and katG listed in **Table 3.2**. The concentration of amplified product was estimated using Nandrop spectrophotometer and equal concentration was loaded in to wells of 1.5% agarose gel. The changes in

band intensity were estimated using ImageJ software which was assumed to be direct measure of candidate gene expression.

For enzyme extraction, *S.* Typhi was grown in presence and absence (control) of bile overnight at 37°C. The cells were centrifuged at 12000g for 20 minutes at 4°C. The cell pellet was washed thrice and re-suspended in 300µl of 0.1M Tris/HCL (pH 7.5). The cells were then sonicated followed by centrifugation at 10000g for 20 minutes at 4°C to remove the cell debri. The protein (crude-fraction) in the cell lysate was quantified at 280nm using a NanoDrop Lite spectrophotometer (Thermo Scientific).

- SOD enzyme assay: The reaction mixture (300µl) contained 100µl of crude enzyme, 120 µl of 50mM phosphate buffer (pH8.3), 30µl of 300µM nitroblue tetrazolium, 10µl 186µM phenazine methosulphate, 20µl NADH (780µM) and 20µl deionized water. The reaction was initiated by addition of NADH followed by incubation at 30°C for 90 seconds. For terminating the reaction, 100µl of glacial acetic acid was added, and the mixture was stirred vigorously with 400µl of n-butanol. The mixture was allowed to stand for 10 minutes and centrifuged to recover the butanol layer. The color intensity of the chromogen in the butanol layer was measured at 560nm using a spectrophotometer (Shimadzu UV-2450) with appropriate controls. One unit of enzyme activity was defined as amount of enzyme concentration required to inhibit 50% of NBT reduction and SOD activity was expressed in milliunits per mg of protein (Bhargava *et al.*, 2014).
- Catalase activity: The catalase activity was studied by monitoring in vitro decomposition of H_2O_2. A reaction mixture containing 100µl of crude enzyme, 500µl 15mM H_2O_2 and 400µl of 50mM phosphate buffer (pH 7.0) was analyzed every 10 seconds for 5 minutes at 240 nm. One unit of catalase activity was defined as the amount of enzyme that decomposed 1mM H_2O_2 per min per mg at room temperature. The activity was expressed as units per mg protein (Bhargava *et al.*, 2014).

Volume activity (U/mL)= (ΔAs-ΔAB)X Total Reaction Volume(mL) X df / 0.0436 X Sample Volume(mL)	
CAT activity per mg protein= **Volume activity**/ Concentration of protein (mg/mL)	
0.0436	Extinction coefficient of H_2O_2 at 240nm
df	Dilution factor
ΔA_{240} /min = A240@1min-A240@5min/4min	

3.3.8 Oxidative stress and quorum sensing in *S*. Typhi

Salmonella Typhi cell free extract (CFE) was used for quorum sensing studies. The *S*.Typhi AI-2 molecule in the extract was detected colorimetrically using a method described previously by Wattanavanitchakorn *et al* (Wattanavanitchakorn *et al*., 2014). *S*.Typhi isolates and its quorum-sensing mutant (*luxS*::Km) was grown in presence and absence of cell free extract at 37°C on shaker for 12 hours. Enzyme assays for SOD and catalase were performed as described earlier in this study. The survival of *S*.Typhi and its quorum sensing mutant was also checked in the presence of H_2O_2. The cells were exposed to150mM H_2O_2 for 20 minutes at 37°C followed by treatment with 4% sodium thiosulfate. The viable cells were then enumerated using 0.005% TTC dye and as CFU by spreading appropriate dilutions on LB agar plates, and the percent survival was calculated as the ratio of CFU in treated to the CFU in untreated culture (Bhargava *et al.*, 2014). The changes in antioxidant enzymes and survival to oxidative stress were also estimated for the *luxS*::Km mutant complemented with the wild-type *luxS* gene.

3.3.9 Estimating persister cell population in response to bile

To determine antibiotic induced persister cell occurrence, log phase cultures of *S*.Typhi were treated with antibiotics (Ciprofloxacin and ampicillin) at 37°C. For time-dependent assay, samples were withdrawn at various time intervals (0, 2, 4, 8, 12, 16, 24 h) following antibiotic challenge (MIC), serially diluted and spread on LB agar plates for persister cell counts. The effect of bile on persister cell formation was analyzed by growing *S*. Typhi in presence of sub-lethal bile concentration, followed by challenge with antibiotic as described above. The appropriate dilution of cells was then spread on LB agar plates to determine the surviving persister cells (Herna´ndez *et al*., 2012; Bhargava *et al.*, 2014).

3.3.10 Biofilm formation

Pure culture biofilms were formed by inoculating overnight grown culture of *S*. Typhi and its quorum sensing mutant into polystyrene well plates containing LB media supplemented with varying (0%, 1%, 1.5 %, 2.5%) bile concentrations followed by crystal violet staining assay (Crawford *et al*., 2008). The organisms were also grown in presence and absence of *S*. Typhi cell free extract. The *luxS*::Km mutant complemented with the wild-type *luxS* gene was used to check if the phenotype was reversed to the original form.

(c) Significance of co-infection during adaptation in gallbladder

3.3.11 Plasmid construction and gene deletions

The promoter region of *rck* operon lies between the ORF6 and the pefI (8101bp to 8941bp) of *S.* Typhimurium (Gene bank accession no. L08613) (Michael et al., 2001) which was amplified using the primers mentioned in **Table 3.2** bearing the EcoRI and BamHI restriction sites. Simultaneously, the eGFP fragment was cleaved from the pEGFP N3 (mammalian expression plasmid) using XbaI and BamHI. The amplified fragments and the cleaved eGFP fragment were run on 1.5% agarose gel followed by extraction using Qiagen QiaQuick Gel Extraction Kit as described in previous chapter. The linear fragment obtained was further digested using EcoRI and BamHI followed by ligation with the eGFP fragment to obtain the P_{rck}-eGFP transcriptional fusion. The fragment was then cloned into pUC18 that was previously cleaved using the restriction enzymes EcoRI and XbaI. The resulting product was then run on 0.8% agarose gel and extracted using Qiagen QiaQuick Gel Extraction Kit. The insertion in the extracted recombinant plasmid was confirmed by performing a PCR using the primers for P_{rck}-eGFP to obtain a ~1500bp amplified product that was visualized using 1% agarose gel. The *rck* promoter is unidirectional, and the directional cloning approach used in this construction ensures the proper orientation of the transcriptional fusion in to the candidate expression vector. The plasmid was then propagated in *E.coli DH5α* and transformed into *S.* Typhi (Clinical isolate T1 and its *sdiA* mutant) for expression analysis. The *sdiA*::kan deletion was carried out using λ-red mutagenesis (described in section 3.3.2) with primer pairs mentioned in **Table 3.2**.

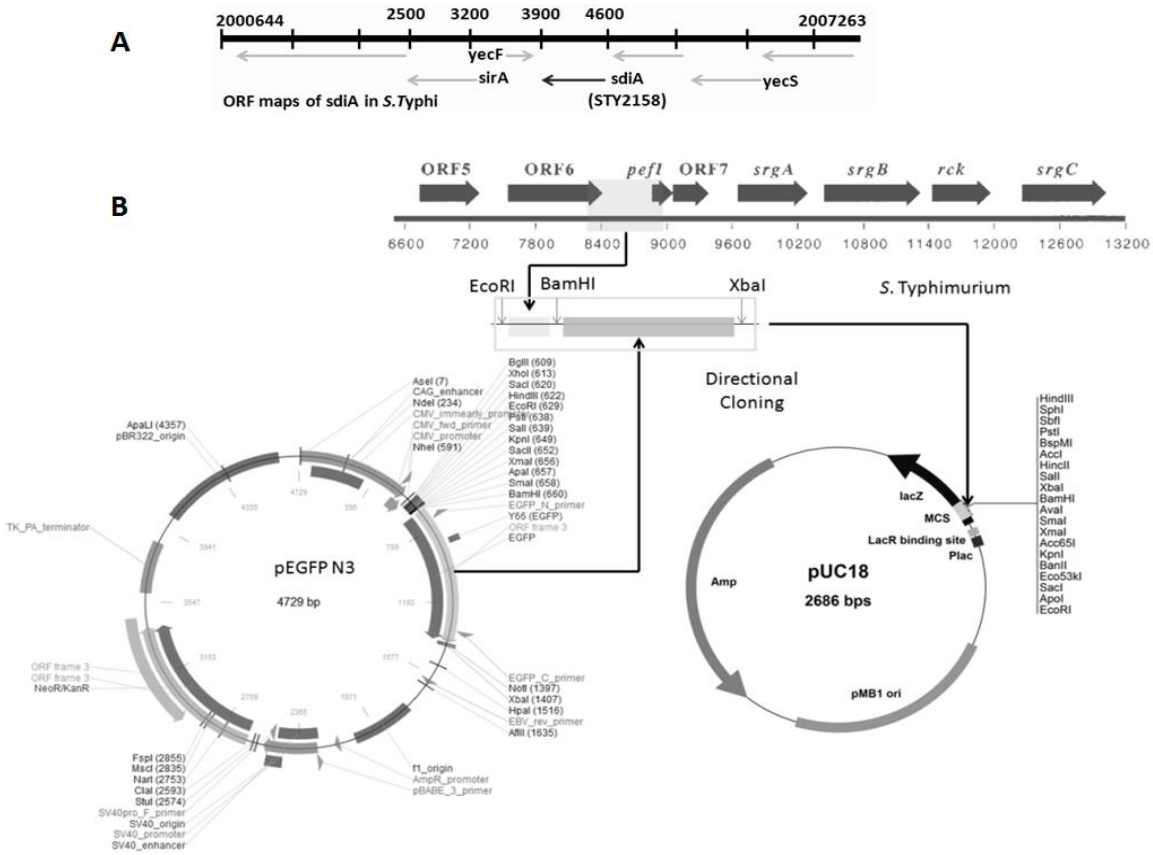


Figure 3.3 Construction of P_{rck}-eGFP transcriptional fusion. A represents the ORF map of the *sdiA* gene in *S.* Typhi. **B** shows the construction of the P_{rck}-eGFP transcriptional fusion using directional cloning approach.

3.3.12 Characterisation of P_{rck}-eGFP whole cell biosensor

For extraction of AHL's we use a method previously described by Kumar et al with a few modifications. Briefly, *P. aeruginosa* was grown overnight (stationary phase cultures are preferred) at room temperature followed by centrifugation at 6000 x g for 10 minutes. The supernatant was transferred to a new tube and equal volume of 0.5% acetic acid was added. The solvent was then evaporated and the residue was dissolved in milliQ water (stored at -20°C until analyzed).

The P_{rck}-eGFP transcriptional fusion was characterized based on its ability to detect and respond to AHL molecules within the environment. The reporter strains were grown till a OD of ~0.4 followed by addition of extracted AHL. The bacteria were then allowed to grow for 1 hour followed by measurement of fluorescent intensity against the OD at 600nm. Different dilutions of the AHL solution (1:2, 1:3, 1:4, and 1:5) were used to test the response of the biosensor to varying concentrations of AHL. For analysing the *sdiA* expression in response to AHL, the RNA was extracted from the bacteria as described

earlier followed by PCR using the primers mentioned in **Table 3.2** for *sdiA* gene. The concentration of amplified product was estimated using Nandrop spectrophotometer and equal concentration was loaded in to wells of 1.5% agarose gel. The changes in band intensity were estimated using ImageJ software which was assumed to be direct measure of candidate gene expression. Also an SDS-PAGE analysis was performed to determine the change in concentration of the eGFP protein in bacterial lysates (intracellular extracts were prepared followed by SDS-PAGE as described previously in section 2.3.9). (Walawalkar YD et al. 2013)

3.3.13 Determine *S.* Typhi existence in a mixed population using biosensor

To check the effectiveness of the biosensor in mixed bacterial populations, the reporter *S.* Typhi strain was co-cultivated with other organisms (like *K. pneumoniae* or *S. aureus* or *P. aeruginosa* or *E.coli or Enterobacter* spp. or *Vibrio* spp.). Equal volumes of both the co-cultivating organisms (each having a cell density of 10^8CFU/ml) were inoculated in LB media and incubated overnight at 37°C on shaker. In addition the response of the reporter strain to bile was also studied by supplementing the media with bile alone (sub-lethal concentration). This was followed by fluorescence intensity measurement with excitation wavelength at 488nm and emission at 509nm, and subsequent plating on ampicillin (100µg/ml) plates to determine the *S.* Typhi CFU/ml in mixed bacterial population. For mixed-culture biofilms, the organisms were grown in polystyrene tubes coated with cellulose for 48 hours. The loosely adherent cells were removed by three washing with PBS (pH 7.0), and the firmly adhered biofilm cells were then extracted alternating cycles of vortexing (20 seconds) and sonication (4 cycles of 30 seconds on and 30 seconds off) with sonicator probe outside the tube in a water bath. The fluorescence intensity was then determined, and the viable cell counts of recovered *S.* Typhi was estimated by plating on LB agar plates with ampicillin (100µg/ml). Also, the effect of bile on *S.* Typhi in mixed-culture biofilm was evaluated by supplementing the media with 3% bile where necessary. The co-existence of other organism used (in medium and biofilms) was detected by plating samples on LB agar (morphological differences and total colony counts) and using fluorescence microscopy. (Bhargava *et al.,* 2014)

3.3.14 Significance of *sdiA* in mediating antibiotic resistance

Disk diffusion assay (as per CLSI, formerly the National Committee for Clinical Laboratory Standards or NCCLS) was performed using antibiotic sensitivity discs (Himedia) for chloramphenicol (30µg), ampicillin (10µg), trimethoprem-sulfamethoxazole (TMP-SMZ) (5µg), ceftriaxone (30µg), ciprofloxacin (5µg), ofloxacin (5µg) and nalidixic acid (30µg). Briefly, *S.* Typhi and its mutant strain (*sdiA*::kan) were evenly spread using a sterile swab on Mueller-Hinton (MH) agar and allowed to dry for 10 minutes under aseptic conditions. The agar plates were incubated in inverted position at 37°C for 16 to 18 hours after which the zones of inhibition were identified by gross visual inspection and the diameters were recorded to the nearest mm.

3.3.15 Statistical analysis

Data is represented as means ± standard deviations with at least three biological replicates. An unpaired t-test was used to determine the significance and a two-tailed p-value of <0.05 was considered to be statistically significant.

3.4 Results and discussion

3.4.1 Adaptation and pre-adaptation of *S.* Typhi to bile

Salmonella Typhi isolates shows a constant level of resistance to human bile, which can be enhanced by adapting the organism to lower concentration of bile as shown in **Figure 3.2**. To determine the concentrations which permit *S.* Typhi growth in high bile concentrations, organisms were grown up to their MIC (1% to 6%) of bile and then transferred to increasing concentrations of bile above MIC. As depicted by **Figure 3.2A**, *S.* Typhi initially growing in 3%-5% of bile was able to tolerate higher concentrations of bile. The *S.* Typhi strains that tolerated lethal bile concentration were transferred to LB media (without bile) and grown overnight. Aliquots from these were used to determine the MIC of bile. The resistance for all isolates, except for the one tolerating 5% bile initially (MIC increased to 7%) were reduced back to original characteristics of *S.* Typhi isolates. The increase in MIC may possibly be due to permanent adaptation of few organisms to slightly higher concentration (6%) of bile. Thus *S.* Typhi adaptation to bile is reversible and indicates ability to possess mutants which can be isolated and studied further. These studies were on par with some of the

previous findings reported by Hernández et al. in 2012, where they even show a transcriptome analysis of *S. enterica* grown in presence of bile. They reveal that the epigentic-type modification may be attributed to alterations in expressions of genes having roles in stress response (rpoS and cspD), membrane architecture (ompC and ompD) and efflux systems (arcD) and *Salmonella* pathogenicity (SP-1 and SP-2). The authors also identify mutations in certain genes like yrbK and rlbB (involved LPS transport) of permanent bile-resistant mutants that may boost bile-resistance. (Hernández et al, 2012)

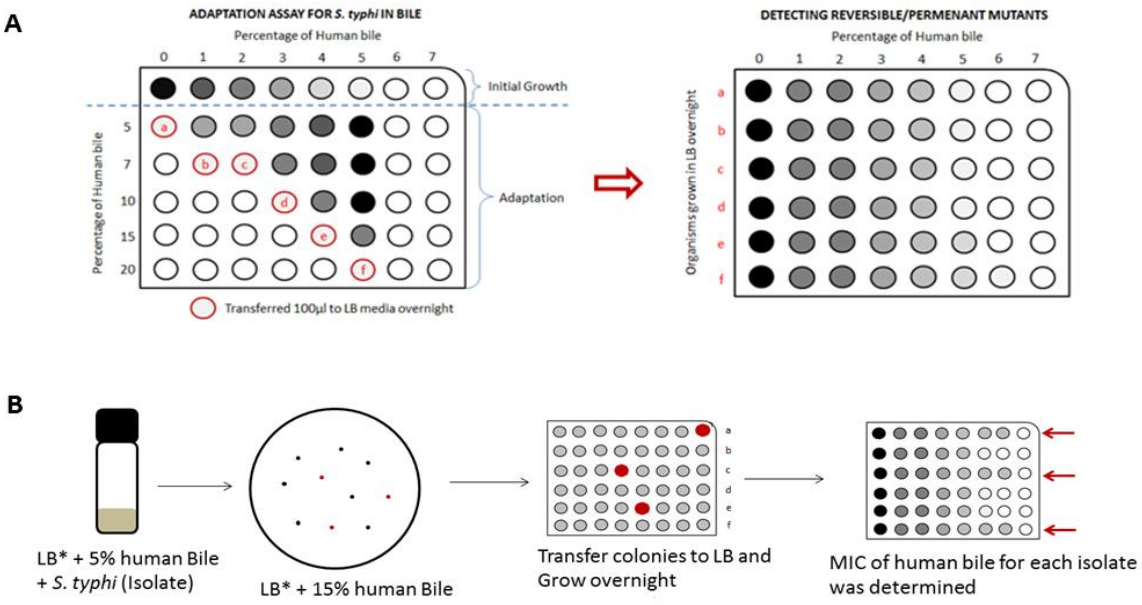


Figure 3.4 Adaptation of *S.* Typhi to bile. Adaptation assay was performed to isolate bile resistant colonies. (**A**) Determining the change in MIC for *S.* Typhi pre-adapted to concentration of bile up to their MIC. The *S.* Typhi cells able to tolerate high concentration of bile were checked for reversible nature by growing in LB without the selective agent and rechecking their MIC. (**B**) Detecting the occurrence of bile-resistant cells (Stable and unstable) using Luria-Delbruk's fluctuation test for *S.* Typhi isolates. The analysis was performed by exposing *S.* Typhi to lethal bile concentration with and without prior adaptation to low bile concentration.

	***S.* Typhi grown in LB**	***S.* Typhi grown in LB+bile**		
	Separate culture tubes	**Single culture tube**	**Separate culture tubes**	**Single culture tube**
No. of samples	20	20	20	20
Mean	18.3	8.2	56.15	36.85
Variance	556.43	110.58	53.6	39.42
Occurrence of bile-resistance colonies	4.5×10^{-10}	3.9×10^{-10}	2.8×10^{-6}	1.7×10^{-6}

Table 3.3 Fluctuation analysis of bile-resistant colonies.

Bile-resistance can be acquired by mutations which may be inherited or induced. Cells with inherited mutations of spontaneous origin (those which are not involved in loss of function) can easily be recovered by plating them on lethal concentrations of bile (Luria & Delbruck, 1943; Hernández et al, 2012) **(Figure 3.2B)**. For *S.* Typhi growing initially in LB and plated on lethal concentration of bile, the fluctuation analysis revealed a higher variance in occurrence of bile resistant colonies across different culture tubes as compared to the variance obtained within one single culture tube. When *S.* Typhi was adapted to a concentration of bile determined from MIC, the bile resistant colonies appeared at a similar frequency with minimal variance between independent and single culture. The number of survivors was distributed in a way where the mean was almost equal to the variance satisfying the Poisson distribution. The frequency of occurrence of bile resistant colonies in *S.* Typhi cultures was much lower (10^{-10}) when compared to the bile-adapted cultures of *S.* Typhi (10^{-6}) suggesting the role of bile in modulating bacterial persistence **(Table 3.2)**. Upon non selective growth of these bile resistant colonies in LB alone followed by a MIC assay to bile, it was observed that many of these were unstable mutants in case of bile adapted *S.* Typhi with only a few in actual *S.* Typhi cultures. Thus, inherited bile resistance may be mostly permanent (stable) whereas initial exposure to lower bile concentrations may adapt the organisms to survive in bile-rich environment only till the selective agent is present (unstable). The variance and frequency of stable bile resistant colonies also followed a similar phenomenon as described above in both cases. **Figure 3.3** characterizes the bile resistant colonies based on their resistance to bile **(Figure 3.3A)** and further depicts ability of the bile resistant colonies to tolerate varying bile concentrations **(Figure 3.3B)**. Also the bile resistant colonies can successfully grow in varying bile concentrations as shown in **Figure 3.4.** This justifies some of the previous

studies reporting that the bacteria can not only tolerate but also grow in bile by undergoing temporary or permanent changes in its behaviour (Begley et al., 2005).

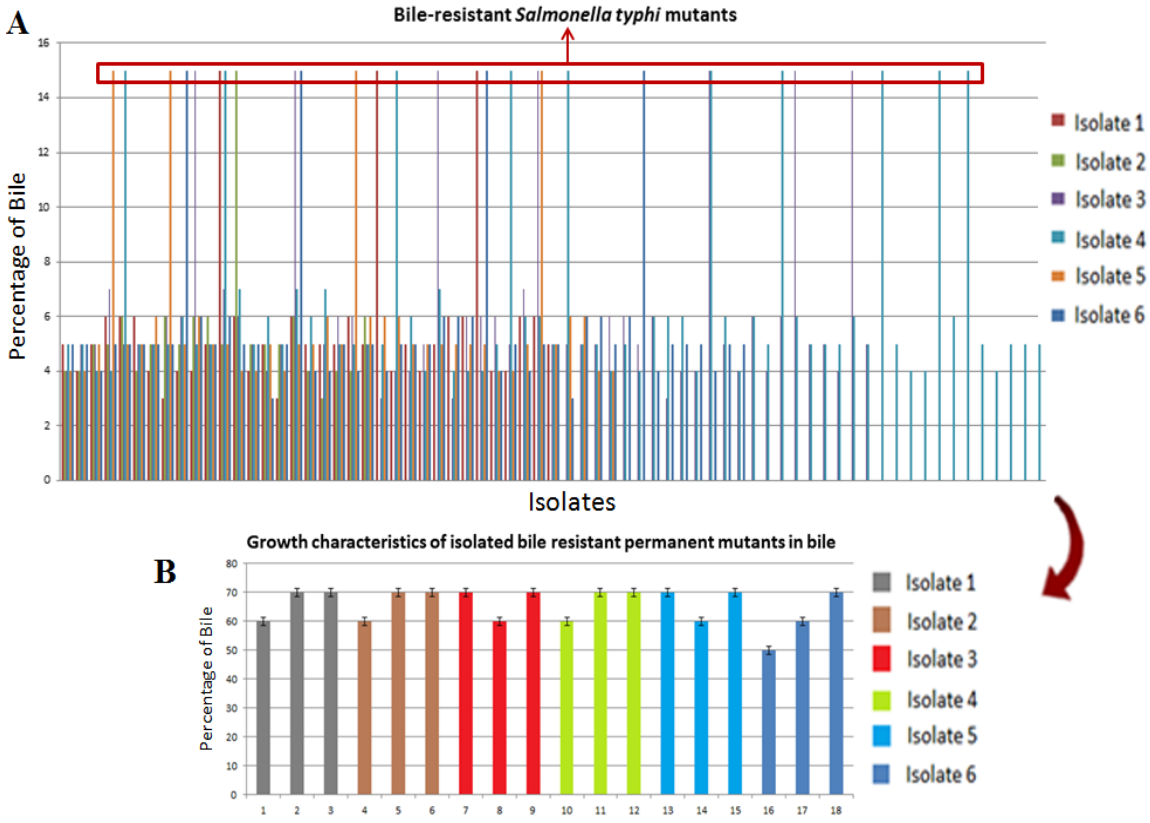


Figure 3.5 Characterising the stable bile resistant *S.* Typhi colonies. The stable bile resistant colonies obtained for each isolate upon pre-adaptation to bile was estimated. **A** represents of stable bile resistant colonies obtained for each isolate as determined from their MIC to bile. **B** shows the ability of the bile-resistant colony to tolerate varying concentration of bile**.** *(Isolate 6: control strain CT18)

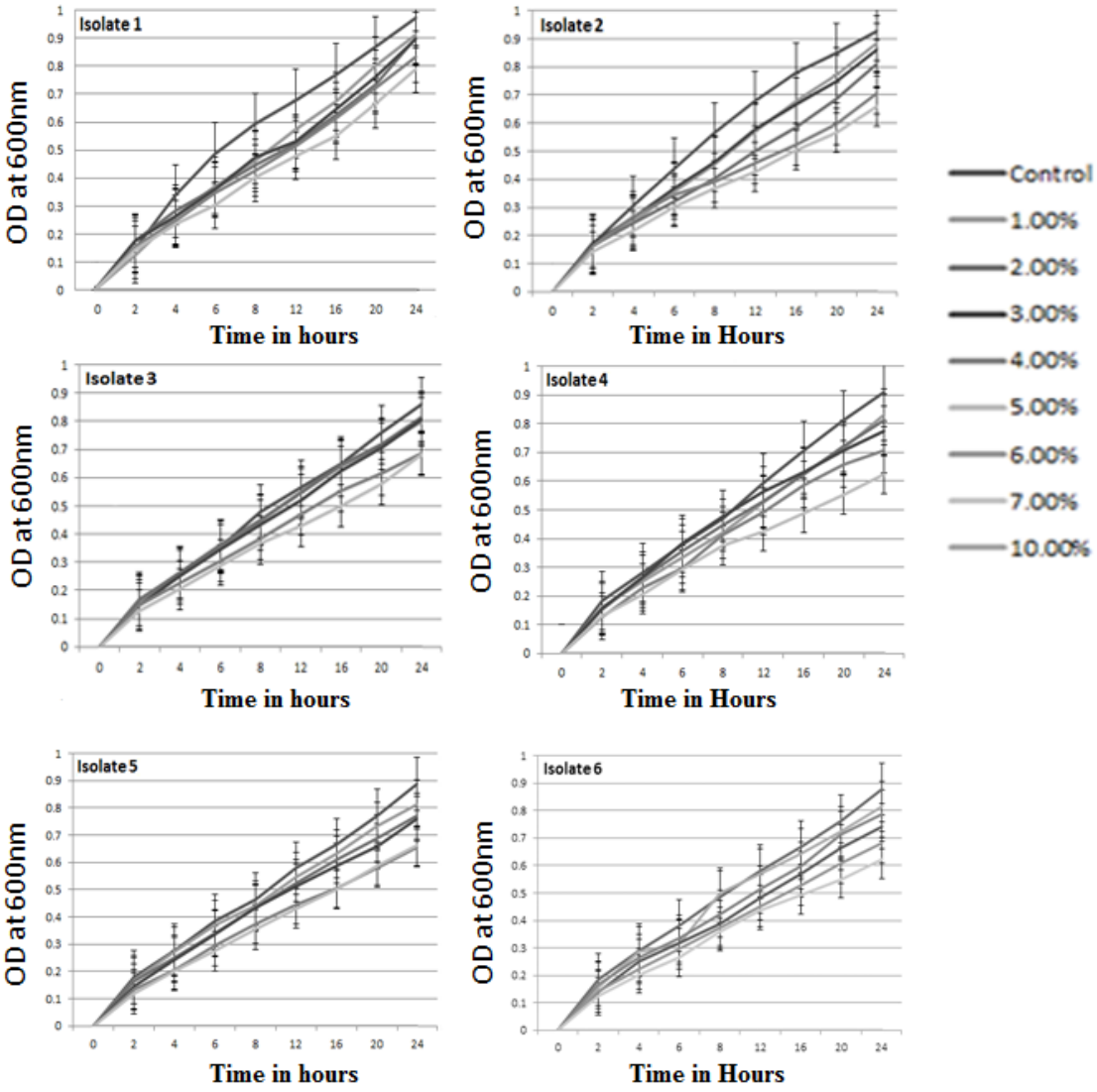


Figure 3.6 Growth of bile-resistant *S.* Typhi in varying bile concentrations. The growth of stable bile-resistant mutants from different isolates (isolates 1-6) was monitored in the presence of higher bile concentrations. *(Isolate 6: control strain)

3.4.2 Synthesis and detection of quorum sensing molecule AI-2 in *S.* Typhi Several factors affect the production of extracellular AI-2 in bacteria. We determine the stage in bacterial growth phase which is optimum for AI-2 production using culture supernatants of selective Gram positive and Gram *negative* organisms. It was observed that organisms grown in PW media significantly ($p<0.05$) produce AI-2 molecules during the late-log phase to early stationary phase of their growth **(Figure 3.5)**. The optimum time for AI-2 production was between 8-12 hrs for all the organisms, before and after which the level of AI-2 dropped significantly. These findings were almost similar to some of the earlier studies which report that optimum AI-2 production occurs between the mid-log to early-stationary phase and that it varies with different bacteria

(Wattanavanitchakorn *et al*., 2014).. Thus the cell density and media components play a key role for AI-2 production in bacteria.

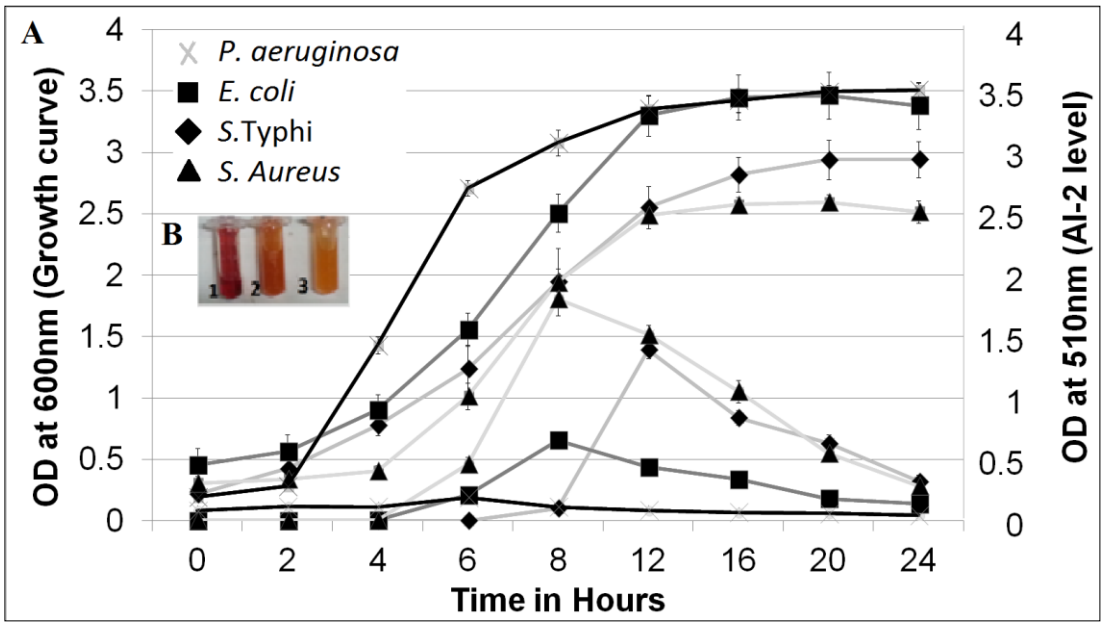


Figure 3.7 Bacterial growth characteristic for optimal AI-2 production. A represents the growth curve of different organisms in PW media and the respective AI-2 molecules produced and detected calorimetrically. B shows the orange-red colour developed during the assay as a response to AI-2 molecules. **Tube 1** shows the positive control (Ascorbic acid); **Tube 2** represents *S.* Typhi bacterial supernatant; **Tube 3** shows negative media control. The data are means of three independent biological replicates with SD and SE.

For detection of AI-2, we adopt a method previously developed by Wattanavanitchakorn et al., which ensures that reduction of metal ions is brought about by AI-2 alone and not by any other potential interfering agents or metabolites (Wattanavanitchakorn *et al*., 2014). It was seen from our study that *S. aureus* showed a ~10-fold increase compared to ~9 and ~6 fold increase shown by *S.* Typhi and *E.coli* respectively **(Figure 3.6)**, a similar change observed in previous report by Wattanavanitchakorn et al. **(Table 3.4)**. The lower production of AI-2 by respective bacterium in our study may be attributed to the use of cell free supernatant which has been shown contain lower AI-2 level compared to the colony rinses.

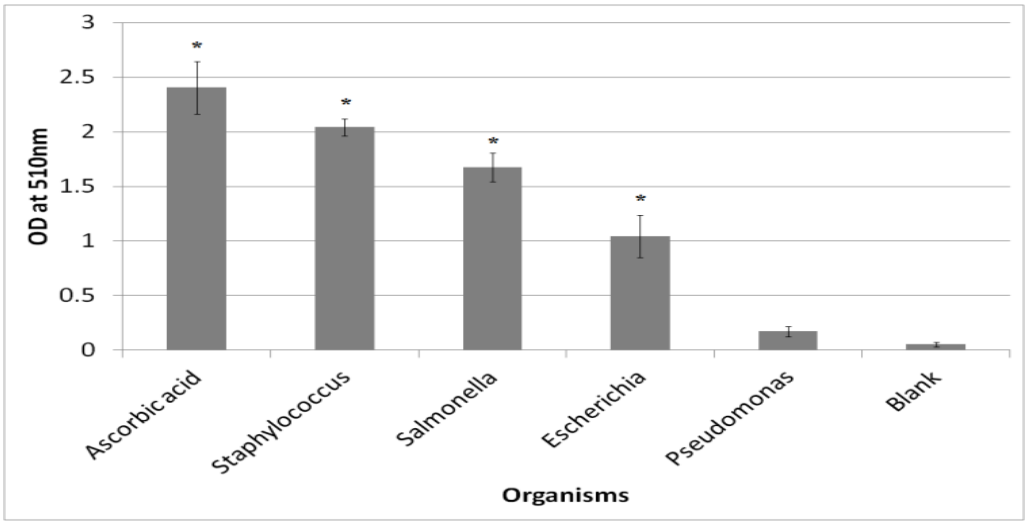


Figure 3.8 Colorimetric detection of AI-2 molecules in bacterial supernatants. The AI-2 produced by various organisms as determined by colorimetric estimation. The data are means of three independent biological replicates with *p value <0.05.

AI-2	Bacteria	Colorimetric detection (OD at 510nm)						Chemosensor detection (F.U.)	
		Previous report by Wattanavanitchakorn *et al.*				Present study			
		CFE	Fold change	Colony rinse	Fold change	CFE	Fold change	CFE	Fold change
+	*Staphylococcus*	NA	NA	0.33 ± 0.005	33	2.04 ± 0.079	12	343.38	13
+	*Salmonella*	0.12 ± 0.01	12	0.30 ± 0.008	30	1.68 ± 0.134	9.88	336.24	12
+	*Escherischia*	NA	NA	0.19 ± 0.007	19	1.04 ± 0.194	6.11	292.67	11
-	*Negative control*	0.00 ± 0.000	1	0.00 ± 0.000	1	0.17 ± 0.047	1	30.61	1

Table 3.4 Comparative analysis of methods used to detect AI-2 molecules

Since *Pseudomonas* does not possess the *luxS* gene and does not produce the AI-2 molecules, it was used as a negative control for our experiments. As an alternative method for detection of such molecules in biological systems, we directly test the applicability of the ‘‘turn-on’’ fluorescent chemodosimeter previously developed by Chatterjee and group (Khandare et al. 2013). This is because the sensor has been shown to be cost-effective and selective, with high sensitivity for detection of target molecules. . For this purpose, all the bacteria were grown in PW media till their late-log to early-stationary phase and the culture supernatant (as described above) were used directly for detection. The sensor successfully detected AI-2 in these cell-free supernatants in a pattern similar to that shown during colorimetric detection, illustrating good performance of the proposed sensing system **(Figure 3.7A)** in qualitative estimation. It was observed that *S. aureus* produced highest level of AI-2 with *E.coli* being least producer. The *S.* Typhi gallbladder isolate was also shown to produce AI-2. Considering the difficulties in obtaining the standard AI-2 molecules and assuming that

AI-2 is nearly similar to ascorbic acid in both structure and reducing ability (Wattanavanitchakorn *et al*., 2014), we further quantify the AI-2 levels in the samples using ascorbic acid as a standard. The AI-2 concentration was estimated in relation to the ascorbic acid concentration as shown in **Figure 3.7B**.

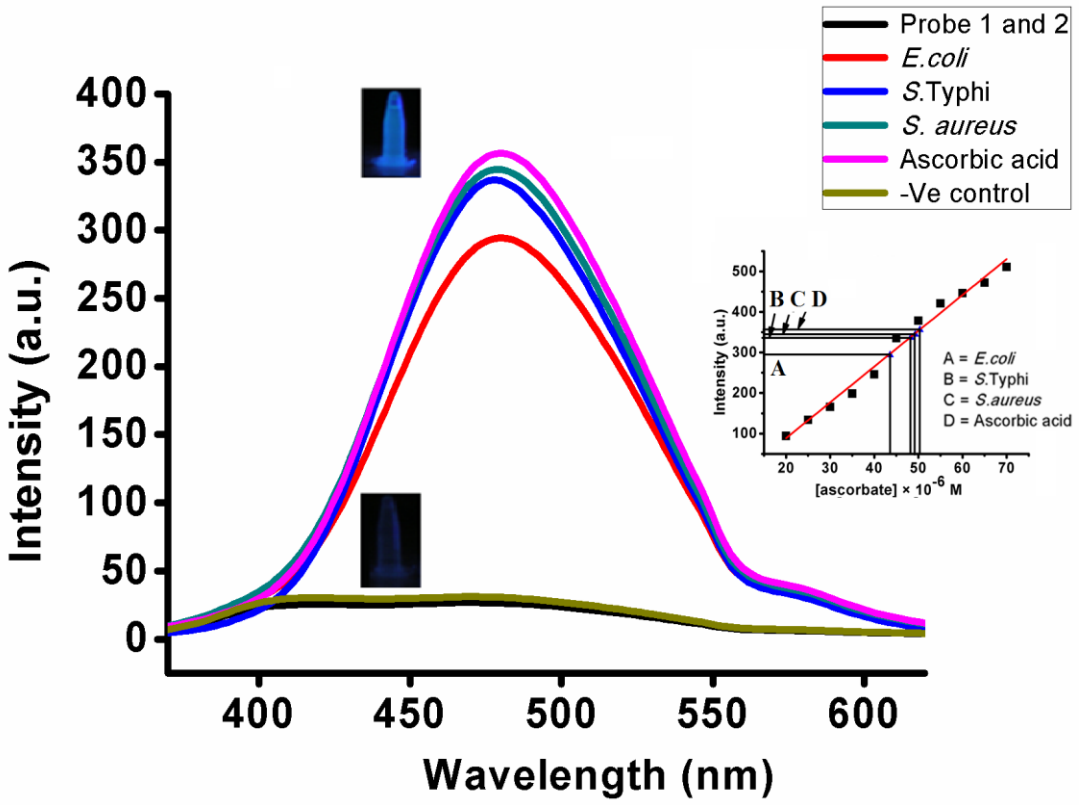


Figure 3.9 Fluorimetric estimation and quantification of AI-2 molecules in bacterial supernatants. **A** represents response of probe 1 and 2 upon addition of bacterial supernatants. **B** shows the probable concentration of AI-2 ions in bacterial supernatants extrapolated against the standard ascorbic acid curve.

3.4.3 Response of *S*. Typhi to bile

Bile is a yellow green viscous liquid produced by the liver and stored in the gallbladder. Along with its ability to digest fats, bile is also known for its bactericidal activity. Since *S*. Typhi is shown to chronically persist within the gallbladder (Nath et al., 2010), the effect of bile on the growth of *S*. Typhi was checked, and the MIC of bile was found to be 4% with sub-lethal concentration taken as 3%. *S*. Typhi clinical isolates grown in the presence of sub lethal bile concentration showed growth inhibition ranging from 4-14% **(Figure 3.8)**, indicating *S*. Typhi could survive bile stress. Also the presence of bile induced ROS generation within *S*. Typhi which led to a >2 fold-higher ($p<0.001$) fluorescence in bile-treated cells compared to untreated cells stained with DCFDA **(Figure 3.9)**.

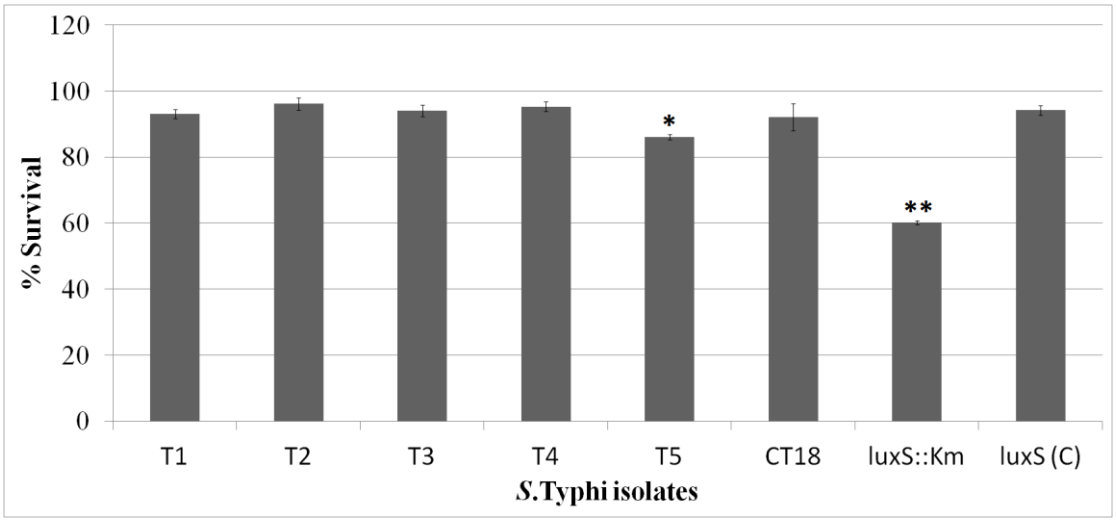


Figure 3.10 Survival profile of *S*.Typhi isolates in presence of sub-lethal bile. Survival profile of *S*.Typhi isolates in presence of sub-lethal concentration of bile (3%). Profiles for Clinical isolates (T1-T5) obtained from gallbladder of patients undergoing cholecystectomy, CT18-standard strain, *luxS*::Km quorum sensing mutant and mutant complemented with wild-type *luxS* [*luxS*(C)] are shown. The level corresponding to 100% survival in non-treated samples varied between 1 to 9.99 X 10^{11} CFU/ml for different isolates. Data represents means of at least three biological replicates, *p<0.05, **p<0.01.

Bile is composed mostly of bile salts which have been shown to effectively induce oxidative stress within bacteria. Thus, the levels of anti-oxidants were evaluated to understand the protective mechanism adapted by *S*. Typhi during bile-stress **(Figure 3.10)**. Four clinical isolates (T1-T4) including CT18 showed a > 2-fold and >1.6 fold increase in SOD ($p<0.05$) and catalase ($p<0.001$) activities respectively. The T5 isolate showed a significant 2-fold increase in SOD activity ($p<0.05$) with no significant change in the catalase activity **(Figure 3.10 A&B)**, thus reflecting a significant growth inhibition (14%) in presence of bile compared to other clinical isolates of *S*. Typhi. Also the gene expression analysis justified the above observation revealing up-regulated expression of anti-oxidative enzymes as a response to bile stress **(Figure 3 C&D)**.

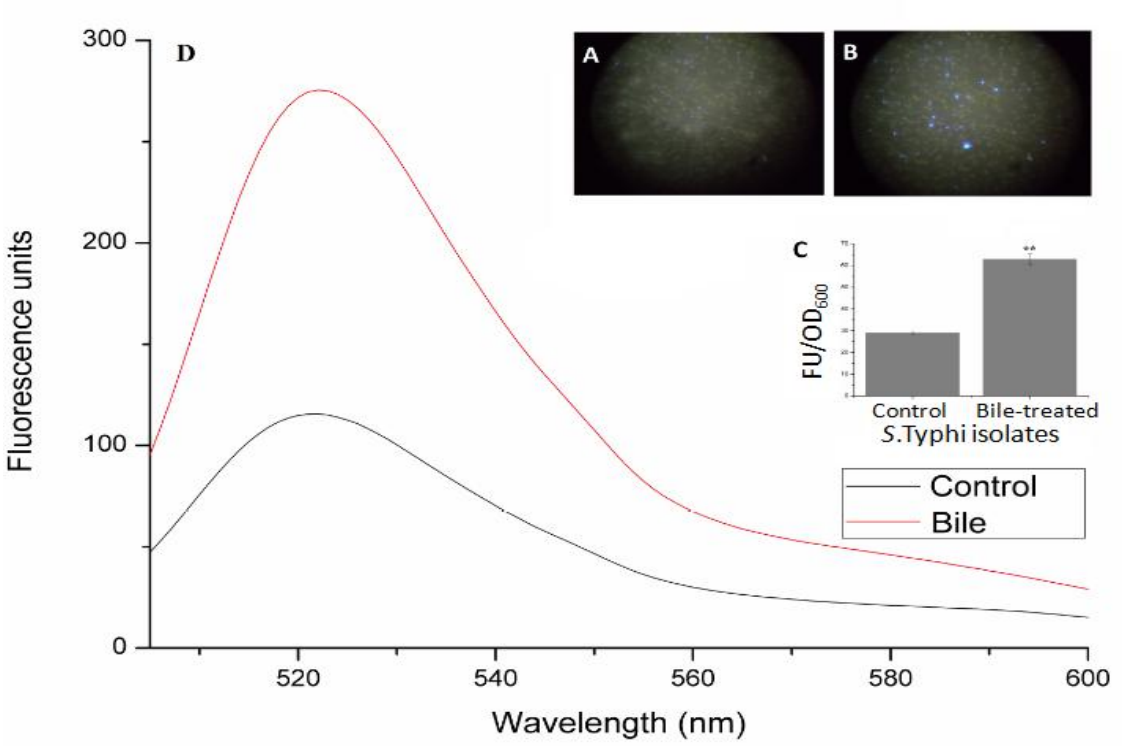


Figure 3.11 ROS generation in *S.* Typhi as a response to bile stress. A and **B** show the fluorescence images of DCFDA-stained *S.* Typhi in presence and absence of bile respectively. **C** and **D** represent the fluorescence quantification data in non-treated and bile-treated samples. Data represents means of at least three biological replicates, *p<0.001.

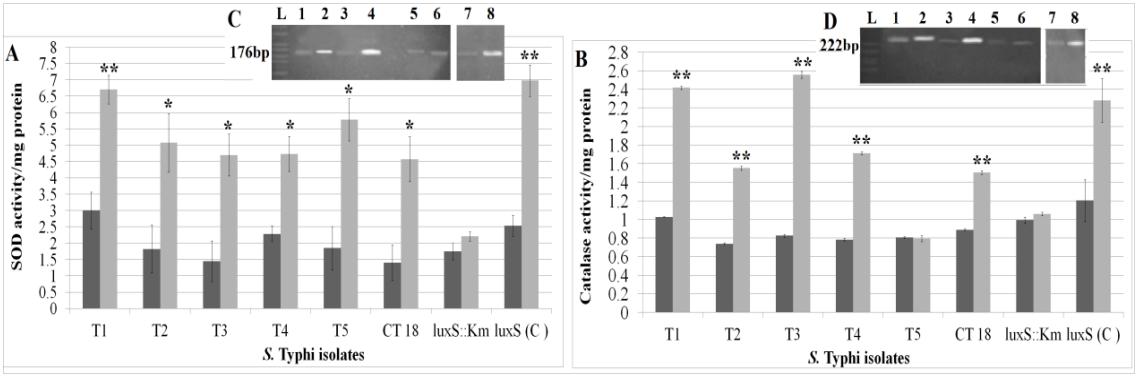


Figure 3.12 SOD and catalase activities in *S.*Typhi isolates. SOD and catalase activities in *S.*Typhi isolates. The changes in SOD (a) and catalase (b) activity was analyzed in T1-T5 clinical isolates, CT18, *luxS*::Km mutant and mutant complemented with wild-type *luxS* [*luxS*(C)] in presence (▒) and absence (■) of bile. Data represents means of at least three biological replicates, *p<0.05, **p<0.001.The relative change in gene expression of these enzymes was also checked using reverse transcriptase PCR assay (c and d) for CT18 (lane 1 and 2), gallbladder clinical isolate (lane 3 and 4) and *luxS*::Km QS- mutant (lane 4 and 6) in presence (lane 2, 4, 6) and absence of bile (lane 1, 3, 5) respectively. (L: Genedirex 50bp DNA ladder).

3.4.4 Biofilm formation by *S*. Typhi during bile stress

Bile is shown to influence biofilm formation in pathogenic bacteria and some indigenous commensals (Crawford et al., 2008; Begley et al., 2009). Certain bacteria including *Salmonella* show significantly enhanced ability to form biofilm in response to bile acids. It is also reported that biofilm formation is partially dependent on quorum sensing signalling molecule AI-2 in *Salmonella Typhimurium* (Yoon and Sofos, 2008; Jesudhasan et al. 2010). All the isolates used in this study adhered well to the polystyrene-plates and showed significant biofilm formation in presence of bile (data not shown). After 16 hour incubation, it was seen that the biofilm forming ability was impaired in *luxS*::Km mutant compared to CT18 which showed significantly higher biofilm mass (>1.2-fold) at all bile concentrations **(Figure 3.11)**. The biofilm formation was dependent on concentration of bile in both CT18 and *luxS*::Km mutant, with a >1.2 fold increase ($p<0.05$) at sub-lethal concentration compared to biofilms formed in absence of bile. Interestingly, complementing the media with exogenous cell free extract (containing the AI-2 molecules) did not significantly affect biofilm formation in the *luxS*::Km mutant. Thus disruption of the *luxS* coding sequence may imply interference with the MicA expression, a small non-coding RNA molecule essential for proper biofilm formation in *Salmonella* (Kint et al., 2010) **(Figure 3.12)**. Furthermore, complementing the mutant with the wild-type *luxS* gene restored the wild-type mature-biofilm forming ability.

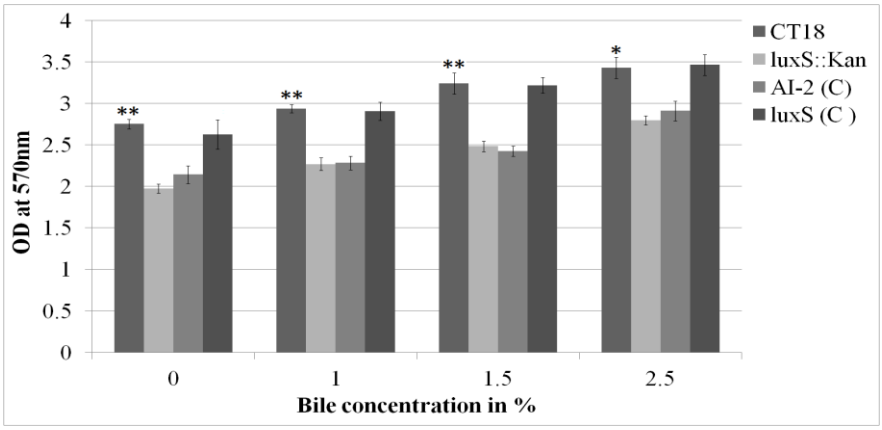


Figure 3.13: QS-system in regulating the biofilm formation in *S*. Typhi. QS-system in regulating the biofilm formation in *S*.Typhi. The biofilm formed by CT18 and *luxS*::Km mutant was quantified using crystal violet assay in presence and absence of varying bile concentration. The mutant was complemented with exogenous wild-type *S*.Typhi CFE [AI-2 (C)] and wild-type *luxS* gene [*luxS* (C)] to demonstrate any changes in its ability to form biofilms in vitro. Data represents means of at least three biological replicates, *$p<0.05$, **$p<0.01$.

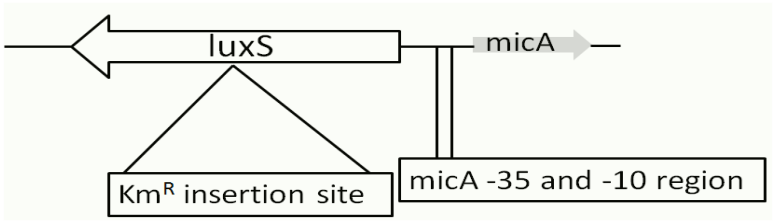


Figure 3.14: ORF map of the *luxS* gene in *S*. Typhi

3.4.5 Role of quorum sensing in *S*. Typhi during bile-stress

Bile induces generation of ROS in *S.* Typhi affecting the viability and growth of the organism. The organism counters this stress effect by enhanced production of anti-oxidant enzymes namely SOD and catalase. Bile has been reported to significantly up-regulate the expression of these enzymes (Herna´ndez *et al*., 2012). Interestingly, the production of anti-oxidant enzymes is shown to be dependent on quorum-sensing system in certain bacteria (Hassett *et al*., 1999; Bhargava *et al.*, 2014). Thus, in the present study we reveal for the first time that the *S.* Typhi quorum-sensing machinery is necessary for efficient production of SOD and catalase in response to bile stress.

The quorum sensing mutant (*luxS*::Km) showed 40% growth inhibition in response to bile-stress when compared to its control CT18 which showed 8% inhibition **(Figure 3.8)**. Complementing the mutant with the wild-type *luxS* restored its resistance to bile with nearly 6% growth inhibition. Viable cell counts on exposure to H_2O_2, for CT18 (5.54±0.16 log CFU/ml) and *luxS*::Km (3.22±0.1 log CFU/ml), showed 68 and 39% survival compared to the unexposed cells (8.22±0.1 log CFU/ml was taken as 100%). This was also confirmed by a TTC assay which showed >3 fold-higher ($p<0.001$) survival in CT18 compared to the quorum sensing mutant.

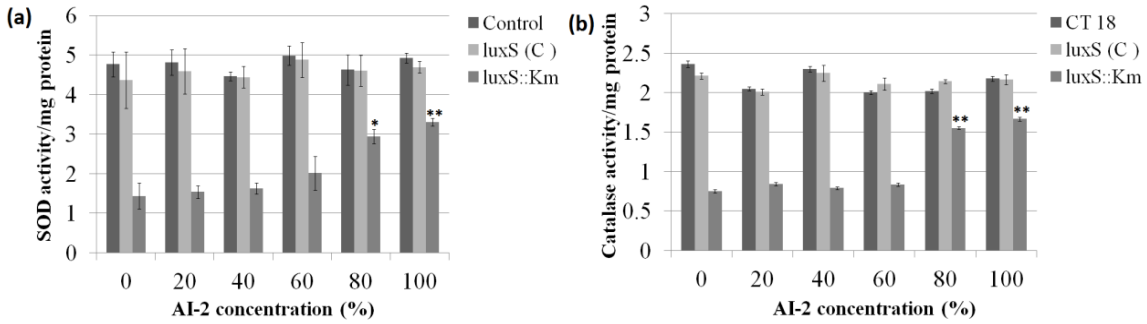


Figure 3.15: QS-system in regulating SOD and catalase activity in *S*. Typhi. (a) and catalase (b) activities in *S*. Typhi CT 18, the QS-mutant *luxS*::Km and mutant complemented with wild-type *luxS* gene was estimated in presence of varying *S*. Typhi CFE concentrations containing the AI-2 molecule. Data represents means of at least three biological replicates, *$p<0.05$, **$p<0.001$.

On exposure to bile, the SOD and catalase activity in *luxS*::Km mutant did not show a significant increase compared to other isolates that showed a >2-fold increase ($p<0.05$ and $p<0.001$ respectively) **(Figure 3.10 A&B)**, which may be the reason for increased sensitivity of the mutant to bile. This was even confirmed with gene expression analysis as shown above **(Figure 3.10 C&D)**. On adding the wild-type cell-free extract exogenously, the SOD and catalase activities of the mutant increased significantly by > 2-fold in presence of bile, although lower percentage of CFE (0-60%) was ineffective **(Figure 13 A&B)**. Both, CT18 and the mutant complemented with wild-type *luxS* gene did not show any significant alteration in both the enzymes on addition of exogenous cell-free extract, as they already harbours the functional quorum sensing system. Thus the quorum sensing system is important for expression of the SOD and catalase enzymes in *S.* Typhi during oxidative stress induced by bile. Targeting such systems and communication molecules in bacteria can sufficiently enhance the bactericidal activity of agents inducing oxidative stress in *S.* Typhi.

3.4.6 Bile-induced tolerance to antibiotics and persister cell formation

The ability of bacteria to resist bactericidal agents like bile and other antibiotics can be linked with emergence of persister cells (Hassett et al., 1999; Wood et al., 2013). These persisters possess non-heritable, epigenetic changes responsible for multidrug tolerance and chronicity of infections. Stress responses function as mediators for generation of persister cells. This study reports that *S.* Typhi shows persister cell formation in response to ciprofloxacin and ampicillin (both of which have been shown to effectively eradicate the chronic state and are also effective in our strains) (Gonzalez-Escobedo et al., 2011), which in turn differs across *S.* Typhi isolates and type of drug used. Exposure of *S.* Typhi CT18 and the clinical isolate to MICs of ciprofloxacin and ceftriaxone in a time-dependent manner showed a typical bi-phasic killing pattern with rapid decline in sensitive population followed by a plateau comprising of the antibiotic tolerant persister cells **(Figure 3.14 A&B)**. Exposure of the cells to bile-induced stress significantly increased the persister cell population by >3-fold for both ciprofloxacin and ceftriaxone. This is of clinical relevance as bile is stored within the gallbladder where it may possibly assist the persistence of *S.* Typhi leading to chronic infection with multi-drug tolerance. By growing in LB overnight, these cells were found to be sensitive to these antibiotics confirming the non-heritable nature of the persister cells as shown above.

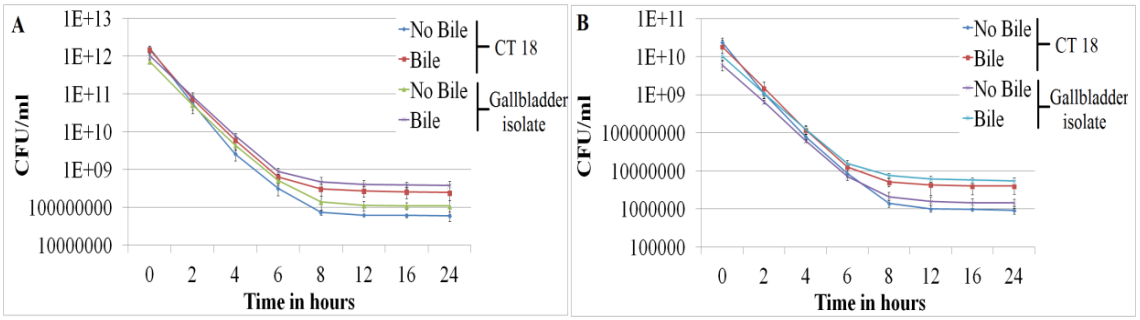


Figure 3.16 Effect of bile on *S*. Typhi persister cell population. Mid-log phase cultures of *S*. Typhi CT 18 and gallbladder clinical isolate were challenged with ciprofloxacin (**A**) and ampicillin (**B**) at MIC in presence and absence of bile. The persister cell population was determined as CFU/ml at different time intervals. Data represents means of at least three biological replicates, $p<0.05$.

3.4.7 Characterization of the P_{rck}-eGFP whole cell biosensor

Quorum-sensing in bacteria typically depends on the *luxI* to synthesize the signal molecules and *luxR* to sense it. *Salmonella, Klebsiella and Escherichia* do not produce the signal molecules but harbour a *luxR* homolog called *sdiA* which responds to AHL produced by other organisms. Chromosomal *sdiA* of *Salmonella* is upregulated as a response to exogenous AHL molecules produced by other organisms, which in turn activates the *rck* promoter (Lee et al., 2009). Thus the activity of the *rck* promoter is indirectly dependent on the amount of AHL molecules in the bacterial surrounding. We exploit this property to construct the P_{rck}-eGFP (environmentally regulated promoter-reporter fusion) containing whole-cell biosensor to detect the AHL molecules and determine the significance of *sdiA* during co-infection. We specifically amplify the region between Orf6-pefI which is shown to contain promoter activity as reported in some of the previous studies. Also, eGFP has been previously shown to be a good reporter system in transcriptional fusions with enhanced fluorescing ability (Walawalkar YD et al. 2013). The approach of directional cloning ensures the proper orientation of target genes within the construct and vectors. **Figure 3.15A** represents the visualisation of plasmids and constructs used in the construction P_{rck}-eGFP transcriptional fusion. The resulting over-expression of ~29kDa eGFP protein in induced reporter strains was confirmed by SDS-PAGE analysis as depicted in **Figure 3.15B**. The fusion was successfully transformed into *S.* Typhi CT18 strain that served as the reporter strain for our studies. The primary benefit of developing such a biosensor would be its ability to tract bacterial behaviour within mixed-bacterial population and deduce the possible role of quorum sensing mediated by *sdiA* in

bacteria. Our findings support some of the earlier studies that bacteria can co-exist in the human gallbladder (Hazrah et al. 2004; Capoor et al., 2008). Interestingly, we were even able to recover *S.* Typhi and *E.coli* from the gallbladder of the same patient. This is a first kind of report that tries to understand the significance of co-infection mediated QS in assisting *S.* Typhi persistence in the gallbladder-like environment.

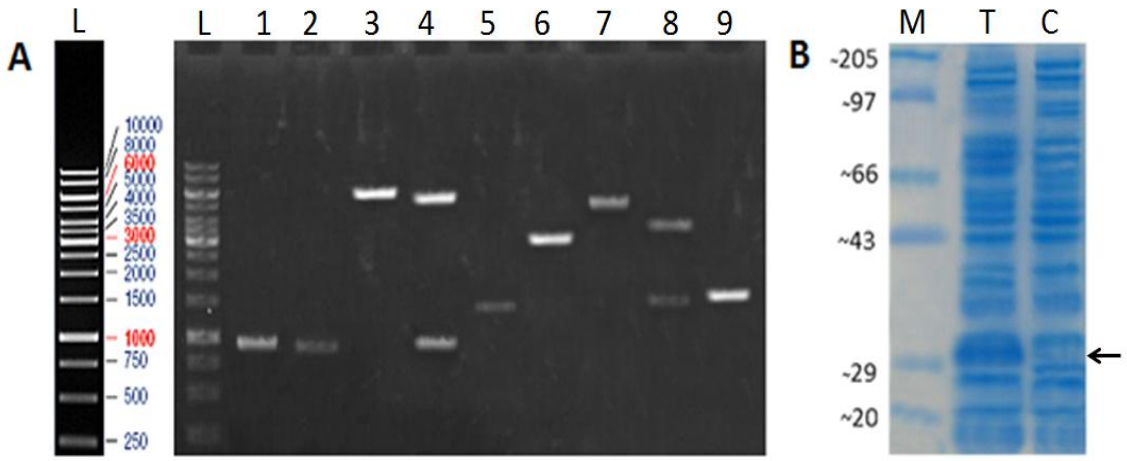


Figure 3.17 Construction of the P_{rck}-eGFP transcriptional fusion. A represents visualisation of candidate plasmids and gene fragments on 1.2% agarose gel. Lane 1: PCR amplified *rck* promoter region (8101bp to 8941bp); Lane 2: purified eGFP fragment excised from pEGFP N3 plasmid using BamHI and XbaI; Lane 3: pEGFP N3 plasmid; Lane 4: Restriction digestion of pEGFP N3; Lane 5: purified P_{rck}-eGFP fragment; Lane6: pUC plasmid; Lane 7: pUC18 vector with P_{rck}-eGFP insert; Lane 8: Plasmid digested with EcoRI and XbaI; Lane 9: PCR amplified P_{rck}-eGFP insert. **B** represents the SDS-PAGE profile of bacterial lysates. T: Test (induced); C:Control (uninduced). Arrow shows the ~29 kDa eGFP protein over-expression in induced cultures as a response P_{rck} promoter activity. L: 50bp Genedirex DNA ladder; M: Broad range protein molecular weight marker (Genetix).

We assume that the promoter senses the AHL signals produced by organisms in a concentration-dependent manner as seen in **Figure 3.16**. The fluorescence reading was normalized to the bacterial density in the medium by using the formula $G=F/OD_{600}$. It was observed that the reporter strain showed significant ($p<0.001$) fluorescence compared to its mutant (*sdiA*::kan). Thus, *sdiA* is necessary for sensing AHL molecules and to regulate the *rck* operon.

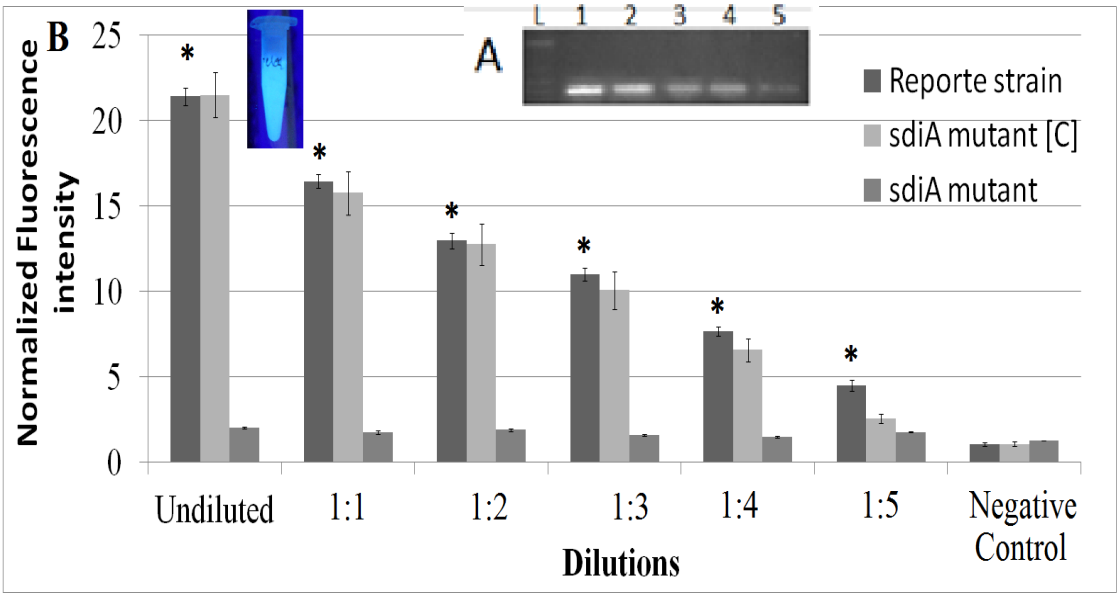


Figure 3.18 Response of P_{rck}-eGFP transcriptional fusion to AHL. A represents the *sdiA* gene expression in *S.* Typhi isolate as response to exogenous AHL molecules produced by *P. aeruginosa*. Lane 1: undiluted; Lane 2-5: 1:1, 1:2, 1:3, 1:4 dilutions respectively. L: 50bp Genedirex DNA ladder. **B** represents the activity of whole cell biosensor to the AHL molecules in presence and absence of *sdiA*. The data is representative of at least three biological replicates with *p<0.01.

3.4.8 Significance of *sdiA* mediated QS in *S.* Typhi biofilm formation

The *sdiA* is a 240-amino acid protein that belongs to the *luxR* family of transcriptional regulators. The *Salmonella sdiA* is regulated by factors like deprivation of iron and presence of AHL in the environment. In our study, the expression analysis revealed up-regulated levels of *sdiA* gene expression in response to exogenous AHL molecules, as shown in **Figure 3.16A**. Microarray analysis has reported significant differences in genes regulated by *sdiA* with many functions left unexplained. In addition, mutations in this gene lead to alteration in biofilm forming ability of *E.coli*. Some of the key functions identified of the *sdiA* are enhancing MDR by regulating drug-efflux pumps, acid tolerance and represses expression of virulence factors. Interestingly, some animals have even shown the activation of *S.* Typhimurium AHL-type QS during their passage and colonisation in the intestine, indicating the effective way to detect intestinal environment. This was not seen in humans suggesting that the intestinal microbiota did not produce correct type or concentration of AHL (Bassler, 2002). Thus it is necessary to understand the significance of this AHL-mediated quorum sensing system in diverse environments.

Our results show that *S.* Typhi can co-exist with bacteria like *K. pneumoniae, S. aureus, P. aeruginosa, E.coli, Enterobacter* spp. *and Vibrio* spp. Thus, we try to understand the role of inter-species quorum sensing in assisting *S.* Typhi persistence. *P. aeruginosa* was shown to produce highest amount of AHL (~10-fold and ~7-fold increase in florescence in planktonic and sessile phase respectively) in the medium and in the mixed-culture biofilm followed by *Vibrio* (~6.3-fold and ~5.5-fold) and *Enterobacter* spp. (~7-fold for both planktonic and sessile phase), as efficiently indicated by the biosensing ability of the reporter *S.* Typhi strain **(Figure 3.17 A&B)**. *E.coli, K. pneumoniae and S.aureus* did not show AHL production in mixed-cultures. Also the *sdiA* mutant strain failed to detect the AHL in the medium and biofilms **(Figure 3.17 A&B)**. The activity of the promoter in response to the AHL molecules was visualized under fluorescence microscope as shown in **Figure 3.18**. Interestingly, the stimulation of *sdiA* by AHL produced by co-existing organisms did not significantly alter *S.* Typhi counts ($p>0.05$) in the biofilm or in the planktonic phase of growth. The *S.* Typhi numbers reduced depending on the type of organism present during co-existence, as depicted in **Figure 3.19A**. For instance, the presence of *P. aeruginosa* significantly ($p<0.001$) reduced *S.* Typhi colony counts by ~1.5 fold compared to presence of *E.coli* which showed the highest *S.* Typhi count of 9.85±0.08 log CFU/ml during co-existence. This was true for all other organisms used and the total log CFU/ml for the medium varied between ~11.5 to 13.5 log CFU/ml depending on the organism. Though lower bacterial counts were observed in subsequent biofilm studies, similar relationship was seen in *S.* Typhi counts obtained from mixed-culture biofilms, as shown in **Figure 3.19B**. These observations were on par with some of the earlier studies reporting that *sdiA* has no effect on biofilm formation in *E. coli* K-12 or *S.* Typhimurium (Lee et al., 2009). Furthermore, AHL production did not significantly assist bacterial growth because significant *S.* Typhi counts were seen in planktonic and sessile phase of growth during co-existence with AHL non-producers (i.e. *E.coli, K. pneumoniae and S.aureus*) (**Figure 3.19 A&B)** .

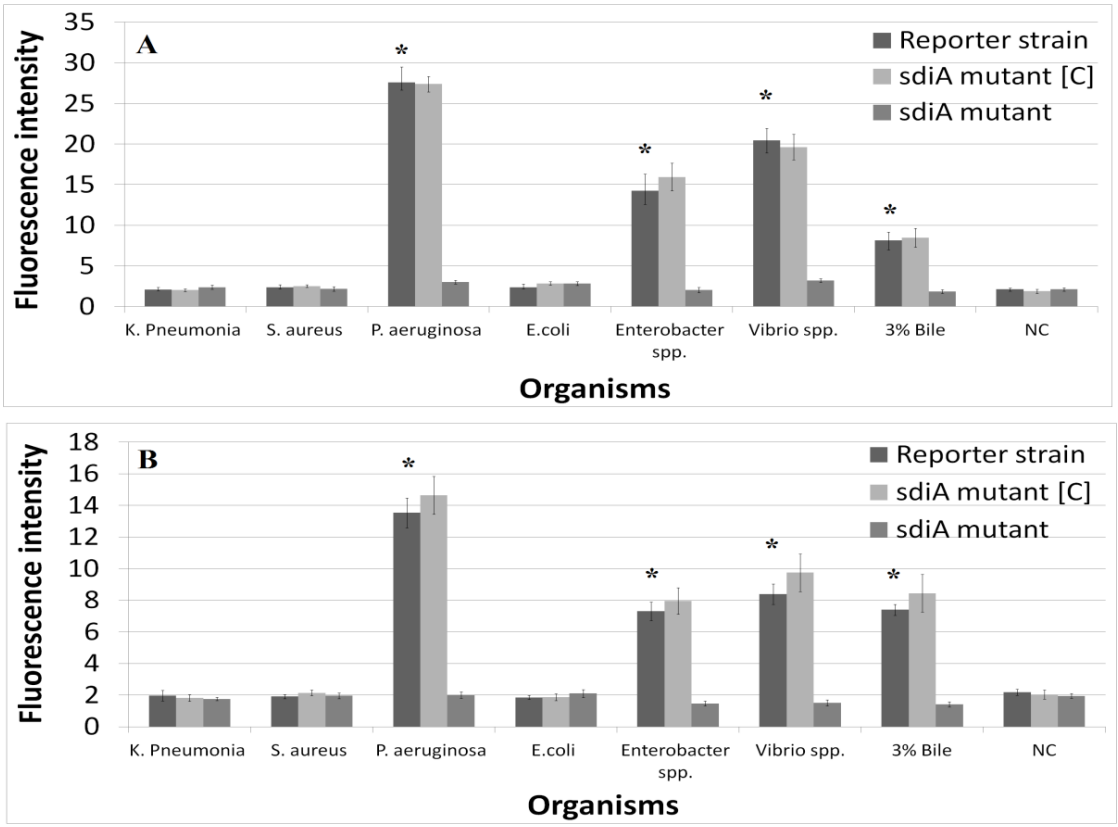


Figure 3.19 Detection of AHL molecules in mixed-cultures. The *S.* Typhi reporter strain was grown in the presence of other organism to detect the AHL molecules produced in the medium (**A**) and in biofilms (**B**). The data is representative of at least three biological replicates with *p<0.001. [C] represents mutant complemented with the wild-type *sdiA* gene.

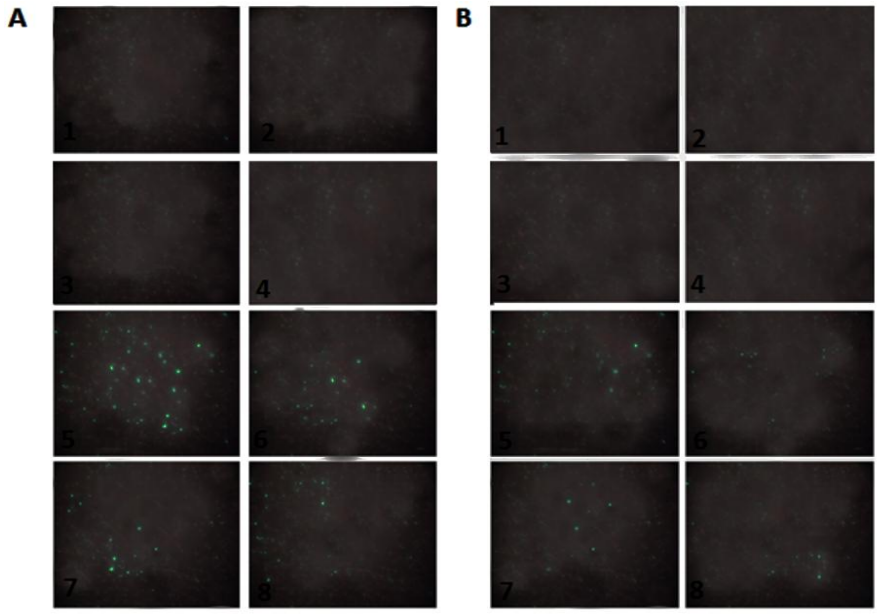


Figure 3.20 Fluorescence images (10X) of mixed-bacterial populations. A represents bacterial population in the medium, whereas **B** represents the bacterial population in biofilms. 1: Negative control; 2-4: AHL non-produces (*E.coli, K. pneumoniae and S.aureus* respectively); 5-7: AHL produces (*P. aeruginosa, Vibrio* spp. and *Enterobacter* spp. respectively) 8: 3% Bile.

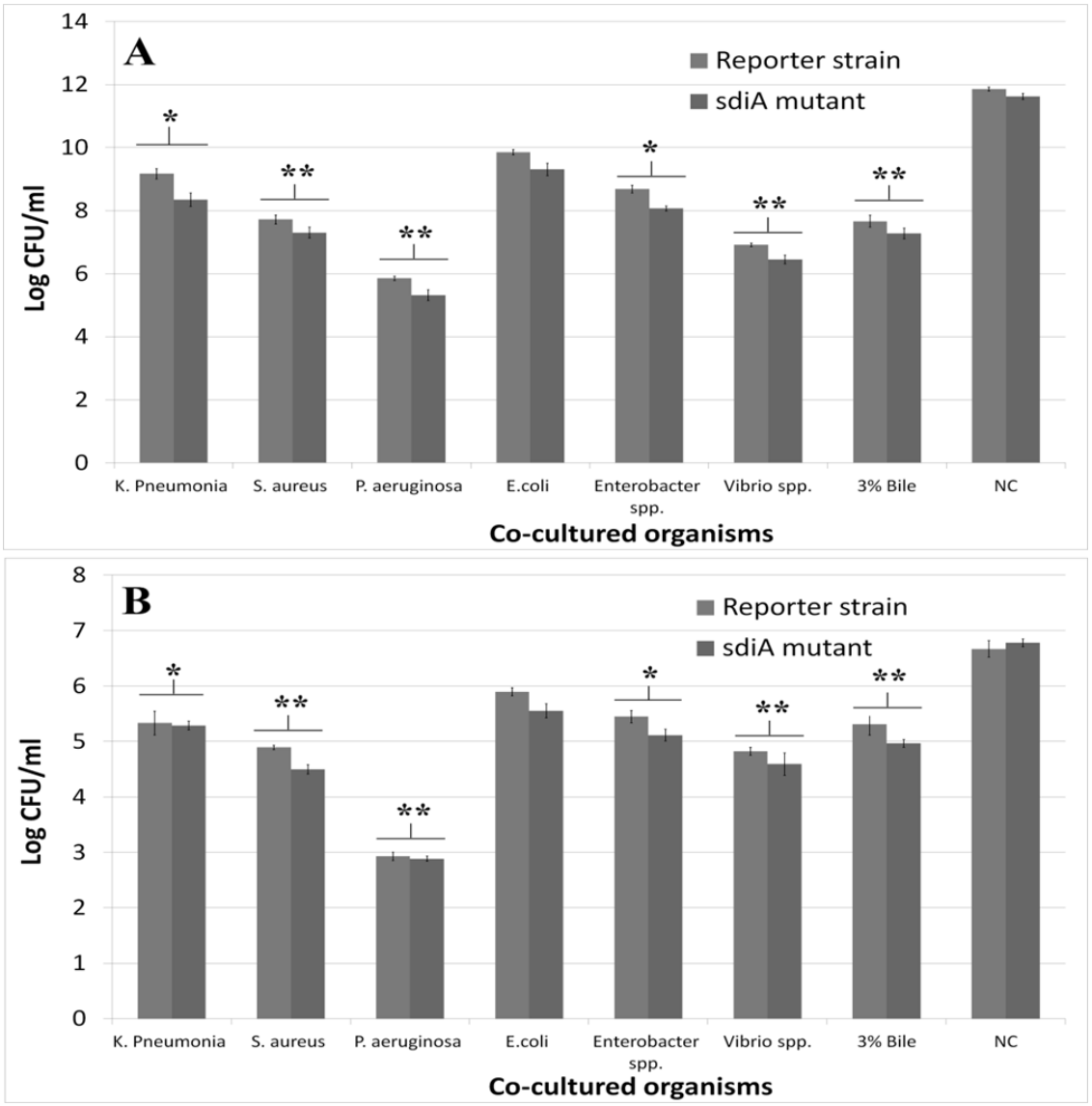


Figure 3.21 *S.* Typhi counts in mixed-cultures. The *S.* Typhi reporter strain was grown in the presence of other organism, and the changes in *S.* Typhi counts in the medium (**A**) and in biofilms (**B**) were shown as log CFU/ml. The data is representative of at least three biological replicates with *p<0.05 and **p<0.001.

Furthermore, our findings report that bile alone can significantly (p<0.001) up-regulate the *sdiA* expression. This does not again confer significant advantage to *S.* Typhi growth or biofilm formation. But, the mutant *sdiA* did show increased susceptibility to certain previously tested drugs. As seen in **Figure 3.20**, a significant increase (p<0.01) in susceptibility was observed towards ofloxacin, ceftriaxone and nalidixic acid. The organism was still resistant to chloramphenicol and TMP-SMZ as per the CLSI chart values. These results may be attributed to one of the previously identified *sdiA* function i.e. regulating the drug efflux mechanisms for multi-drug resistance in bacteria (Lee et al., 2009).

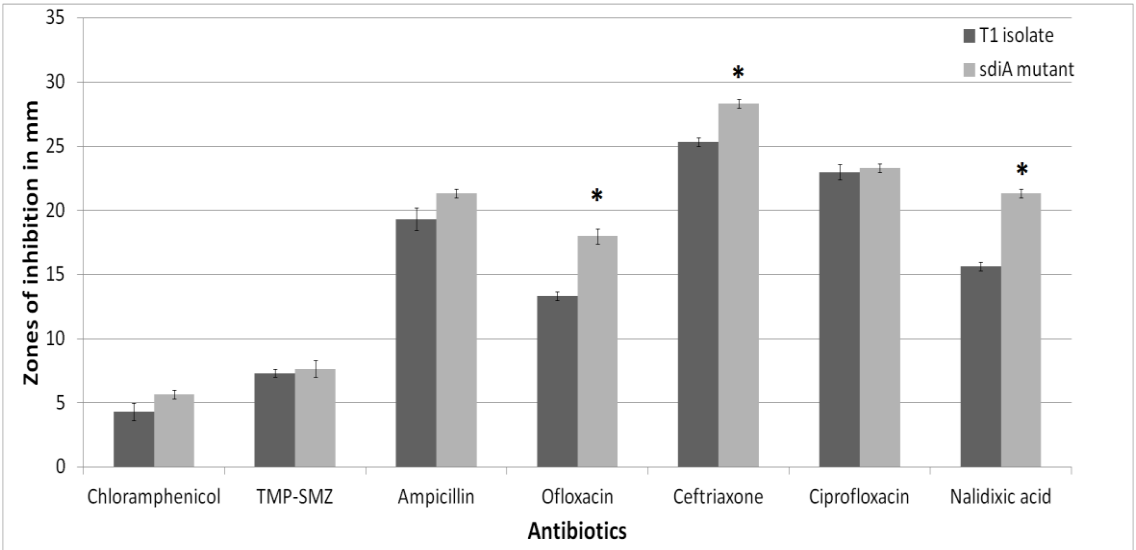


Figure 3.22 Significance of QS in *S.* Typhi drug resistance. The changes in susceptibility towards antibiotics was analysed using disc-diffusion assays. The data is representative of at least three biological replicates with *p<0.01.

Thus, our studies reveal that *sdiA* does not significantly impose competitive properties to *S.* Typhi in mixed-culture populations, but may have a role in regulating resistance to drugs mediated via bile present in the gallbladder.

3.5 Conclusion

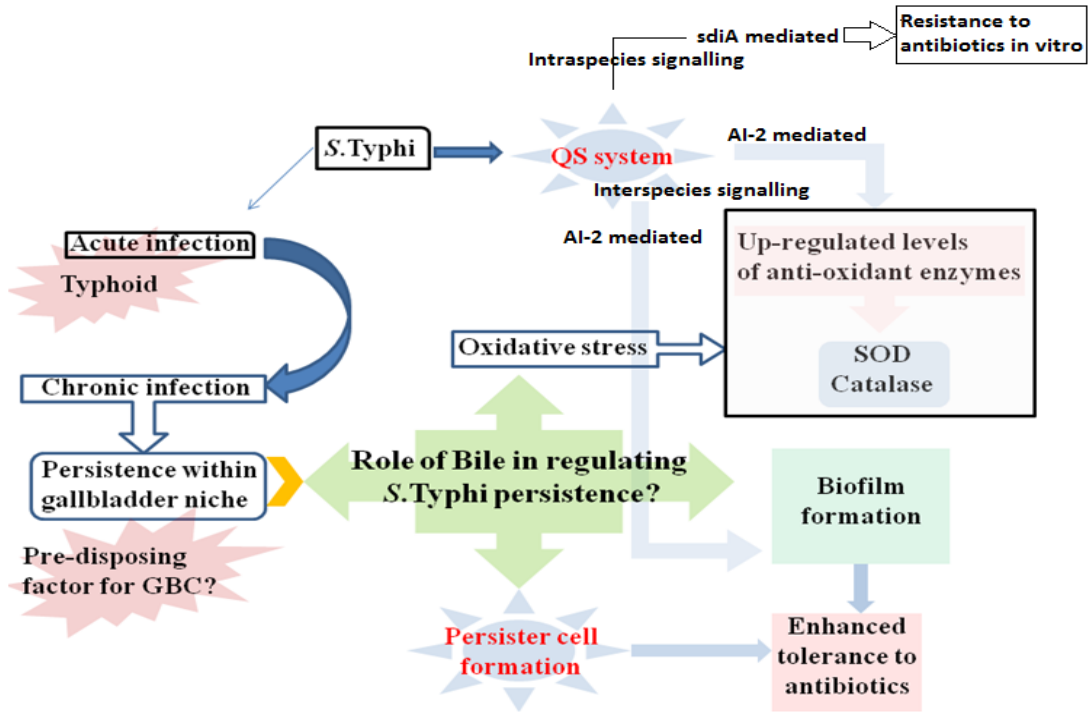


Figure 3.23 Bile-mediated mechanisms in regulating *S.* Typhi persistence

The present study reveals the adaptive mechanisms in *S.* Typhi in response to in-vitro bile-generated oxidative stress and presence of co-infection with other bacteria **(Figure 3.23)**. We show for the first time that the intra-species (mediated by *luxS*) QS system in *S.* Typhi plays a significant role in regulating the anti-oxidative enzymes as a response to bile stress. Also the phenotypes necessary for bacterial persistence within the gallbladder, like biofilm formation, was dependent on this interspecies QS system harboured by *S.* Typhi. Since, our earlier studies report the coexistence of *S.* Typhi with *E.coli* within gallbladder of the same patient; we also investigate the role of inter-species QS system in *S.* Typhi mediated by the *sdiA*. By developing a whole cell biosensor, we monitor the changes in *S.* Typhi behaviour in presence of other organisms. The subsequent results show that such inter-species QS does not significantly confer an advantage to growth or biofilm formation in *S.* Typhi, but it is shown to play a role in resistance towards drugs. Such resistance to antibiotics was further attributed to the persister cell generation which was enhanced by the presence of bile. Other than these epigenetic like modifications shown in presence of bile, this study also shows that *S.* Typhi can undergo irreversible changes during its adaptation to bile.

CHAPTER-IV

Role of *S.* Typhi in Regulating Host Mechanisms-Implications of RhoA/MLCK and NFkβ Signaling

4. Role of *S.* Typhi in Regulating Host Mechanisms- Implications of RhoA/MLCK and NFkβ Signaling

4.1 Abstract

The asymptomatic chronic carriage of *S.* Typhi in the gallbladder is a challenge for diagnosis and treatment in typhoid endemic regions. Such carriers have been proposed to be pre-disposing factors for gallbladder cancers. Besides escaping from the phagocytic attack, the organism has evolved sophisticated virulence mechanisms to enter non-phagocytic cells. The modulation of Rho family GTPase activity in the host brings about localized remodeling of actin cytoskeleton to internalize the bacteria. Also the bacteria are shown to have an effect on the NFkβ signaling, thus regulating pro-inflammatory responses within the host. These changes are brought about by *Salmonella* effectors and toxins, as strategies for invasion and intracellular persistence within the host. But in this process, the host signaling may be altered significantly to further cause complications and lead to pre-malignant and malignant lesions. In this study we try to understand the role of chronic *S.* Typhi persistence in regulating RhoA and NFkβ expression in the gallbladder. Both, the activation of RhoA and NFkβ have been shown to have implication in epithelial to mesenchymal transition (EMT) and carcinogenesis respectively. The presence of *S.* Typhi did not significantly alter RhoA and NFkβ expression in tissue specimens, with over-expression mostly correlated to the chronic inflammation due to gallstones. Interestingly, *in vitro* experiments involving gallbladder epithelial cell infections showed a significant increase in expression of these genes along with increase in expression of MLCK (a downstream effector protein involved in control of actin dynamics and leads to EMT). But, there was no significant loss in E-cadherin expression-a molecular marker for epithelial to mesenchymal transition. Furthermore, patients suffering from gallbladder cancer did show increased expression of rhoA and NFkβ along with loss in expression of the E-cadherin (CDH1) indicating EMT. Though the expression of these components may be of prognostic significance in gallbladder disease progression, they do not provide direct evidence of *S.* Typhi chronic persistence within gallbladder.

4.2 Introduction

Salmonella Typhi can chronically persist within the gallbladder following acute phase of infection without any symptoms, and is proposed to be one of the pre-disposing factor for gallbladder cancers (Capoor et al., 2008; Nath et al., 2010). Extensive research in understanding *S.* Typhi acute phase of infection has been carried out, and there is paucity of data related to mechanisms underlying the role of the bacteria in bringing about transformation of host cells during chronic persistence. The actin cytoskeleton manipulation is a prevailing theme in host-pathogen interactions (Hänisch et al., 2011). Also, pro-inflammatory responses mediated by chronic bacterial persistence have been shown to significantly alter variety of host mechanisms (Nath et al., 2010). These changes are brought about by various effectors and toxins produced by *S.* Typhi (Guerra et al., 2011; Lei et al., 2016). The Rho family of small GTPases has been shown to be ideal targets for *Salmonella* effector proteins and is specifically necessary for internalization of the bacterium into non-phagocytic cells. The effectors generally includes the sop proteins regulated via the secretion systems (previously described in chapter 1), and toxins like CDT that activate RhoA and its downstream components. Along with functions like cell cycle progression and regulating cytoskeletal dynamics, several Rho GTPases have oncogenic activities *in vitro* and can promote cancer progression with metastatic dissemination (Van Aelst et al., 2002; Serizawa et al., 2015). This correlates with their increased expression seen in different cancers and GTPases have also been shown to be involved in all stages of cancer. For instance, RhoA is upregulated in gastric, colon, liver, bladder, breast, testicular, skin cancers (Parri and Chiarugi, 2010). The up-regulated RhoA expression further leads to activation of MLCK via rock which contributes to migratory nature of cancer cells (Parri and Chiarugi, 2010; Serizawa et al., 2015). Thus, these molecules along with their downstream components may have prognostic significance in disease progression and make them suitable targets for therapeutic agents.

Furthermore, *Salmonella* epithelial interactions are shown to activate pro-inflammatory NFkβ signaling pathways in the host. For instance, *Salmonella* infection directly increases GSK-3β activity that degrades β-catenin, thus consequently increasing the concentration of NFkβ (Duan et al., 2007). In addition, RhoA is also shown to regulate NFkβ signalling pathway in certain diseases (Xie et al., 2013). NFkβ is shown to be

central coordinators of adaptive and innate immune responses, but recent studies have report their critical role in cancer development and progression (Karin, 2006). Interestingly, these proteins provide a mechanistic link between inflammation and cancer, and control the ability of pre-neoplastic and neoplastic cells to resist apoptosis (the aid in tumor angiogenesis and invasiveness) (Karin, 2006). Thus, the pathways that regulate NFkβ expression and the subsequent downstream components to the NFkβ signalling provide attractive targets for therapeutic agents. This also supports observations that malignancy is preceded by chronic inflammation (DeNardo et al., 2008).

In this study we report for the first time the role of Chronic *S.* Typhi persistence in regulating the expression of RhoA and NFkβ, both *in vitro* and *in vivo*. We determine their prognostic significance, and also show their association with EMT and cancer progression.

4.3 Materials and methods

4.3.1 Sample collection, Histopathological analysis and Primers

A total of 250 patients undergoing cholecystectomy for gallbladder disease from February 2012 to March 2015 at Safdarjung Hospital (New Delhi, India) and SMRC Hospital (Goa, India) were enrolled for this study. For normal specimens, we consider 50 human cadavers (without any gallbladder disease) from the Department of Forensic Science-Goa Medical College and Hospital (Goa, India). The consent was obtained from the patients, and the study was approved by the IHEC (Statement No.IHEC-31/13-14). Human gallbladder tissue, bile and gallstones were obtained from diseased and non-diseased patients as described in Chapter 1. For primary culture of gallbladder epithelial cells (GBEC), freshly excised normal gallbladder tissue obtained from cadavers was immediately transported to the laboratory in sterile DMEM medium (Himedia) at 4°C followed by isolation procedure as described 4.3.3.

For histopathological classification, the specimens were fixed in formalin followed by routine H&E staining (These procedures were carried out at the respective hospitals from where the samples were obtained). (Rosai 2011; Kumar et al., 2013)

For RNA related studies, gallbladder tissue samples were collected in RNA*later*® (Thermo Fisher Scientific) solution and stored at -80°C. The primer pairs used in this study are listed in **Table 4.1**.

Gene	Sequence
rhoA	**FP**: 5' TGCAATGCACTTTCAGCCAC 3'
	RP: 5' AGCAGAATCCAAACTCCGCA 3'
NFkβ	**FP**: 5' TGGGTGCTTCTGCTTTGCAT 3'
	RP: 5' GCCTGTGTCCTCTCCTTCATT 3'
MLCK	**FP**: 5' GCATCAAGTACATGCGGCAG 3'
	RP: 5' TTTTTCTGCATTGAGCGGGC 3'
CK19	**FP**: 5' GCCTCGCCATGACTTCCTAC 3'
	RP: 5' GGTACCAGTCGCGGATCTTC 3'
CDH1	**FP**: 5' TTGTCCCTCCTCCACGATCA 3'
	RP: 5' ATGACAAGTGCGAGACAGCC 3'
H1-d (*fliC*)	**FP**: 5' TCGTTTGAGGATAAAAACGGT 3'
	RP: 5' CAGTTTGAGCAACGCCAGTA 3'

Table 4.1 List of primer pairs used in this study

4.3.2 Detection of *S*. Typhi DNA using PCR assay

The gallbladder tissue obtained were subjected to PCR assay for detection of *S.* Typhi specific gene fragment as described earlier in chapter 1. Briefly, the DNA was extracted from 25–30 mg of sample using the NucleoSpin® Tissue Kit as per manufacturer's instructions. The template DNA was subjected to PCR amplification using primers specific to H-1d region of flagellin gene fragment of *S.* Typhi **(Table 4.1).** The amplified gene fragment was visualized using 1.5% agarose gel. The fragment was eluted using QiaQuick Gel Extraction Kit (Qiagen), cloned in pGEM-T Easy vector system (Promega) and sequenced to confirm desired gene amplification. (Sambrook and Russell, 2001; Hazrah et al., 2004; Nath et al., 2008)

4.3.3 Isolation of gallbladder epithelial cells

The isolation of gallbladder epithelial cells was performed as described by Miquel et al with few modifications (Miquel et al., 2003). Briefly, the isolation was carried out within 30-45 minutes post-surgery. The mucosa was rinsed carefully with DMEM to remove any adherent mucous and bile. The tunica mucosa was placed in 0.125% collagenase solution prepared in DMEM for 15 minutes at 37°C. The mucosa was

abraded every 5 minutes using a scalpel and flushed with DMEM. The resulting cell suspension was immediately subjected to centrifugation at 1000 rpm for five minutes. An aliquot of the cells seeded in 24mm petridish containing DMEM medium with 10% FBS and 0.5% antibiotics. The plates were incubated at 37°C with 5% CO_2 and monitored every day for colonies of cells. The cells were then transferred to 24 well plates containing coverslips for immunostaining assays and bacterial infection assays.

4.3.4 Immunofluorescence staining

The coverslips with adhered GBECs were immunostained with CK19. For staining, coverslips were blocked for 30 min at 37°C in the blocking buffer (1X PBS, 5% heat inactivated horse serum, 0.2% Tween-20) in a humid chamber containing water-soaked Whatmann paper. Coverslips were then incubated with primary antibody (Santa Cruz Biotechnology) diluted in blocking buffer (1:100) for 1 hour at 37°C in the humid chamber. Coverslips were washed three times for 5 min each with PBS/Tween on a shaker at room temperature and then blocked again for 5 min at 37°C in blocking buffer in the humid chamber. Coverslips were then incubated with secondary antibody (GFP-Tagged) diluted in blocking buffer (1:200) for 1 hour at 37°C in a humid chamber and washed once for 5 min with PBS/Tween at room temperature on a shaker, keeping coverslips covered to avoid contact with light to prevent photobleaching. Then coverslips were washed 1 X 5 min with PBS/Tween + 1ul of 4',6-diamidino-2-phenylindole (DAPI-Sigma) at room temperature on a shaker, again keeping coverslips covered, followed by washing 1 X 5 min with PBS/Tween at room temperature on a shaker, keeping coverslips covered. Antifade solution (0.5% p-phenylenediamine (PPD)-Sigma in 20mM Tris, pH 8.8 + 90% glycerol) was added and coverslips were used to cover the cells that are fixed on the coverslips. Finally, images were captured using a Nikon fluorescence microscope and slides stored at -20°C for future usage. (Miquel et al., 2003; Edward et al., 2011; El-Malah et al., 2014)

4.3.5 Infecting the gallbladder epithelial cells

Isolated gallbladder epithelial cells were seeded into 24-well tissue culture plates containing washed coverslips at a density of 2 x 10^5/ml in volumes of 0.5 per well. The cells were observed every 24 hours to ensure sub-confluent monolayers (85-90% confluent) on coverslips for the infection assay. This process took around 3-4 days, and, 24 hours before the assay medium was exchanged with serum free medium with or

without 0.3% bile. *S.* Typhi and its mutant were grown in LB and LB+amp respectively up to 2 x 10^7 CFU/ml that represents multiplicity of infection of 100:1 (bacteria:cells). The bacteria were then washed three times in normal saline (0.9%), and re-suspended in the medium used to grow the gallbladder epithelial cell line (DMEM with 10% serum and no antibiotics) which ensures removal of any secreted molecules that may be deleterious like cytotoxins. Bacteria were then added in volumes of 10µl to epithelial cells (each well contained and isolated GBEC in 490µl of DMEM + 10% FBS and no antibiotics) incubated for 4 hours in cell culture incubator at 37°C with 5% CO_2 to allow bacterial entry to occur. The cells were then incubated for 120, 180, 240 minutes at 37°C under 5% CO_2 (Edward et al., 2011; El-Malah et al., 2014).

4.3.6 Bacterial internalisation assays

Monolayers of infected GBEC were washed three times with warm DMEM to remove extracellular bacteria and incubated for 2 hours in the DMEM containing 50 mg/ml gentamicin to kill the remaining extracellular bacteria. The infected epithelial cells were washed 2 times with DMEM followed by the addition of 100µl of 1% Triton X-100 to each well for lysis. The cells were incubated for 10 minutes at room temperature and then 900µl of LB media was added. The media was gently pipetted to homogenise the solution followed by plating on LB media using the drop-plate technique (Edward et al., 2011; El-Malah et al., 2014).

4.3.7 Monitoring attachment of *S.* Typhi using microscopy

To check for bacterial attachment to GBEC, we use Giemsa's staining method previously described by El-Malah et al. Briefly, the media in the well was removed and the cells were washed with PBS. This was followed by addition of 100% methanol for 1 minute. The supernatant was discarded and Giemsa's stain solution was added for 30 minutes. The stain was then discarded and the cells were washed three times with sterile distilled water and left to air dry. The cells were then observed under 100x oil immersion lens. (El-Malah et al., 2014)

4.3.8 Reverse-Transcriptase PCR assay

Total RNA was extracted with RiboZol™ (Amresco, USA) reagent from *S.* Typhi infected epithelial cells and whole gallbladder tissue samples of patients suffering from gallbladder disease as per manufacturer's instruction*. RT-PCR was carried out using

Revert Aid First Strand cDNA Synthesis Kit and random hexamer primers as per the manufacturer's protocol[#]. For every reaction of cDNA synthesis 1µg of purified RNA was used followed by PCR amplification (with specific primers to E-cad, RhoA, NFκβ and MLCK mentioned in **Table 4.1**) within the linear range of amplification (30 cycles). A 10µl aliquot of the amplified product was loaded on 1.2% agarose gel and bands obtained were analysed using ImageJ 1.46r software. The normalized gene expression with a > 1.5 fold increase and a p-value of < 0.01 was considered to be significant.

[*RNA extraction protocol using RiboZol: For tissue samples, we add 1ml of RiboZol to 50mg of tissue followed by homogenization. For cells grown in monolayer, the media was discarded and 1ml of riboZol was added followed by pipetting several times to lyse the cells. Following lysis, the homogenized samples were incubated for 5-10 minutes at RT for complete dissociation of nucleoprotein complexes. 200µl of chloroform was then added per ml Ribozol and the tuves were shaken vigorously for 15 seconds to mix the samples followed by incubation for 2-3 minutes at RT. The samples were then centrifuged at 12000 x g for 15 minutes at 4°C. This produced three layers of separation (lower red phenol-chloroform phase, white interphase and upper aqueous phase) with RNA present in the upper aqueous phase. We transfer around 80% of the aqueous phase into a new RNase free eppendorf tube followed by RNA precipitation by adding 0.5ml iso-propanol. The samples were incubated for 10 minutes at RT and then centrifuged at 12000 x g for 10 minutes at 4°C. A gel-like transparent pellet was observed at the bottom or sides of the tube indicating the precipitation of RNA with size depending on amount of RNA recovered. The supernatant was removed carefully without disturbing the RNA pellet and the pellet was then washed twice in 75% ethanol (prepared in RNase-free water) by vortexing and centrifugation at 7500 x g for 5 minutes at 4°C. Following the final ethanol wash, carefully remove the ethanol and air dry the pellet for 10 minutes (complete drying reduces the solubility of RNA). The RNA was then dissolved in 25µl of RNase free water by pipetting several times followed by incubation at 60°C for 10 minutes to completely dissolve the RNA. The RNA yield was quantified using and quantified using NanoDrop Lite spectrophotometer.]

4.3.9 Immunoblotting Experiments

The infected cells were lysed using RiboZol reagent and the total protein was extracted as per manufacturer's instruction[##]. Samples were then subjected to 10% sodium dodecyl sulphate polyarylamide gel electrophoresis (SDS-PAGE) as described in previous chapters (Sambrook and Russell, 2001). For blotting, the PVDF membrane was cut to the size of the gel and pre-wetted in 100% methanol. The membrane was equilibrated by soaking it in transfer buffer (10% methanol, 24mM Tris-HCl, 194mM glycine) for 20 minutes. The gel sandwich was then prepared as follows: The gel was carefully placed on the pad-blotting paper assembly taking care to see that no air bubbles are present. The surface of the gel was then wetted using transfer buffer followed by gently placing of the soaked PVDF membrane. The sandwich was completed placing filter paper-pad assembly and fixing the cassette firmly together taking care to prevent any air bubbles or movements in the gel. The apparatus was then placed in the tank with transfer buffer and transfer carried out at 30V (90mA) for 6-8 hours. The membrane was then carefully removed, marked and rinsed with distilled water (The membrane was stored in between two dry filter paper pieces wrapped in plastic cover at 4°C). (Sambrook and Russell, 2001; Sakamoto et al., 2011) The blots were then stained and visualized for changes in candidate protein expression.

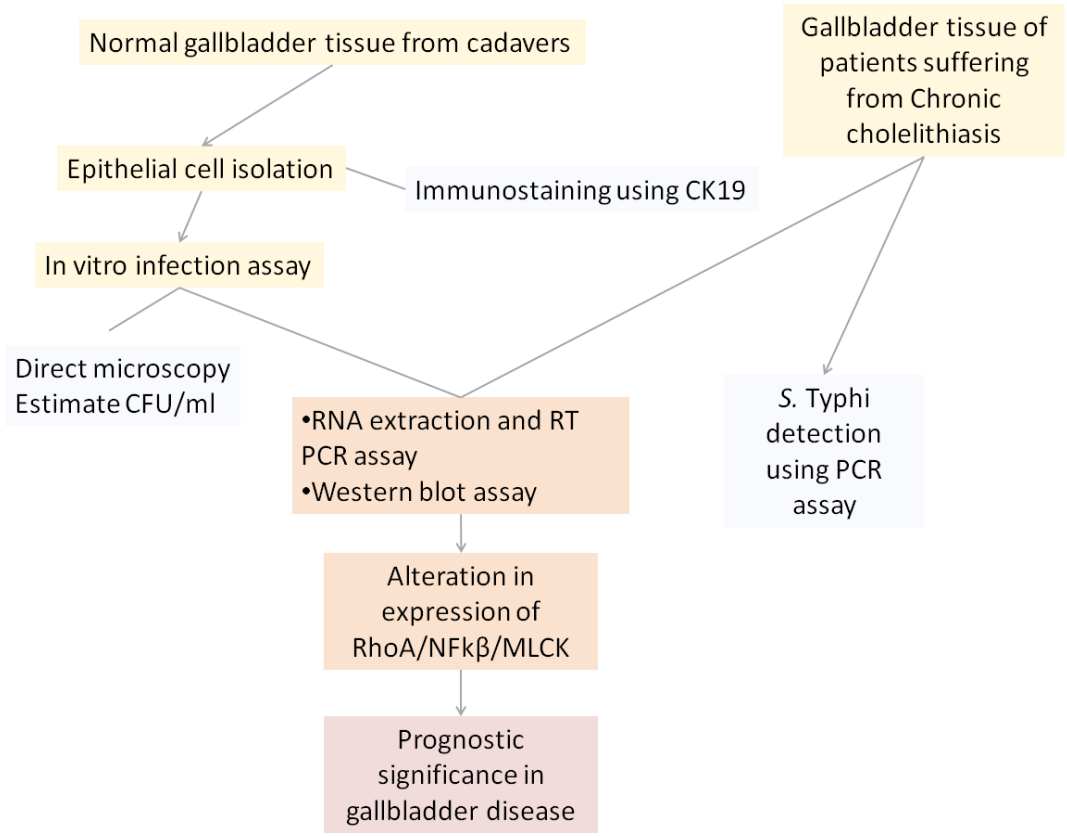
Western blot staining: The PVDF membrane was blocked for 30 minutes in 5% non-fat dry milk in TBST buffer [TBS(Tris, NaCl) containing 0.2% Tween-20] and incubated with a primary antibody in 5% TBST-milk for overnight at 4°C. After washing with TBST (3 x 5 min), membrane was incubated with HRP-conjugated goat anti-rabbit (1:3000), anti- mouse (1:4000) or anti-goat (1:3000) (Santa Cruz) secondary antibodies for 2 hours at room temperature. After washing (3 x 10 min) in TBST, the HRP-labelled antibody was visualized using 3,3',5,5'-tetramethylbenzidine (TMB) solution (Sigma) as per manufacturer's protocol. (Sambrook and Russell, 2001; Sakamoto et al., 2011)

[##Protein extraction using RiboZol: Following the complete removal of aqueous phase for RNA extraction, 0.3 ml of 100% ethanol was added to the interphase/organic phase and mixed by inversion. Followed by an incubation of 3 minutes at RT the tubes were centrifuged at 2000 x g for 5 minutes at 4°C. The phenol/ethanol supernatant obtained was transferred to a new tube and 1.5 ml of iso-propyl alcohol was added. The tube was incubated for 10 minutes at RT followed by centrifugation at 12000 x g for 10 minutes

at 4°C. The supernatant was discarded and the pellet was washed in 0.3M guanidine hydrochloride prepared in 95% ethanol. The tubes were incubated for 20 minutes at RT followed by centrifugation at 7500 x g for 5 minutes at 4°C (The wash step was repeated twice). The final protein pellet was dried and re-suspended in 1% SDS by repeated pipetting followed by centrifugation at 10000 x g for 10 minutes at 4°C. The supernatant containing the protein was transferred to a new tube which was stored at -20°C till analysed.]

4.3.10 Statistical analysis and Data interpretation

All the experiments represent mean values of at least three biological replicates with standard errors. The level of significance for experiments was determined using unpaired students t-test with p value of <0.05 considered to be significant.



Scheme 1. Overview of methodology used in this study

4.4 Results and Discussion

4.4.1 Classification of tissue based on histopathological analysis

The gross anatomy of the gallbladder tissue specimens obtained is shown in **Figure 4.1)**. Routine histology using H&E staining showed that majority of the patients (98.4%) enrolled for this study suffered from chronic cholelithiasis with different degrees of inflammatory responses and manifestations **(Figure 4.2)**. Only 1.6% patients were characterized to have gallbladder cancer with a very low survival rate following surgery.

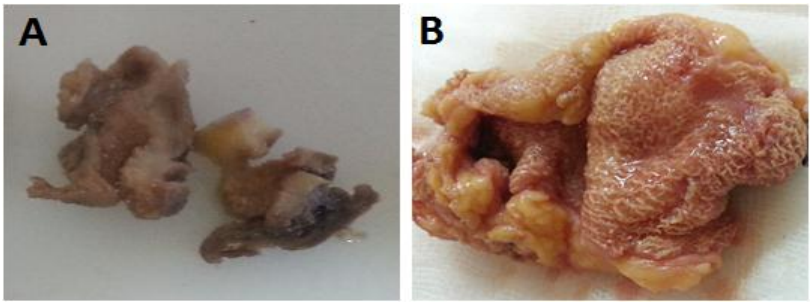


Figure 4.1 Gallbladder anatomy during diseased state. A represents gallbladder cancer tissue that shows abnormal wall thickening and ulceroproliferative growth; and **B** represents chronic cholelithiasis in patients with gross appearance showing reddish mucosa, wall thickening and ulceration.

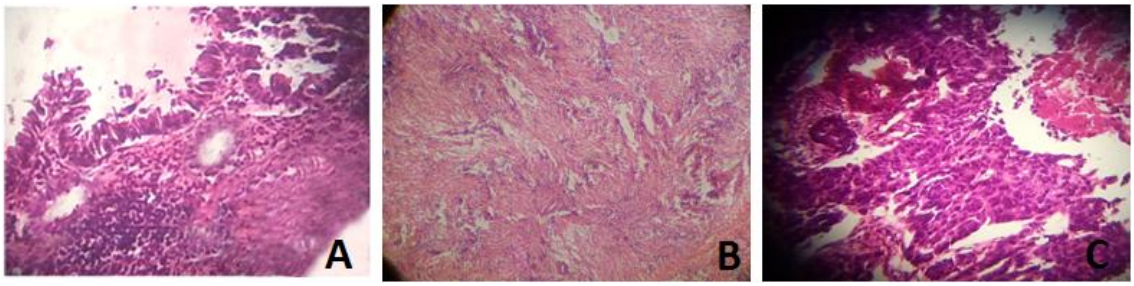


Figure 4.2 Histopathological images (40X) showing gallbladder disease. A represents normal gallbladder pathobiology with intact epithelium and no infiltration or inflammation; **B** shows chronic cholelithiasis with inflammatory infliltration and Rokitansky Aschoff sinus; and **C** depicts adenocarcinoma with proliferating malignant glands and hyperchromatic pleomorphic cell infiltration.

4.4.2 Detection of chronically persisting *S.* Typhi in the gallbladder using PCR

To screen for *S.* Typhi chronically persisting in the gallbladder, we use the PCR assay previously described in chapter 1. On screening of gallbladder tissue, bile and gallstone it was observed that 10.8% (27/250) of the patients suffering from gallbladder disease were positive for *S.* Typhi. No S. Typhi was detected in the patients showing normal

gallbladder pathology. **Table 4.2** shows the relation of S. Typhi occurrence to the diseased state of the gallbladder. Furthermore, presence of *S.* Typhi did not alter the gallbladder histology as shown in chapter 1.

No. of patients	Normal	CC with CS	GBC
Gallbladder pathology	50	246	4
Showing *S.* Typhi chronic persistence	0	27*	0

Table 4.2 Chronic persistence of *S.* Tyhpi in the gallbladder. *p<0.01; CC Chronic cholelithiasis; CS chronic cholecystitis; GBC gallbladder cancer.

Thus, our study shows the frequency of *S.* Typhi during chronic inflammatory stages of the gallbladder to be on par with some of the previous clinical findings. These numbers may vary depending on the region and population under study (Nath et al., 2010). Also, the absence of *S.* Typhi during the cancerous state in this study does not represent the exact scenario, as low numbers of gallbladder cancer samples were obtained. This data further supports the hypothesis that *Salmonella* may have a role to play in gallstone formation and chronic inflammation (Roa et al., 1999; Hazrah et al., 1999).

4.4.3 Isolation and characterisation of human gallbladder epithelial cells

To develop an *in vitro* model of infection, we prepare primary cultures of gallbladder epithelial cells (GBEC) obtained from the normal human gallbladder epithelium as described earlier by Miquel et al. (Miquel et al., 2003). **Figure 4.3** shows the sequential morphological changes occurring during the primary culture of these cells. As observed cell death and apoptosis was observed after 14 days following successful attachment and growth. The cells were further characterized by immunostaining with Cytokeratin 19 (an epithelial cell marker) antibody that typically stains epithelial cells. Most of the cells obtained were positive for CK19 as shown in **Figure 4.4**, indicating their epithelial nature.

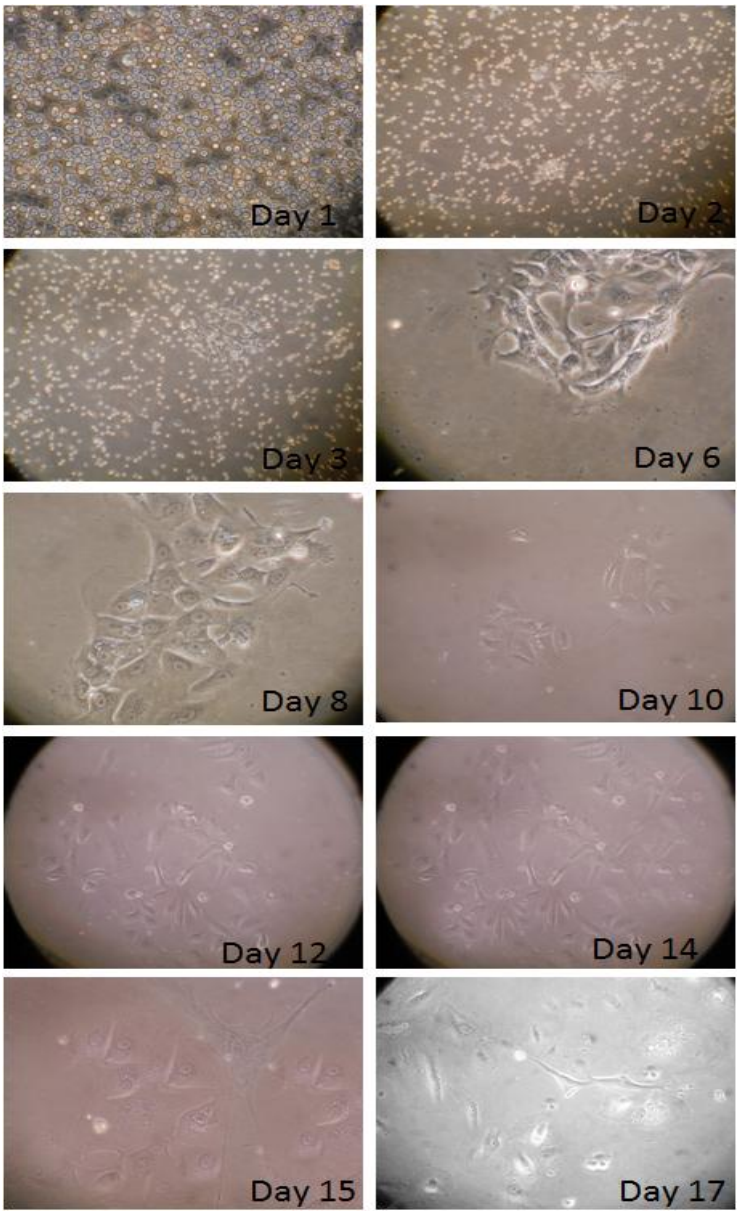


Figure 4.3 Isolation of gallbladder epithelial cells. The cells were isolated from gallbladder tissue and cultured *in vitro*. The images represent the successful attachment and growth of epithelial-like cells isolated during the primary culture.

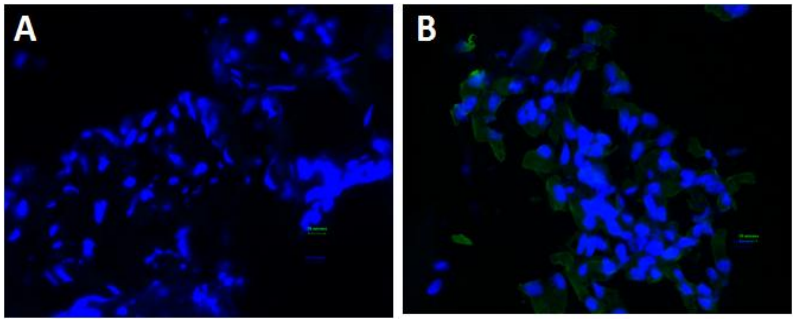


Figure 4.4 Immunostaining of cultured primary cells. The cells obtained on primary culture were stained using the GFP-tagged CK19 antibody to identify their epithelial nature. **A** represents the negative control whereas **B** represents the isolated cells positive for CK19 antibody.

4.4.4 In-vitro infection of isolated GBEC with *S.* Typhi gallbladder isolate

We develop an *in vitro* model system to understand the molecular changes brought about by *S.* Typhi in the gallbladder epithelium. Following the addition of *S.* Typhi gallbladder isolate to primary cultures of GBEC, the bacteria successfully adhered on to the cells by 2 hours as seen in **Figure 4.5**.

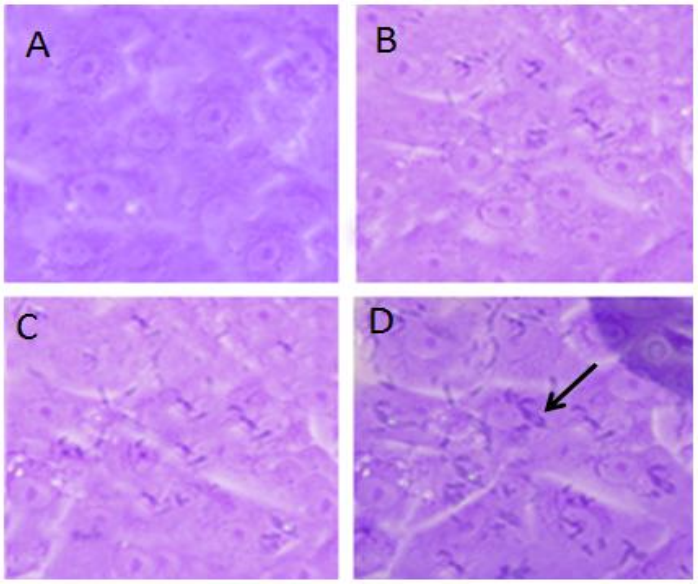


Figure 4.5 Adhesion of *S.* Typhi to GBEC. The GBEC were monitored at 0 (**A**), 120 (**B**), 180 (**C**) and 240 (**D**) minutes following infection with of *S.* Typhi gallbladder isolate. The arrow represents the bacteria stained using Giemsa stain followed by visualisation under the microscope at 100X (oil immersion)

To monitor *S.* Typhi internalisation and its intracellular replication in GBEC, we perform bacterial colony counts following lysis of epithelial cells **(Figure 4.6)**. As seen in **Figure 4.6A**, no bacteria were obtained till 3 hours following infection. Bacteria were recovered at 4 hours post-infection with counts of log 3.2±0.1 CFU/ml. The counts increased significantly ($p<0.01$) till 6 hours (~4-fold increase) indicating successful replication of the bacteria in the host. After 6 hours, no change in the bacterial counts was seen with nearly similar CFU/ml values recorded at 360 and 420 minutes **(Figure 4.6B)**.

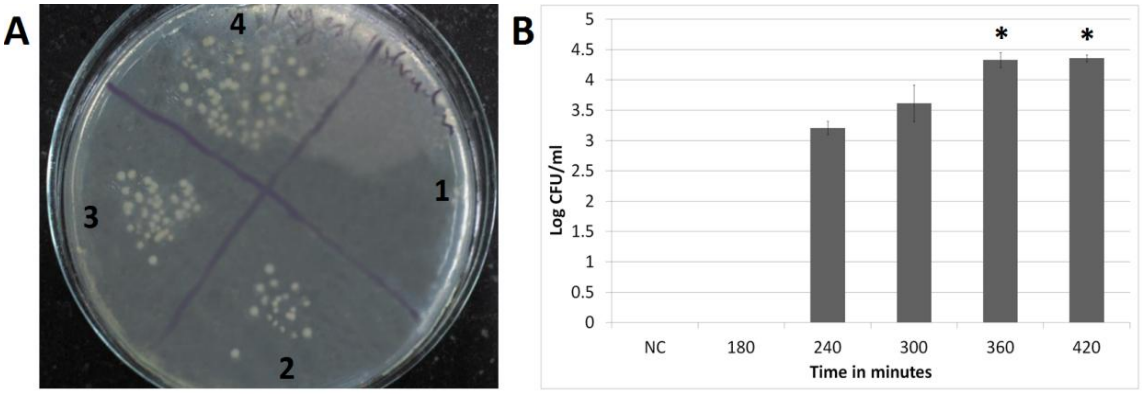


Figure 4.6 Invasion and intracellular replication of S. Typhi in GBEC. A&B represents the variation in bacterial colony counts determined by the drop-plate method. **1-4** shows the colony counts for 180, 240, 300, 360 minutes respectively. The data is mean of three independent biological replicates with *p<0.01. NC represents negative control.

Salmonella exhibits a variety of effector proteins and virulence factors that facilitates their survival in the host environment. The highly variable and sophisticated mechanisms employed by the organisms not only help them to survive in pahgocytic cells, but also internalize and replicate within non-phagocytic cells. (Guerra et al., 2011; Lei et al., 2016)

4.4.5 Implications of RhoA/MLCK and NFkβ as a prognostic marker

Both, the activation of RhoA (Serizawa et al., 2015) and NFkβ (Karin, 2006) have been shown to have implication in EMT and carcinogenesis respectively. RhoA regulates the MLCK expression via ROCK which contributes to the invasive property of cancer cells (Parri and Chiarugi, 2010), and NFkβ controls the ability of pre-neoplastic and neoplastic cells to resist apoptosis (Karin, 2006). These factors have been associated with cancer progression based on alterations in their expression seen in various cancers. RhoA and NFkβ are regulated by number of factors, with one of them being bacterial infections. *Salmonella* produces a variety of effector molecules and virulence factors that play a role in regulating these mechanisms in the host for successful replication and survival (Guerra et al., 2011; Lei et al., 2016).

Our findings related to gene expression of RhoA/MLCK and NFkβ reveal no significant alterations in the presence of *S.* Typhi in the gallbladder tissue of patients suffering from chronic cholelithiasis **(Figure 4.7A)**. But *in vitro* infection studies of isolated GBECs show a significant ($p<0.001$) up-regulation in expression of these genes **(Figure 4.7B)**. A nearly 2-fold increase was seen the expression of RhoA/MLCK

and NFkβ in GBEC infected with *S.* Typhi **(Figure 4.8 A&B)**. These changes in expression were further analyzed at protein level using western blot that showed similar results, as depicted in **Figure 4.9 A&B**. There was no loss in E-cadherin expression in both the gallbladder disease and GBEC indicating no involvement of EMT. Also a significant expression ($p<0.01$) of the epithelial cell marker CK19 was observed in the GBEC compared to the significantly lower levels (decrease by ~1.8 fold) in tissue derived genetic material. Furthermore, in comparison to patients suffering from chronic cholelithiasis, the cancer patients showed significantly higher ($p<0.05$) expression of RhoA/MLCK and NFkβ in gallbladder tissue with a significant loss in E-cadherin expression, indicating epithelial to mesenchymal transition **(Figure 4.10 A&B)**. Thus, we assume that the expression of RhoA/MLCK and NFkβ may have a possible prognostic value in determining disease progression of the gallbladder.

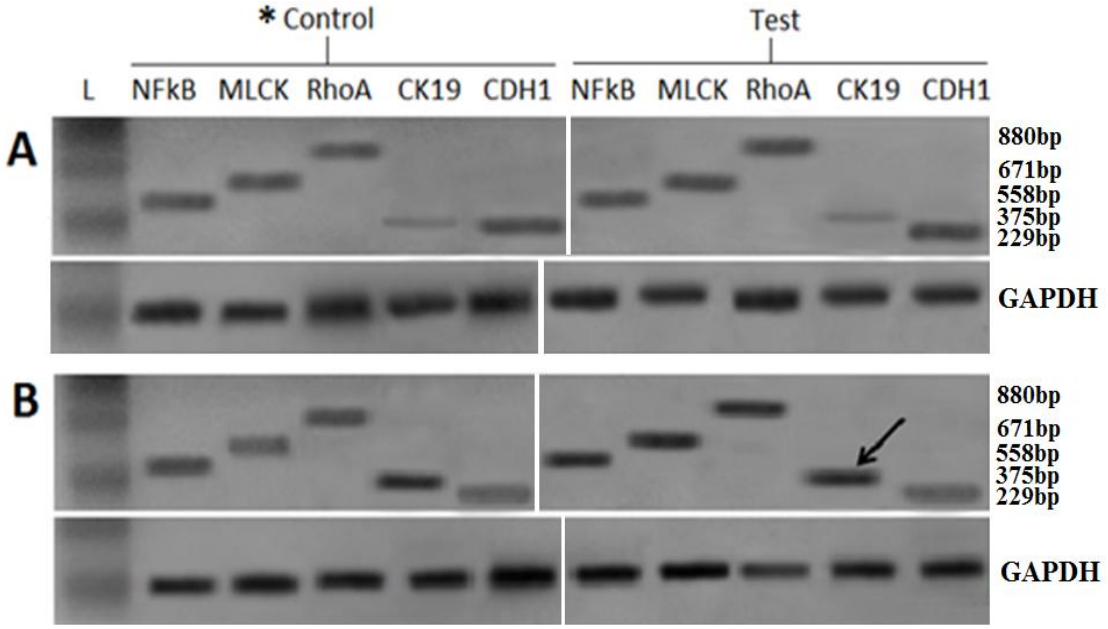


Figure 4.7 Gene expression analyses of candidate genes. The changes in gene expression were analysed using RT-PCR assay in tissue samples **(A)** and in isolated GBEC **(B).** The changes in expression were analysed using ImageJ software. L: 50bp Genedirex DNA ladder. Arrow represents up-regulated expression of CK19 in epithelial cells. *Control for tissue is normal gallbladder samples and that for GBEC is cells without infection of *S.* Typhi. GAPDH is the loading control.

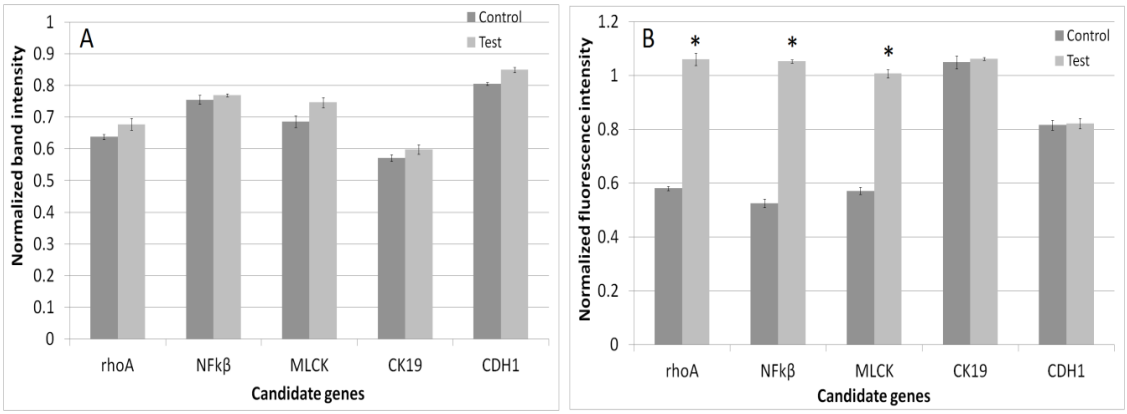


Figure 4.8 Changes in gene expression in presence of *S.* Typhi. The change in gene expression for candidate genes was determined based on the band intensities obtained in tissue samples (**A**) and in GBEC (**B**). A change of <1.5 fold was considered to be significant increase with *p<0.01. The data is mean of at least three biological replicates with SD and SM. *Control for tissue is normal gallbladder samples and that for GBEC is cells without infection of *S.* Typhi.

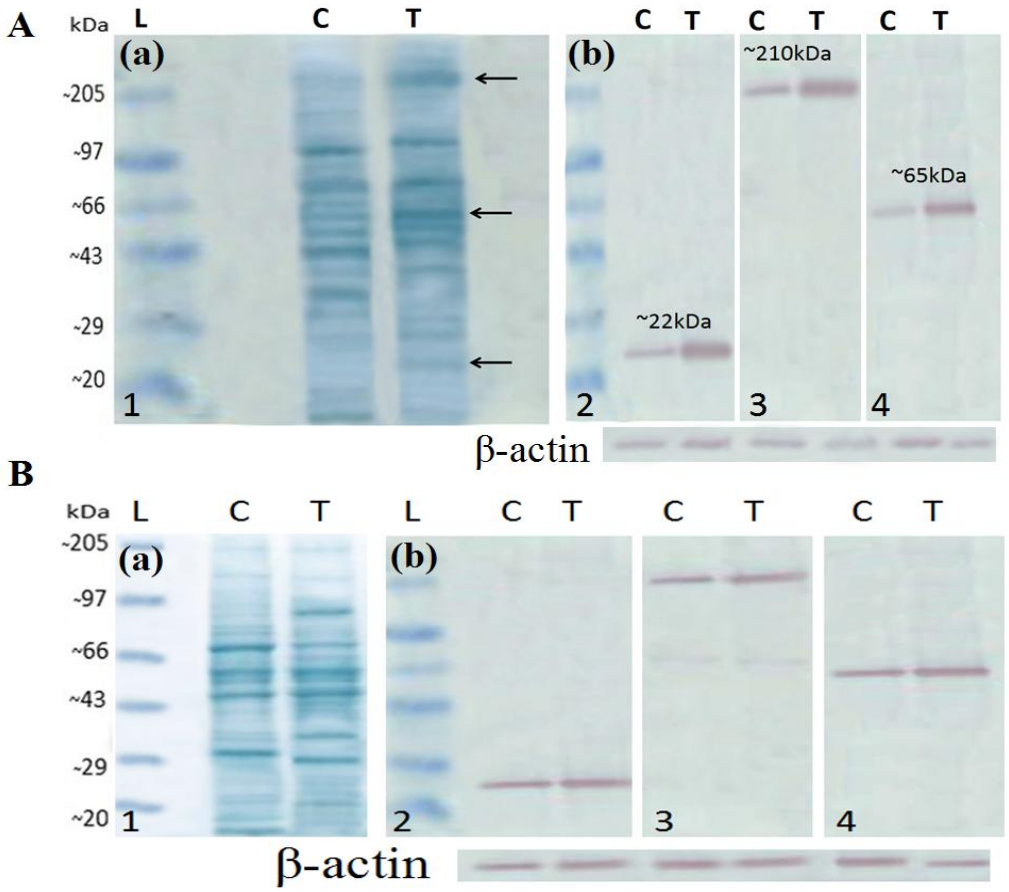


Figure 4.9 SDS PAGE and Western blot analysis of RhoA/MLCK and NFkβ. Changes in protein expression was evaluated using SDS PAGE **(a)** and western blot assay **(b). A** represents expression analysis in isolated GBEC, whereas **B** shows the expression in tissue samples. *Control (C) for tissue is normal gallbladder samples and that for GBEC is cells without infection of *S.* Typhi. T: Test samples. ~22kDa, ~65kDa and ~210kDa bands corresponds to RhoA, NFkβ and MLCK respectively. β-actin is the loading control.

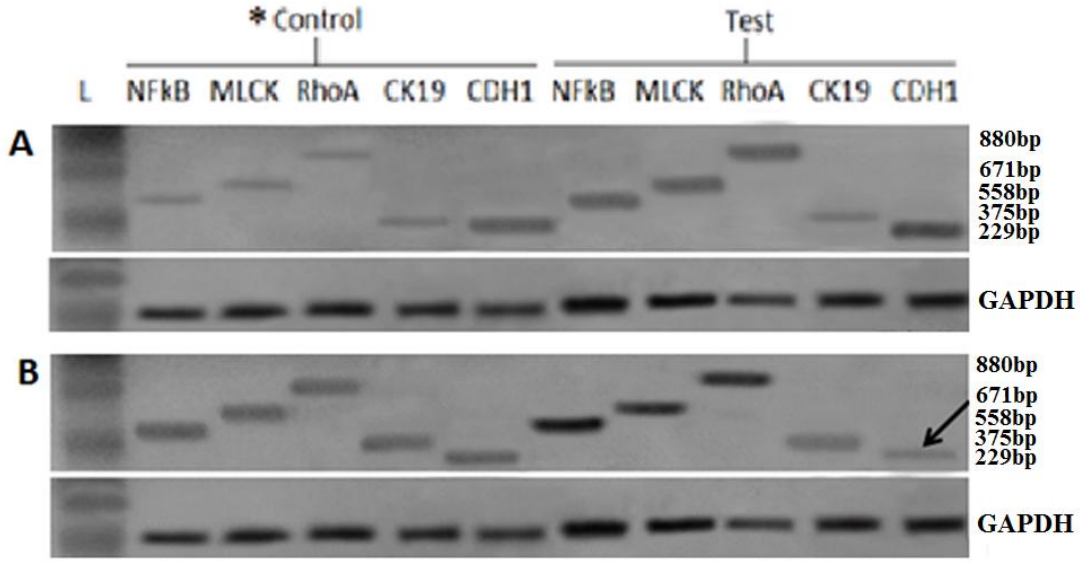


Figure 4.10 Alteration in gene expression in Cancer patients. The changes in gene expression were analysed using RT-PCR assay in chronic cholelithiasis **(A)** and in GBC **(B).** L: 50bp Genedirex DNA ladder. *Control for **A** is normal gallbladder samples and that for **B** is patients with chronic cholelithiasis. Marker arrow shows down-regulation in expression of CDH1 gene. GAPDH is the loading control.

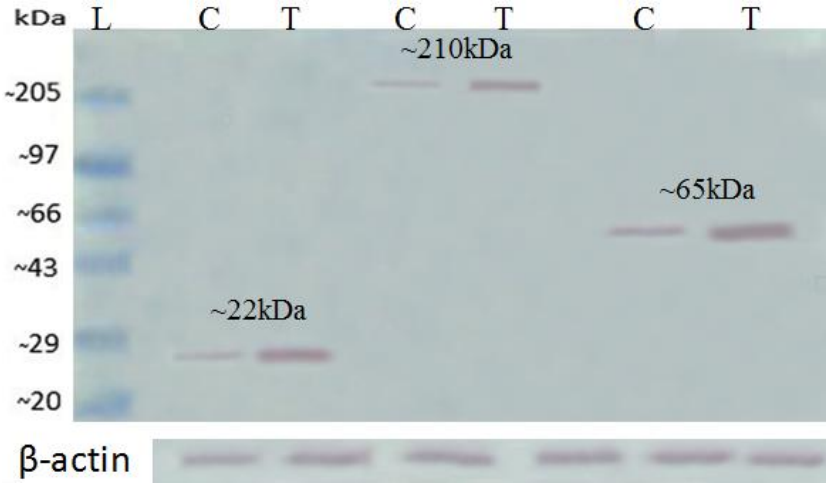


Figure 4.11 SDS PAGE and Western blot analysis of RhoA/MLCK and NFkβ in cancer patients. Changes in protein expression was evaluated western blot assay**.** *Control (C) is gallbladder samples showing chronic cholelithiasis T: Test samples showing gallbladder cancer. ~22kDa, ~65kDa and ~210kDa bands corresponds to RhoA, NFkβ and MLCK respectively. β-actin was used as loading control.

Thus, understanding the expression of RhoA or NFkβ and their downstream signalling components could help in prognosis and are potential targets for drug development. This would in turn help in preventing gallbladder cancers, the incidences of which are on a rise in regions around the world including India.

4.5 Conclusion

In conclusion, this study tries to evaluate the significance of RhoA/MLCK and NFkβ expression during gallbladder disease progression. We reveal that the chronic persistence of *S.* Typhi within the gallbladder of patients undergoing cholecystectomy did not significantly alter the expression these molecules or their downstream components. But, *in vitro* infection assay using GBEC showed successful adhesion and intracellular replication of the bacteria with significant up regulation of both RhoA/MLCK and NFkβ. These results can be attributed to bacterial numbers, and the organism may adapt to a dormant-state without actively being involved in regulating these components in the gallbladder. Interestingly the expression of these proteins was better correlated with different stages of gallbladder disease progression, with cancer patients showing significantly higher expression compared to patients with chronic cholelithiasis. Thus, we assume that RhoA/MLCK and NFkβ may be of prognostic value during the inflammatory stages of the gallbladder and is significantly over-expressed in gallbladder cancers.

Summary

Salmonella enteric serovar Typhi chronically persists within the gallbladder and is considered as a predisposing factor for gallbladder cancers. It is necessary to screen such carriers and understand the distribution of such carriers within a population with high incidences of gallbladder diseases. We screen for *S.* Typhi chronic persistence in gallbladder of patients undergoing cholecystectomy, using microbiological and PCR assays. *S.* Typhi isolated on culture was further characterised by determining its tolerance to bile, quantifying its biofilm- forming ability using crystal violet assays and estimating its drug susceptibility using disc diffusion tests. The culture method was positive for *S.* Typhi in only 5% of the total population, whereas PCR assay showed positive results for 11.5% of the patients, indicating low efficiency of culture based method to screen organisms in gallbladder. Though a very few cancer samples were obtained in this study, none of the three patients suffering from gallbladder carcinoma were positive for *S.* Typhi. Interestingly, the gallbladder was also shown to be colonized by other enteric and non-enteric organism, and some patients even showed polymicrobial infection in the gallbladder. The *S.* Typhi isolates showed significant biofilm formation in presence of bile and were resistant to first line drugs like chloramphenicol and TMP-SMZ. Also, the organism showed intermediate susceptibility to fluoroquinolones and cephalosporins, being susceptible only to ampicillin and ciprofloxacin *in vitro*.

Bile salts are shown to enter the bacterial cells and regulate several gene loci involved in oxidative stress, cell and membrane protein synthesis, efflux mechanisms and other survival mechanisms. In this study we report that bile led to the generation of reactive oxygen species (ROS) in *S.* Typhi, which in response showed a significant increase in the production of anti-oxidative enzymes namely superoxide dismutase (SOD) and catalase. The work for the first time reports that the AI-2 mediated quorum-sensing system of *S.* Typhi plays a key role in regulating the level of these enzymes during oxidative stress generated by bile. Furthermore, in the presence of ciprofloxacin and ampicillin, *S.* Typhi formed persister cells which increased > 3 fold when media was supplemented with bile. Other than these epigenetic like modifications shown in presence of bile, this study also shows that *S.* Typhi can undergo irreversible changes during its adaptation to bile. These findings show that *S.* Typhi adapts a protective

mechanism and shows increased persistence against antibiotics in the presence of bile which could be of clinical significance in the case of chronic bacterial persistence within gallbladder.

In addition, *S.* Typhi is also shown to sense quorum sensing signals produced by other organism via the *sdiA*. On this basis we develop a whole cell biosensor (P_{rck}-eGFP transcriptional fusion) to detect the AHL molecules produced by other organisms in mixed cultures, and try to find out the behaviour response of *S.* Typhi in presence of other organism. Though our findings show that *sdiA* is upregulated as a response to AHL molecules and bile, it did not confer any significant advantage to *S.* Typhi growth and biofilm formation. Interestingly, *sdiA* was found to be necessary in enhancing the resistance to certain drugs like ofloxacin, ceftriaxone and nalidixic acid.

The asymptomatic chronic carriage of *S.* Typhi in the gallbladder is a challenge for diagnosis and treatment in typhoid endemic regions. *Salmonella* effectors and toxins bring about various molecular alterations within the host so as to facilitate the survival and dissemination of the organism. Also, such alteration of the host signaling may be significant enough to further cause diseases like cancer. In this study we try to understand the role of chronic *S.* Typhi persistence in regulating RhoA and NFkβ expression in the gallbladder. Both, the activation of RhoA and NFkβ have been shown to have implication in EMT and carcinogenesis respectively. The presence of *S.* Typhi did not significantly alter RhoA and NFkβ expression in tissue specimens, with over-expression mostly correlated to the chronic inflammation due to gallstones. Interestingly, *in vitro* experiments involving gallbladder epithelial cell infections showed a significant increase in expression of these genes along with increase in expression of MLCK (a downstream effector protein involved in control of actin dynamics and leads to EMT). Furthermore, patients suffering from gallbladder cancer did show increased expression of rhoA and NFkβ along with loss in expression of the E-cadherin (CDH1) indicating EMT. Though the expression of these components may be of prognostic significance in gallbladder disease progression, they do not provide direct evidence of *S.* Typhi chronic persistence in gallbladder.

Future prospects

- We reveal that bile plays a key role in regulating a number of mechanisms in *S.* Typhi, as evident from the *in vitro* phenotypic changes. Some of the possible mechanisms have been explained, with most of them still lacking proper evidence.
- Our study indicates that communication in bacteria may play a major role in chronic persistence within the gallbladder. Thus, further analysis on the target genes regulated by these systems is necessary to understand mechanisms underlying chronic bacterial persistence.
- The gene knockout strains generated during this study can be used to understand their additional roles in regulating bacterial mechanisms and adaptation.
- We develop a whole cell biosensor with possible application in monitoring bacterial behaviour during polymicrobial infections.
- We identify RhoA/MLCK and NFkβ with significant prognostic value during the gallbladder disease progression. Thus, further understanding in regulation of downstream components and genes by these transcriptional factors is necessary. This would in turn help in identifying possible therapeutic targets which could prevent gallbladder cancers.

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APPENDIX I

1.1 Resolving gel preparation

(12%): Constituents	Amount (mL)
Water	5.0
30% Acrylamide- Bisacryl amide mixture	6.0
1.5 M Tris pH 8.8	3.8
10 % SDS	0.15
10% APS	0.15
TEMED	0.006

1.2 Stacking gel preparation (5%)

Constituents	Amount (mL)
Water	3.4
30% Acryl Amide-Bisacryl amide mixture	0.83
1.0 M Tris pH 6.8	0.63
10 % SDS	0.05
10% APS	0.05
TEMED	0.005

1.3 Running Buffer (10 X) for SDS-PAGE

Components	**Amount**
Tris	250 mM
Glycine	1.92 M
SDS	10 %

1.4 Loading Dye

Components	**Quantity**
Bromophenol blue	25 mg
Sucrose	4 g
SDS	10 %
Water	10 mL

1.5 Staining Solution for SDS

Components	Quantity
R250 Coomasie Blue	0.025%
Acetic acid	30 mL
Ethanol	50 mL
Water	100mL

1.6 TE Buffer

Components	Quantity
Tris HCL (pH 8.0)	10mM
EDTA (pH 8.0)	1mM

1.7 50X TAE Buffer

Components	Quantity(per 100mL)
Tris Base	24.2gm
Glacial Acetic acid	5.71mL
0.5M EDTA	10mL

APPENDIX II

1.1 Preparation of Competent cells. (Sambrook and Russell, 2001)

- Inoculate 100ml of Luria-Bertani broth with a single colony of organism (16-20hrs old) and incubate the flask at 37^{o}C for 4hrs on a shaker with vigorous shaking (300 cycles/min). (Grow the cell till you get OD600 = 0.1-10^{8} cells/ml)
- Aseptically transfer the cells to a sterile centrifuge tube (falcon's tube). Keep the cells on ice for 15 minutes (cool to 4^{o}C)
- Centrifuge the tubes at 4000rpm to recover the cells for 10min at 4^{o}C.
- Decant the supernatant media from the tube and drain any traces of media.
- Re-suspend each pellet in 10ml of sterile ice cold 0.1 M $CaCl_2$ and keep the tubes on ice.
- Recover the cells by centrifugation at 4000rpm for 10min at 4^{o}C.
- Decant the supernatant and drain the traces of fluid.
- Re-suspend the pellet in 2ml of ice cold 0.1M $CaCl_2$ (containing 1% glycerol in total volume of CaCl2) for 50ml original culture.
- Aliquot 200µl in eppendorfs and store at -80^{o}C. Thaw on ice whenever required for transformation.

1.2 Transformation of Cells (Sambrook and Russell, 2001)

- **Add DNA** (less than **50ng** in a volume of **10µl** or less) to **200µl of competent cells**. Mix the contents well using the micropipette. Store the tube **on ice for 30min**
- Transfer the tube to a water bath preheated to **42^{o}C** for exactly **90 seconds**. (Don't shake the tubes)
- Quickly transfer the tubes to an ice bath and **chill for 2min.**
- Add **800µl** of **sterile LB** to the tube and **incubate** the culture for **45min at 37^{o}C water bath** for bacteria to recover and to express antibiotic resistant marker encoded by plasmid.
- Transfer appropriate volume (200µl per plate) of transformed cells onto LB plates containing appropriate antibiotic for selection. Spread the culture on the agar surface.
- Leave the plates at RT till the liquid is absorbed and then incubate at 37^{o}C for 24hrs.

APPENDIX III

List of Publications

1. **Yogesh D Walawalkar**, Kanishka Tiwary, Tannishtha Saha and Vijayashree Nayak. Significance of Microsatellite Instability and Gene Methylation as a Prognostic Biomarkers during Gallbladder Cancer Progression: A Review. J Cell Sci Ther 2015, 6:196.
2. **Yogesh D. Walawalkar** and Vijayashree Nayak. Role of Bile in Regulating Small RNAs in *Salmonella typhi* –A Factor for Chronic Infection within Gallbladder? International Journal of Current Microbiology and Applied Sciences 2015, 4(2); 183-197.
3. **Yogesh D. Walawalkar**, Rajni Gaind & Vijayashree Nayak. Study on *Salmonella* Typhi occurrence in gallbladder of patients suffering from chronic cholelithiasis—a predisposing factor for carcinoma of gallbladder. Diagnostic Microbiology & Infectious Diseases 2013, 77(1): 67-73.

APPENDIX IV

List of conferences and workshops

1. Presented poster the 34th Annual Convention of Indian Association for Cancer Research (International Conference-"Cancer Research: from Bench to the Bedside") Jaipur, 2015
2. Attended workshop on Flow Cytometry in Oncology (Diagnosis of Acute Leukaemias/Diagnosis of Chronic Leukaemias/Assessment of MRD in Haematological Cancer) held by Indian Association of Cancer Research (IACR), Jaipur-2015
3. Attended Workshop on Cancer Mutation Testing & SNPs with Real Time PCR (Non Small Cell Lung Cancer / CML) helad by IACR, Jaipur-2015
4. Presented poster at International Conference titled "Bacterial Expressions" held at NCBS-TIFR, Bangalore 2013
5. Attended international symposium on molecular pathology held by molecular pathology association of India (MPAI), New Delhi-2013

APPENDIX V

Brief Biography of the Candidate

Personal Details

Name	Mr. Yogesh D. Walawalkar
Education	M.Sc. Microbiology, Mumbai University (2011)
	B.Sc. Microbiology, Goa University (2009)
Email	yo.genetech@gmail.com
	p2012016@goa.bits-pilani.ac.in

Research Experience

- Junior Research Fellow at BITS, Pilani (2011). Worked on project entitled 'Correlating chronic *S.* Typhi infection with gallbladder cancers'
- Trainee at Syngenta India Limited, Goa-India (2011)
- Worked on a project entitled 'Engineering whole cell biosensors' in collaboration with IIT Bombay (2009).
- Summer training at Dept. of Chemical Engg.- IIT-Bombay (2010)
- Animal Cell and Tissue Culturing, Ruia College, Mumbai-India (2009)
- Pathology lab training (Observership) at P.D. Hinduja National Hospital and Medical research centre, Mumbai (2010)

No. of Publications **03 (As first author)**

No. of Conferences **05**

APPENDIX VI

Brief Biography of the Supervisor

Personal Details

Name	Dr. Vijayashree Nayak
Designation	Associate Professor
Affiliation	Birla Institute of Technology & Science, Pilani
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Contact	0832-2580161

Dr. Vijayashree Nayak, persued her Ph.D form MAHE Manipal 1998 and has ctively been involved in teaching and research since then in various universities and colleges within the country. Currently, her lab broadly focuses on couple of aspects like molecular mechanisms in Cancer and therapeutic applications of Tissue engineering. Current goal of her research is to obtain a detailed molecular understanding of cell cycle regulation loss and epithelial mesenchymal transition (EMT) in certain cancers. From various known factors responsible for progression towards cancer, her work is particularly concerned with the role of chronic infections and bacterial mediated carcinogenesis. Her interest also lies in developing novel scaffolds for cell culture in tissue engineering applications and drug screening for anticancer activity. Currently she is involved in couple of project sanction by DST, India and BRNS.